

Active Sodium and Potassium Transport in High Potassium and Low Potassium Sheep Red Cells

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ABSTRACT The kinetic characteristics of the ouabain-sensitive (Na + K) transport system (pump) of high potassium (HK) and low potassium (LK) sheep red cells have been investigated. In sodium medium, the curve relating pump rate to external K is sigmoid with half maximal stimulation ($K_{1/2}$) occurring at 3 mM for both cell types, the maximum pump rate in HK cells being about four times that in LK cells. In sodium-free media, both HK and LK pumps are adequately described by the Michaelis-Menten equation, but the $K_{1/2}$ for HK cells is 0.6 ± 0.1 mM K, while that for LK is 0.2 ± 0.05 mM K. When the internal Na and K content of the cells was varied by the PCMBMS method, it was found that the pump rate of HK cells showed a gradual increase from zero at very low internal Na to a maximum when internal K was reduced to nearly zero (100% Na). In LK cells, on the other hand, no pump activity was detected if Na constituted less than 70% of the total (Na + K) in the cell. Increasing Na from 70 to nearly 100% of the internal cation composition, however, resulted in an exponential increase in pump rate in these cells to about $\frac{1}{6}$ the maximum rate observed in HK cells. While changes in internal composition altered the pump rate at saturating concentrations of external K, it had no effect on the apparent affinity of the pumps for external K. These results lead us to conclude that the individual pump sites in the HK and LK sheep red cell membranes must be different. Moreover, we believe that these data contribute significantly to defining the types of mechanism which can account for the kinetic characteristics of (Na + K) transport in sheep red cells and perhaps in other systems.

INTRODUCTION

There exist among sheep two types of individuals: those having red cells with high potassium (HK) and low sodium content (85 mmole K, 15 mmole Na per liter cells), and others having red cells with low potassium (LK) and high sodium content (12 mmole K, 88 mmole Na per liter cells). Tosteson and

Hoffman (1960) investigated active and passive Na and K transport in these HK and LK cells in an effort to define the membrane basis for the genetically determined difference in cation composition. It was found that while the passive permeability of the cells to Na was about the same, the passive permeability to K was approximately two times greater in LK than in HK cells. Moreover, the rate of active (Na + K) transport (pump) in HK cells was four times as great as in LK cells.

In this investigation, the kinetic characteristics of the (Na + K)-pump activity in HK and LK cells were studied in order to determine whether the difference in pump rates could be adequately explained by a difference in the number of pump sites in the membrane of each cell type, or whether it was necessary to postulate differences in the characteristics of the individual pump sites. Previous investigations have shown that quantitative correlations can be made between the (Na + K)-adenosine triphosphatase (ATPase) activity and pump rate (Tosteson et al., 1960; Tosteson, 1963; Brewer et al., 1968) and between ouabain-binding sites and pump rate (Dunham and Hoffman, 1969) in HK and LK cells. These findings are compatible with the hypothesis that the difference between the (Na + K)-pump rates is due to a difference in the number of identical sites in the HK and LK membranes. However, data reported in this paper indicate that the apparent affinity of the pump for external and internal K and Na is different in HK as compared with LK sheep red cell membranes. Moreover, both types of sheep red cells are clearly distinguishable in this regard from human red cells (Post and Jolly, 1957; Whittam and Ager, 1965). We conclude that at least one of the membrane components which comprise the pump is different among the three cell types.

Another important observation in these experiments was the apparent independence of the activation of the pump by internal and external cations in both types of sheep red cells. Thus, altering the concentrations of Na and K within the cell changed the maximum pump rate but not the apparent $K_{1/2}$ for external K. We believe that this observation substantially reduces the number of possible models which can account for the kinetic characteristics of active Na-K transport.

MATERIALS AND METHODS

Preparation of Cells

The sheep used in this investigation were crossbreeds of Suffolk, Hampshire, and Rambouillet sheep. All LK animals reported upon were shown to lack the M antigen (Lauf and Tosteson, 1969).

Blood was obtained by jugular venipuncture and was collected into ice-cold heparinized flasks. After a sample was taken for hematocrit and hemoglobin determinations, the cells were washed four times in either $MgCl_2$ wash solution (120 mM $MgCl_2$ saturated with $MgCO_3$ and filtered, pH approx. 8) or in 150 mM NaCl, and

the buffy coat was discarded. When required, the internal cation composition was varied by a method similar to that devised by Garrahan and Rega (1967).

Packed cells were added to an ice-cold solution containing 87% isosmotic XCl , 3% isosmotic X -phosphate (pH 7.4), 10% 300 mM sucrose, 2 mM $MgCl_2$, and 0.2 mM parachloromercuribenzenesulfonate (PCMBs, Sigma Chemical Co., St. Louis, Mo.). X represents K, Na, or a combination of the two. Isosmotic indicates 282 mosmolar, which is the osmolarity of 150 mM NaCl. The hematocrit of the suspension was 0.5–2%. The cells were incubated at 4°C in this medium for 16–24 hr, being resuspended by gentle swirling several times during this period. The cells were then spun down the supernatant was aspirated and they were resuspended in a similar

TABLE I
EFFECT OF PCMBs TREATMENT ON K INFLUXES IN HK AND LK SHEEP
RED BLOOD CELLS WITH NORMAL (K)_i AND (Na)_i

Sheep	Treatment	(K) _i	(Na) _i	${}^iM_K^T$	${}^iM_K^L$	${}^iM_K^P$
		<i>mmole/liter cells</i>			<i>mmole/(liter cells-hr)</i>	
LK 2582	PCMBs	7.0±0.3	93.9±2.4	0.64±0.04	0.49±0.02	0.15±0.04
LK 2582	Control	6.9±0.3	89.6±3.5	0.67±0.02	0.57±0.02	0.10±0.03
HK 2562	PCMBs	73.6±1.4	24.2±0.5	0.59±0.02	0.07±0.00	0.52±0.02
HK 2562	Control	72.4±0.9	19.9±0.1	0.52±0.02	0.05±0.00	0.47±0.02

Cells were incubated 21 hr in PCMBs solution containing a K/Na ratio appropriate to maintain their original composition (10% K for LK, 80% K for HK). The cells were then incubated in a dithiothreitol solution at 37°C for 45 min, washed, and influxes were measured in an Na medium containing 5 mM K. Control cells were treated in the same manner, but PCMBs and dithiothreitol were omitted from the suspensions. The figures indicate the mean and standard deviation of three determinations. The symbols used in the table are defined in Table II (Exp. 63).

medium containing 5 mM dithiothreitol (DTT, Calbiochem, Los Angeles, Calif.) and 11 mM glucose instead of the PCMBs. The resuspended cells were incubated at 37°C for 45 min and were washed four times in media similar to those in which the cation fluxes were to be measured.

The ratio of Na to K in the PCMBs solution was that desired as the final cell Na/K ratio, corrected for the ions contained in the fresh cells. The Na/K ratio in the DTT solution was similar except that, when small but finite amounts of K were desired in the cells, K was omitted from the medium to prevent transport of additional K into the cells during the DTT incubation.

During the PCMBs-DTT treatment total hemolysis was less than 2%. The total Na + K in the altered cells was within the range for normal cells (95 ± 10 mmole/liter of fresh cells). Table I indicates that the active and passive unidirectional K influxes in cells treated with PCMBs and DTT were not significantly different from those measured in controls with similar internal Na and K.

Experimental Procedures

All flux measurements were made on fresh or PCMBs-treated cells suspended in media isosmotic with 150 mM NaCl. The hematocrit was always between 0.5 and 2%.

Constituents of the media for individual experiments are given in the legend of each figure.

⁴²K INFLUX EXPERIMENTS Packed cells were resuspended in appropriate flux medium containing all components except K, and were incubated for 15–30 min at 37°C in either 25- or 50-ml Erlenmeyer flasks. The flasks were agitated at about 150 rpm, and were covered with small plastic cups to prevent evaporation or contamination. At the end of the initial incubation period, warm isotonic medium containing sufficient ⁴²K-labeled KCl to give the desired final concentration was added. Routinely, samples were taken at 30 and 90 min after the addition of the isotope, although in some experiments the time intervals were shortened.

Sampling was accomplished by pouring or pipetting aliquots of the cell suspension into cold 12-ml polycarbonate centrifuge tubes and then placing them directly into a previously chilled Sorvall SS-24 rotor. Sampling of 24 flasks required about 5 min, but the order of sampling was always the same so that the time intervals between the first and second sample from any two flasks in a single experiment never varied by more than 30 sec. The samples were spun at 8000 *g* and 4°C for 2 min (Sorvall RC-2B centrifuge, Ivan Sorvall, Inc., Norwalk, Conn.). The supernatants were decanted into vials for later analysis. The cells were then washed four times in 70 vol of ice-cold MgCl₂ wash solution.

²²Na EFFLUX EXPERIMENTS Cells were loaded with ²²Na by including the isotope in the PCMBBS solution. The experimental procedure was the same as for ⁴²K influx measurements, which were usually carried out simultaneously on the same cells.

NET Na AND K FLUX AND ATP HYDROLYSIS EXPERIMENTS Net K influx was measured only on cells with low K content so that increases in cell K were large compared to the initial values. This also insured that the error due to K efflux was kept small. Net Na efflux determinations were only done in media initially containing no Na for the same reasons. The experimental procedure was similar to that for ⁴²K influx measurements, except that six to nine samples were taken over a period of up to 4 hr.

In experiments in which ATP hydrolysis was measured, duplicate samples were taken and analyzed as described below.

Analytical Procedures

pH was measured using a Radiometer pH meter (Radiometer Co., Copenhagen, Denmark) equipped with a thermostated microelectrode unit. Osmotic pressure of incubation media was adjusted to be isosmotic with 150 mM NaCl as determined by freezing point depression (Osmette Precision Osmometer, Precision Systems, Framingham, Mass.). Microhematocrits were determined in quadruplicate on fresh whole blood (International Equipment Company, Needham Heights, Mass., model MB microcapillary centrifuge, optical comparator designed by T. J. McManus). Measurements of ⁴²K and ²²Na activity were determined by detection of gamma emission using a Packard Autogamma System (Packard Instrument Co., Inc., Downers Grove, Ill.). Hemoglobin concentrations were determined by measuring optical density at 540 nm

using either a Zeiss PMQ II (Carl Zeiss, Inc., New York) or a Gilford 300-N (Gilford Instrument Company, Oberlin, Ohio) spectrophotometer. Na and K were determined by atomic absorption flame photometry (Perkin-Elmer model 303, Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.). Samples for ATP estimation were deproteinated in cold 6% perchloric acid. The assay was performed by measuring light emission when the neutralized sample was mixed with properly prepared firefly lantern extract (Worthington Biochemical Corp., Freehold, N. J.). The method has been described previously in detail (Allen, 1967; McManus et al., manuscript in preparation).¹

TABLE II
DEFINITION OF SYMBOLS

$[B]_o$	mmole B per liter of medium (mM), where B represents Na, K, etc.
$[B]_i$	mmole B per liter of cell water (mM), where B represents Na, K, etc.
$(B)_i$	mmole B per liter of fresh packed cells, where B represents Na, K, etc.
Na_i	$(Na)_i / [(Na)_i + (K)_i] \times 100$. That is, the percentage of the total cell alkali metal content which is sodium.
K_i	$100 - Na_i$
${}^iM_K^T$	Total K influx measured as mmole/(liter fresh cells·hr)
${}^iM_K^L$	Ouabain-insensitive or "leak" influx of K in mmole/(liter fresh cells·hr)
${}^iM_K^P$	Ouabain-sensitive or "pump" influx of K in mmole/(liter fresh cells·hr)
$({}^iM_K^P)_{K_o}^{max}$	The maximum value of ${}^iM_K^P$ which can be obtained by increasing $[K]_o$, with other conditions being held constant
	In the flux designations, a prescript o rather than i refers to efflux rather than influx. The subscript "Na" refers to Na rather than K.
$K_{1/2}$	The value of $[K]_o$ at which ${}^iM_K^P = \frac{1}{2}({}^iM_K^P)_{K_o}^{max}$
X_o	Specific activity of isotope in the medium
${}^i k_K^L$	First-order rate constant for ouabain-insensitive K influx (hr^{-1})

Calculations and Presentation of the Data

An explanation of the symbols is found in Table II. All results are expressed per liter of fresh, packed cells in plasma. The volume of packed cells represented by a sample of hemolysate was calculated on the basis of the optical density (at 540 nm) of the hemolysate, and the optical density and hematocrit of whole blood preparations.

The fluxes were calculated by the equations

$${}^iM_K = \frac{\Delta \text{ counts/liter cells}}{(\Delta \text{ time}) X_o} \quad (1 a)$$

$${}^oM_{Na} = \frac{\Delta \text{ counts/liter supernatant}}{(\Delta \text{ time}) X_i} \times \left(\frac{1 - \text{hematocrit}}{\text{hematocrit}} \right) \quad (1 b)$$

These simple equations were chosen because the error which is introduced by them (underestimation of 2% in HK and 10% in LK pump fluxes) is less than the uncer-

¹ McManus, T. J., D. W. Allen, and H. D. Kim. A rapid ultramicroassay for ATP utilizing firefly luminescence and a liquid scintillation counter. Manuscript in preparation.

tainty incurred in correcting for back flux of ^{42}K from cells containing very low K. Pump fluxes (operationally defined for this paper as ouabain-sensitive fluxes) were calculated by subtracting fluxes determined in the presence of 10^{-4} M ouabain from those measured on duplicate cell suspensions in the absence of cardiac glycoside.

In experiments in which it was the independent variable, $[\text{K}]_o$ assumed values which ranged from 0.01 to 20 mM. In these cases, a conventional plot of the ouabain-sensitive flux *versus* $[\text{K}]_o$ was impractical because significant differences in curves in the region where $[\text{K}]_o < 1$ mM became lost in the width of the lines and in the size of the symbols denoting the experimental points. To overcome this difficulty, some of the data have been presented in the form suggested by Eadie (1942). This method consists of plotting the velocity of the reaction (in this case ${}^iM_{\text{K}}^p$) as a function of the velocity divided by the substrate concentration (${}^iM_{\text{K}}^p/[\text{K}]_o$). If the pump rate as a function of $[\text{K}]_o$ is well described by the Michaelis-Menten equation, the Eadie plot yields a straight line. The y -intercept is equal to $({}^iM_{\text{K}}^p)_{\text{K}_o}^{\text{max}}$, and the slope is equal to $-(K_{1/2})$, where $K_{1/2}$ is the value of $[\text{K}]_o$ at which ${}^iM_{\text{K}}^p = \frac{1}{2} ({}^iM_{\text{K}}^p)_{\text{K}_o}^{\text{max}}$.

The Eadie plot has three characteristics which argue in favor of its use rather than the more traditional Lineweaver-Burk plot:

- (a) Undue weight is not given to data obtained at very low substrate concentrations.
- (b) In systems which are well described by the Michaelis-Menten equation, $K_{1/2}$ and $({}^iM_{\text{K}}^p)_{\text{K}_o}^{\text{max}}$ can be deduced independently from the slope and y -intercept of a straight line, respectively.
- (c) In systems which are not well described by the Michaelis-Menten equation, the deviation from linearity is so striking that one is not even tempted to draw a best straight line fit.

So that cells with greatly different maximum pump rates may be compared on the same coordinates, both the ordinate and the abscissa of the Eadie plots have been divided by the appropriate estimate of $({}^iM_{\text{K}}^p)_{\text{K}_o}^{\text{max}}$. This procedure does not alter the slope of the plot.²

In addition to the Eadie plot, which in every case includes all the data obtained in a given experiment, a conventional plot of the pump rate as a function of $[\text{K}]_o$ has usually been given. In this plot, points obtained at high and low values of $[\text{K}]_o$ have sometimes been omitted because their inclusion would require that the scales of the graph be so large or small that the shape of the total curve is obscured. Intermediate points have never been left out.

The symbol Na_i , as defined in Table II, has been used to describe the relative K and Na content of the cells when these parameters were the independent variables in an experiment. This procedure allows the three variables $(\text{Na})_i$, $(\text{K})_i$, and ${}^iM_{\text{K}}^p$ to be plotted in two dimensions by combining the independent variables. The other alternative, plotting fluxes as a function of either $(\text{Na})_i$ or $(\text{K})_i$, places excessive weight on the selected ion, especially when its concentration is high. It should be emphasized

² In the terms of this paper, the Eadie equation may be written

$${}^iM_{\text{K}}^p = ({}^iM_{\text{K}}^p)_{\text{K}_o}^{\text{max}} - K_{1/2} ({}^iM_{\text{K}}^p/[\text{K}]_o).$$

By dividing both the abscissa and ordinate by the y -intercept, $({}^iM_{\text{K}}^p)_{\text{K}_o}^{\text{max}}$, the resultant plot has a y -intercept of 1.0 and a slope of $-K_{1/2}$ (assuming the plot is linear).

that the use of Na_i rather than K_i in the graph and tables in the Results section is arbitrary and should not imply the dominance of $(\text{Na})_i$ over $(\text{K})_i$ as the important parameter.

In the legends, the symbol HK or LK, followed by a number (e.g. HK 2562), indicates the sheep from which the cells used in the experiment were obtained; the symbol \bar{k}_K^L denotes a mean estimate of the first-order rate coefficient for ouabain-insensitive K influx. Thus $\bar{M}_K^L \approx (\bar{k}_K^L)[\text{K}]_o$. The experiments presented are representative of at least two and usually more experiments which gave similar results. An exception to this rule is that the experiments in Fig. 3 *a* and *b* were considered to provide mutual verification, and were performed only once. Whenever possible, data presented in the figures are from experiments performed with both HK and LK cells on the same day.

RESULTS

Fig. 1 *a* is a plot of \bar{M}_K^P as a function of $[\text{K}]_o$ for HK and LK cells in sodium medium. Under these conditions the curves for HK and LK are indistinguishable except for the 3.5-fold difference in magnitude indicated on the ordinate for the two cell types. In sodium medium with 5 mM $[\text{K}]_o$, Tosteson and Hoffman (1960) found that the ratio of the pumps was about four to one. Dunham and Hoffman (1969) measured $(\bar{M}_K^P) K_{K_o}^{\text{max}}$ in sodium medium and found a mean HK/LK pump ratio of 8.4.

Fig. 1 *b* is the Eadie plot of the data shown in Fig. 1 *a*. A deviation from linearity similar to the one shown here for HK and LK sheep cells is qualitatively equivalent to a convex Lineweaver-Burk plot or a "sigmoid" plot of pump rate *versus* $[\text{K}]_o$ previously shown to describe the behavior of the human red cell pump under comparable conditions (Sachs and Welt, 1967; Garrahan and Glynn, 1967 *a*). Nonlinearity of this type is compatible with the hypothesis that two or more external K ions must interact with the pump before transport of the ions can occur. In contrast, an ensemble of pump sites not requiring multiple K interactions, but with differing affinities for $[\text{K}]_o$, would yield a convex Eadie plot.

A similar experiment performed in Na-free medium is illustrated in Fig. 2. A comparison of this figure with Fig. 1 indicates that removal of Na from the medium shifted both the HK and LK activation curves to the left. The linearity of the Eadie plots in Fig. 2 *b* indicates that the dependence of the pumps of both HK and LK cells on $[\text{K}]_o$ in Na-free medium is described adequately by the Michaelis-Menten equation. The most striking feature of Fig. 2 is, however, the appearance of a large difference in the activation curves for HK and LK cells which cannot be eliminated by altering the scale on the ordinate, and hence, cannot be due to a simple difference in the number of pump sites in the membrane of each cell type. This feature is most readily appreciated in Fig. 2 *b*. The slope of the straight line in this plot is equal to $-(K_{1/2})$, where $K_{1/2}$ is the concentration of $[\text{K}]_o$ at which the pump is activated to $1/2$ of its

maximal rate. Since the kinetic characteristics of the $(K)_o$ activation of the K pumps of HK and LK cells are not very different from each other in the presence of high external sodium, a difference in the absence of external sodium requires that either the two pumps differ with respect to both activation by $[K]_o$ and inhibition by $[Na]_o$, or that the difference is in the effect of high $[Mg]_o$ on the pumps. The latter of these possibilities is suggested by the fact that high $[Mg]_o$ regularly reduces the maximum flux by a larger fraction in LK than in HK cells. If this depression occurred primarily at $[K]_o$ above about 0.5 mM, it would produce a difference in the apparent $K_{1/2}$ of the type that was observed.

To answer the question of whether the pumps differed with respect to their interactions with magnesium ions, on the one hand, or the alkali metal ions on the other, $[K]_o$ activation curves were obtained in low-sodium media in which tris(hydroxymethyl)amino methane (Tris) or choline were the major cations. The resultant Eadie plots in Figs. 3 *a* and 3 *b* respectively, indicate that the difference in $K_{1/2}$ for HK and LK pumps persists in the absence of high $[Mg]_o$. Table III summarizes the values of $K_{1/2}$ and $(M_K^E)K_{K_o}^{max}$ obtained in high sodium and in the three Na-free media. The $K_{1/2}$ of each cell type shows considerable dependence on which medium was used, but in Na-free media, the $K_{1/2}$ for LK was always lower than that for HK, and indeed, there was no overlap of the $K_{1/2}$ values for the two cell types. Generally, the maximal pump rate was higher in the presence of sodium than it was in its absence. It is interesting to note that experiments performed in Tris showed less depression of the HK pump than experiments in the other sodium-free media and yet yielded the lowest $K_{1/2}$. This is contrary to what would have been observed if the difference in $K_{1/2}$ was referable to differential depression of the maximum flux in the two cell types. The conclusion which must be drawn, therefore, is that the pumps of HK and LK cells with normal internal cation composition differ in their apparent affinity for $[K]_o$ and in their less easily characterized reaction with the inhibitor-activator $[Na]_o$.

Since the K pump is one aspect of the coupled (Na + K) pump, it was possible that the difference in $K_{1/2}$ for $[K]_o$ was due to the striking difference in cellular Na and K content of HK and LK cells. Indeed, as will be discussed later, the majority of models proposed to account for coupled (Na + K) transport yield formal equations which predict that $K_{1/2}$ for $[K]_o$ is a function of the internal cations.

To investigate this possibility, the PCMBBS procedure was used to produce HK and LK cells containing similar concentrations of Na and K. Figs. 4 *a* and 4 *b* illustrate the conventional and Eadie plots of pump activation by $[K]_o$ in magnesium medium for both HK and LK cells made very low in K_i , the alkali metal content being maintained by increasing Na_i . Again, the Eadie plot yields reasonably straight lines of significantly different slope, with

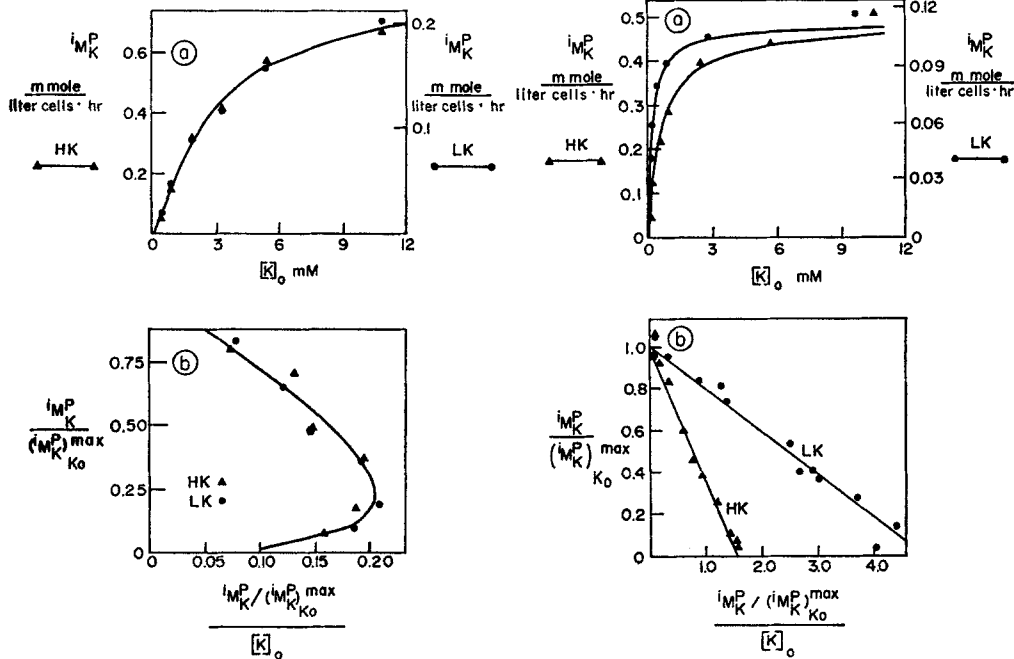


FIGURE 1

FIGURE 2

FIGURE 1 *a*. The activation of the K pump by $[K]_o$ in HK and LK sheep red cells suspended in a sodium medium (Exp. 62). Medium: 135 mM (K + Na)Cl, 15 mM Tris-Cl (pH 7.4), 11 mM glucose, and 2 mM MgCl₂. Samples were taken at 30 and 90 min after addition of isotope.

Cells	(K) _i	(Na) _i	i_{K}^{L}	K _{1/2}	$(i_{K}^{P})_{K_o}^{max}$
HK 177	94 mM	17 mM	0.01/hr	~3 mM	0.84 mmole/(liter cells·hr)
LK 188	11 mM	78 mM	0.04/hr	~3 mM	0.24 mmole/(liter cells·hr)

The curve drawn through the data was calculated from the equation

$$i_{M_K}^P = (i_{M_K}^P)_{K_o}^{max} \times \frac{[K]_o^2}{[K]_o^2 + 2.66 [K]_o + 1.25}$$

which was obtained by fitting a Lineweaver-Burk plot of the HK data in Fig. 8 to a second-order polynomial by the least squares method. The values for $(i_{M_K}^P)_{K_o}^{max}$ in HK and LK cells were determined by minimizing the deviation from the line.

FIGURE 1 *b*. The Eadie plot of the same data normalized by dividing the pump rates by their maxima.

FIGURE 2 *a*. The activation of the K pump by $[K]_o$ in HK and LK sheep red cells in low sodium medium; Exp. 12 (LK) and 32 (HK). Medium: 0.01–20 mM KCl, 15 mM Tris-Cl (pH 7.4) (HK), 10% v/v glycylglycine-MgCO₃ (pH 7.4) (LK), 11 mM glucose, made isosmotic with MgCl₂. Samples were taken at 30 and 120 min after addition of isotope.

the $K_{1/2}$ for LK cells ($0.2 \text{ mM } [K]_o$) equal to about $\frac{1}{3}$ of that for HK cells ($0.6 \text{ mM } [K]_o$). The data obtained in a similar experiment in sodium medium are shown in Fig. 5. The lines in both the conventional and Eadie plots were drawn using constants obtained by fitting the reciprocals of the pump rates and corresponding values of $[K]_o$ obtained for the HK cells to a second-order polynomial by least square analysis. This technique has been used by Sachs and Welt (1967) for analyzing data obtained on human red cells. The scale for LK cells was then chosen to minimize the deviation of the lowest five data points from the HK curve in the conventional plot. It is worth noting that the

TABLE III
EFFECT OF ALTERING THE PRINCIPLE EXTRACELLULAR CATION ON CHARACTERISTICS OF ACTIVE K INFLUX ACTIVATION ON $[K]_o$ IN HK AND LK SHEEP RED BLOOD CELLS

Principal cation	Cell type	$K_{1/2}$	$iM_K^P \text{ max}$	Shape of Eadie plot
		$mM [K]_o$	$mmole/(liter \text{ cells} \cdot hr)$	
Na	LK	~ 3	0.24	Nonlinear
Na	HK	~ 3	0.84	Nonlinear
Mg	LK	0.20	0.11	Linear
Mg	HK	0.63	0.48	Linear
Tris	LK	0.17	0.08	Linear
Tris	HK	0.42	0.73	Linear
Choline	LK	0.29	0.13	Linear
Choline	HK	0.51	0.48	Linear

The data are a summary of the results obtained from the experiments illustrated in Figs. 1-3.

curves in Figs. 1 and 5 are identical except for the differences in absolute magnitudes of iM_K^P .

Table IV gives the values of $K_{1/2}$ and $(iM_K^P)_{K_o}^{\text{max}}$ obtained with high sodium LK and HK cells in sodium and in magnesium media, along with the comparable data for cells of normal cation composition. It is apparent from the table that the differences in $K_{1/2}$ observed in normal HK and LK cells are not referable to differences in internal Na and K. Indeed, the values of $K_{1/2}$, and

Cells	$(K)_i$	$(Na)_i$	i_k^L	$K_{1/2}$	$(iM_K^P)_{K_o}^{\text{max}}$
HK 2562	73 mM	15 mM	0.011/hr	0.63 mM	0.48 mmole/(liter cells·hr)
LK 2551	13 mM	81 mM	0.008/hr	0.20 mM	0.11 mmole/(liter cells·hr)

Curves are of the Michaelis-Menten type.

FIGURE 2 *b*. The Eadie plot of the data in Fig. 2 *a*. Straight lines were fitted to the data by least squares and are equivalent to the corresponding curves in Fig. 2 *a*.

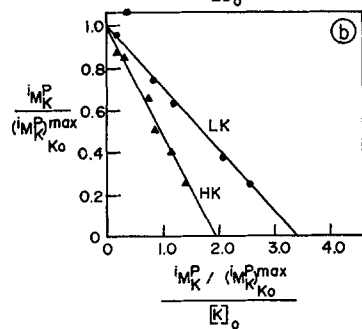
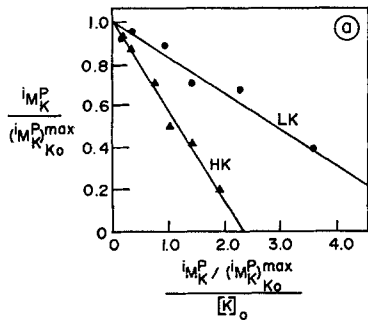


FIGURE 3

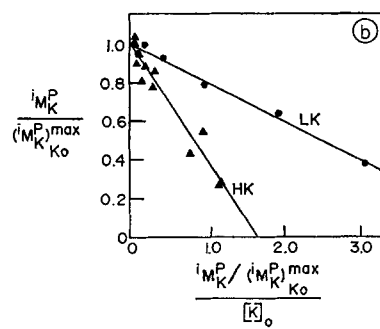
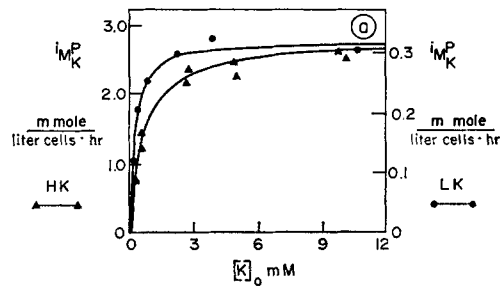


FIGURE 4

FIGURE 3 *a*. The Eadie plot of the activation of pump-K influx in HK and LK sheep red cells in Tris medium (Exp. 74). Medium: 0.1–5 mM $[K]_o$, 11 mM glucose, made isosmotic with Tris-Cl (pH 7.4, 37°C).

Cells	$(K)_i$	$(Na)_i$	i_K^L	$K_{1/2}$	$(i_{M_K^P}^P)_{K_o}^{max}$
HK 177	76 mM	13 mM	0.03/hr	0.42 mM	0.73 mmole/(liter cells·hr)
LK 183	6 mM	90 mM	0.21/hr	0.17 mM	0.08 mmole/(liter cells·hr)

Samples were taken at 20 and 110 min after addition of isotope. Kinetic parameters were obtained by least square fit of the data shown.

FIGURE 3 *b*. The Eadie plot of the activation of pump-K influx in HK and LK sheep red cells in choline medium (Exp. 75). Medium: 0.1 to 5 mM $[K]_o$, 11 mM glucose, 15 mM Tris-Cl (pH 7.4, 37°C), made isosmotic with choline chloride.

Cells	$(K)_i$	$(Na)_i$	i_K^L	$K_{1/2}$	$(i_{M_K^P}^P)_{K_o}^{max}$
HK "S"	80 mM	18 mM	0.01/hr	0.51 mM	0.48 mmole/(liter cells·hr)
LK 2582	12 mM	87 mM	0.10/hr	0.29 mM	0.13 mmole/(liter cells·hr)

Samples were taken at 15 and 75 min after addition of isotope. Kinetic parameters were obtained as in Fig. 3 *a*, except that one point was omitted from the analysis (the one at the upper border of the figure). If this point is included, $(i_{M_K^P}^P)_{K_o}^{max} = 0.14$ mmole/liter cells·hr, and $K_{1/2} = 0.33$ mM for the LK cells.

FIGURE 4 *a*. The activation of the K pump of high sodium HK and LK cells by $[K]_o$ in a sodium-free medium (Exp. 56 (HK), 61 (LK)). The cells were incubated in K-free

the shapes of the $[K]_o$ activation curves generally, seem to be independent of the internal cation composition. Since the difference in $K_{1/2}$ for $[K]_o$ exhibited by HK and LK red cells suspended in Na-free media persists in the absence of significant differences in the internal composition, it is reasonable to conclude that it is due to differences in the membrane components which constitute the pumps in these two genetically distinct types of cells.

The values of $(iM_{K/K_o}^P)^{\max}$ summarized in Table IV indicate that lowering the potassium content in both HK and LK cells stimulates the activity of the pump considerably. To examine this phenomenon more extensively, experi-

TABLE IV
EFFECT OF ALTERATION OF INTERNAL Na^+ AND K^+ ON THE
ACTIVE TRANSPORT CHARACTERISTICS OF
HK AND LK CELLS

Cell type	Na_i	Medium	$K_{1/2}$	$(iM_{K/K_o}^P)^{\max}$
	%		mM $[K]_o$	mmole/(liter cells·hr)
LK	87	Na	~3	0.24
LK	99+	Na	~3	0.38
LK	87	Mg	0.20	0.11
LK	99+	Mg	0.20	0.32
HK	17	Na	~3	0.84
HK	97+	Na	~3	2.78
HK	17	Mg	0.63	0.48
HK	97+	Mg	0.60	2.78

The data are a summary of the results presented in the figures.

ments were performed to characterize the K pump as a function of the relative magnitudes of Na_i and K_i (expressed in the figures as $[(Na)_i/(Na)_i + (K)_i] \times 100$, see Materials and Methods). An experiment of this type done in

PCMBS solution at 4°C for 23 hr (HK) and 13 hr (LK), then were incubated for 45 min at 37°C with DTT. Flux medium: 0.1–20 mM KCl, 15 mM Tris-Cl (pH 7.4, 37°C), 11 mM glucose, made isosmotic with $MgCl_2$.

Cells	$(K)_i$	$(Na)_i$	$i k_{K}^L$	$K_{1/e}$	$(iM_{K/K_o}^P)^{\max}$
HK 2572	<3 mM	—	0.02/hr	0.60 mM	2.78 mmole/(liter cells·hr)
LK 2582	<1 mM	110 mM	0.007/hr	0.20 mM	0.32 mmole/(liter cells·hr)

Samples were taken at 15 and 75 min after addition of isotope. LK controls, cold stored, but not treated with PCMBS or DTT, showed $(iM_{K/K_o}^P)^{\max}$ of 0.048 mmole/(liter cells·hr) and K_i of 0.24 mM $[K]_o$.

FIGURE 4 *b*. The Eadie plot of the data in Fig. 4 *a*. The lines were determined by a least square fit to give the kinetic parameters shown above.

Na medium with 5 mM $[K]_o$ is illustrated in Fig. 6. The HK pump showed a gradual, continuous increase over the entire range from 0 to 100% Na_i . The LK pump, on the other hand, was not significantly different from zero until Na constituted over 70% of the internal cation content, and then the pump showed a dramatic rise to reach a maximum value when K_i was reduced to

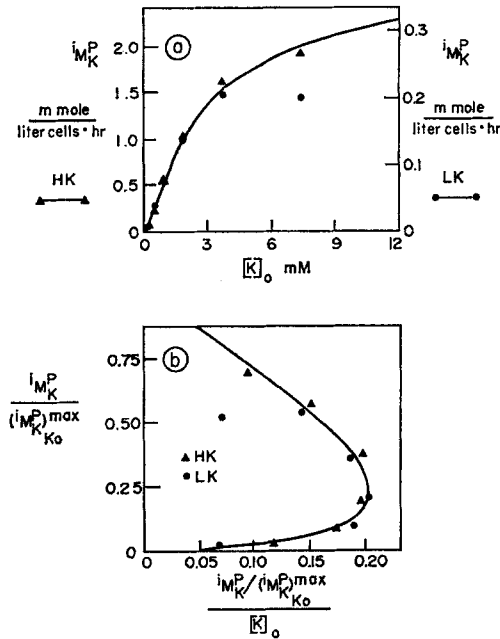


FIGURE 5

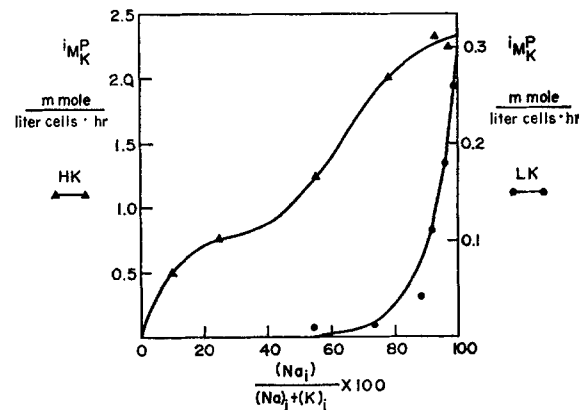


FIGURE 6

FIGURE 5 a. The activation of the K pump of high sodium HK and LK cells by $[K]_o$ in sodium medium (Exp. 77). The cells were incubated in K-free PCMBS solution at 4°C for 19 hr, then were incubated with DTT for 45 min at 37°C. Flux medium: 0.2–7.5 mM KCl, 135–128 mM NaCl, 15 mM Tris-Cl (pH 7.4, 37°C), 2 mM $MgCl_2$, 11 mM glucose, 1.0 g % bovine serum albumin.

Cells	$(K)_i$	$(Na)_i$	i_{K}^L	$K_{1/2}$	$(i_{M_K}^P)_{max_{K_o}}$
HK 2575	<3.0	90	0.01/hr	3	2.78 mmole/(liter cells · hr)
LK 2582	<1.5	95	0.12/hr	3	0.38 mmole/(liter cells · hr)

Samples were taken at 15 and 75 min after the addition of isotopes.

FIGURE 5 b. The Eadie plot of the data in Fig. 5 a. For details see text.

FIGURE 6. The dependence of the K pumps of LK and HK sheep red cells on $(Na)_i$ and $(K)_i$ (Exp. 66). Flux medium: 5 mM KCl, 130 mM NaCl, 2 mM $MgCl_2$, 15 mM Tris-Cl (pH 7.4, 37°C), 11 mM glucose, 0.1 g % bovine serum albumin. Cells: LK 187 $i_{K}^L = 0.042$ –0.054/hr; HK 177 $i_{K}^L = 0.014$ /hr. Samples were taken 15 and 75 min after addition of isotope. The curves were drawn by eye using these and additional data.

less than 1% (i.e., $(K)_i < 1$ mM). These findings suggest a major difference in the mechanism of control of the two types of pump which, in this case, cannot be due to a difference in cation concentration on the *trans* side (outside) of the membrane.

It will be recalled that the conclusion drawn from the data summarized in Table IV was that the shapes of the curves relating iM_K^P to the composition of the external medium were independent of the internal K and Na composition over the range tested. If it is rigorously true that the activation of the pump by

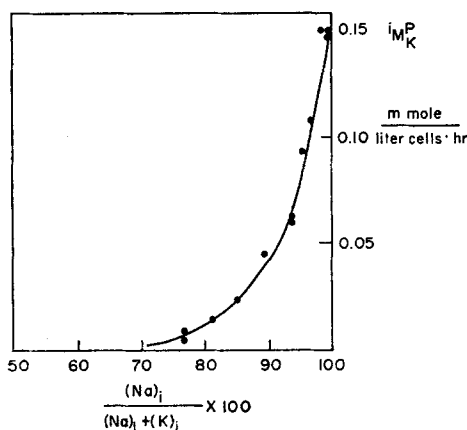


FIGURE 7

FIGURE 7 The dependence of the K pump on LK cells on $(Na)_i$ and $(K)_i$ in Na-free medium (Exp. 64). Flux medium: 1.7 mM KCl, 15 mM Tris-Cl (pH 7.4, 37°C), 11 mM glucose, made isosmotic with $MgCl_2$. $i k_K^L = 0.09$ – 0.11 /hr. Samples were taken at 15 and 75 min after the addition of isotope. Curves were drawn as indicated in text.

FIGURE 8 The dependence of the (K + Na) pump of HK cells on $(Na)_i$ and $(K)_i$ in Na-free medium (Exp. 40). Flux medium: 0.5 or 5.0 mM KCl, 15 mM Tris-Cl (pH 7.4, 37°C), 11 mM glucose, made isosmotic with $MgCl_2$. $i k_M^L = 0.02$ /hr. Samples were taken at 45 and 125 min. Curves were drawn as indicated in text.

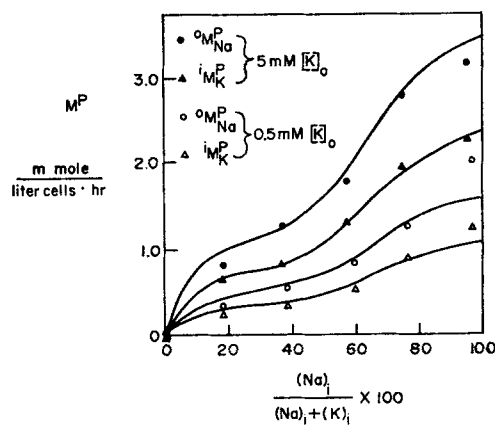


FIGURE 8

external ions is independent of the internal composition, it is a theoretical general consequence (see Discussion) that the shape of curves relating iM_K^P to Na_i must be independent of the composition of the medium. In other words, the HK and LK curves in Fig. 6 should be identical to those obtained in other media if the ordinate scale is multiplied by some constant factor. That this expectation was confirmed by observation is shown in Fig. 7, which represents an experiment performed on LK cells suspended in a magnesium medium containing 1.7 mM $[K]_o$, in contrast to the sodium medium with 5 mM $[K]_o$ used in the experiment shown in Fig. 6. The curve used to describe the data is the LK curve in Fig. 6 multiplied by 0.5.

Fig. 8 illustrates a similar experiment on HK cells. In this case, however, magnesium media containing two different concentrations of $[K]_o$ (0.5 and 5.0

mm) were used, and simultaneous measurements of ${}^iM_K^P$ and ${}^oM_{Na}^P$ were made. The HK curve in Fig. 6, multiplied by constant factors, has been used to fit the data. The factors used were 1.0 and 1.5, respectively, for the ${}^iM_K^P$ and ${}^oM_{Na}^P$ curves obtained at 5 mM K_o , and 0.45 and 0.88, respectively, for ${}^iM_K^P$ and ${}^oM_{Na}^P$ measured at 0.5 mM K_o . The calculated Na/K pump ratio was 1.51 ± 0.25 (SD) for the data obtained at 5 mM $[K]_o$, and 1.37 ± 0.25 for the fluxes at 0.5 mM $[K]_o$.

All of the flux data presented above were measured isotopically. The assumption has been made that ouabain specifically inhibits active, as opposed to passive, K transport. There is evidence for this assumption in many mammalian red cells, including normal HK and LK sheep red cells (Tosteson and Hoffman, 1960). Since some of the pump fluxes reported here are more than three times as great as any that have been reported previously for sheep red cells, and since the actions of ouabain on Na and K transport in human red cells are known to be complex (Hoffman, 1969 and Glynn, 1957), it was thought advisable to reexamine the relation between the ouabain-sensitive unidirectional potassium influx and the energy-requiring, net active transport of Na out of and K into, sheep red cells.

The data plotted in Fig. 6 are the ouabain-sensitive K influxes. The HK ouabain-insensitive K influx was constant at 0.07 ± 0.02 mmole/(liter cells · hr) over the entire range of internal ion composition investigated. The ouabain-insensitive K influx in LK cells in this particular experiment increased monotonically from 0.21 to 0.27 mmole/(liter cells · hr) as Na_i was raised from 55 to 99.6%. In both cases, therefore, the increase in ouabain-sensitive influx reflected an increase in the total K influx. Since the increase in K influx was observed as internal K was diminished, the possibility that the increase was due to an ouabain-sensitive K exchange seems unlikely.

Fig. 9 *a* shows that HK cells which were made high in Na_i , but in which the gradient for K influx was still positive (i.e. $[K]_i > [K]_o$), showed a rate of ouabain-sensitive net accumulation of K which was similar to that measured isotopically in cells of the same composition (Fig. 6). Fig. 9 *b* shows the net Na efflux into magnesium medium measured simultaneously on the same cells. It can be seen that Na efflux was also largely inhibited by ouabain, and since the medium was sodium "free," the possibility of exchange diffusion can be ruled out. The pump fluxes obtained in a parallel experiment in which $[K]_o$ was 10 mM instead of 5 mM are summarized in Table V. Note that the increase in $[K]_o$ increased the pump fluxes only slightly, a result consistent with the kinetic characteristics described in Fig. 4. Table V also gives the calculated Na/K pump ratios. Under the experimental conditions, it can be shown that the error introduced by approximating unidirectional K and Na fluxes by net fluxes was less than 2%. The ratios given are compatible with a two potassium for three sodium pump exchange such as has been frequently described for human

TABLE V
NET K AND Na PUMP FLUXES IN HIGH Na HK CELLS

[K] _o , mμ	[Na] _o = 0 mM	(K) _i = 12-18 mM
	5	10
${}^iM_K^P$, mmole/(liter cells·hr)	1.87	1.96
${}^oM_{Na}^P$, mmole/(liter cells·hr)	2.87	2.86
$\frac{{}^oM_{Na}^P}{{}^iM_K^P}$	1.54	1.46

The data represent the results of single representative experiments. Fluxes were determined by analysis of K and Na in cells and supernatant of nine samples in duplicate taken over 3 hr. Fluxes were computed by least square regression lines (see legend of Fig. 9, Exp. 55).

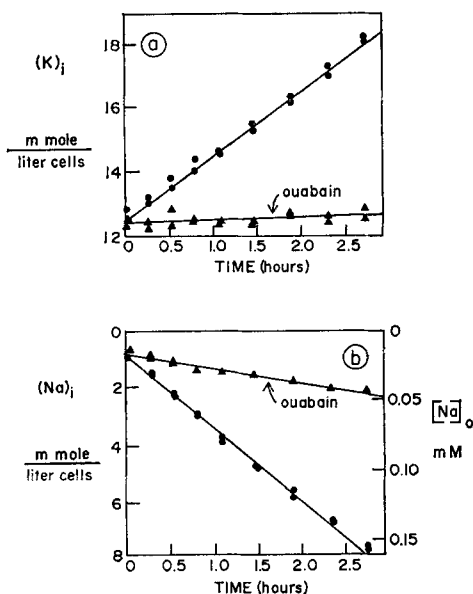


FIGURE 9

FIGURES 9 *a* and 9 *b*. Net Na and K fluxes in high sodium HK cells suspended in Na-free medium with and without ouabain (Exp. 55). Flux medium: 5 mM KCl, 15 mM Tris-Cl (pH 7.4, 37°C), $\pm 10^{-4}$ M ouabain, made isotonic with MgCl₂. Net changes in (K)_i; (Fig. 9 *a*) were calculated from the potassium and hemoglobin content of washed cells. Net changes in (Na)_i were calculated from the appearance of sodium in the supernatant, and the hemoglobin content of the whole suspension. No hemolysis was detected during the flux period.

FIGURE 10. Activation of K and Na pump fluxes in high sodium HK cells in an Na-free medium (Exp. 39). Flux medium: 0.2-2.4 mM KCl, 15 mM Tris-Cl (pH 7.4, 37°C), 11 mM glucose made isotonic with MgCl₂. The cells contained less than 3 mM (K)_i, and approximately 88 mM (Na)_i. ${}^oM_{Na}^L = 0.8-1.1$ mmole/(liter cells·hr) and ${}^iM_K^L \approx 0.02$ /hr. Samples were taken at 40 and 160 min after addition of ⁴²KCl.

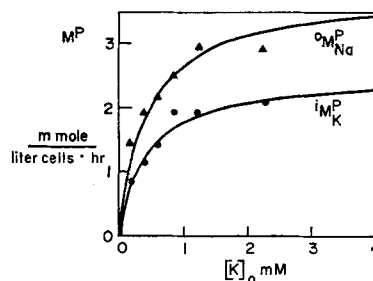


FIGURE 10

erythrocytes. The Na/K pump ratio for sheep red cells with normal cation composition, however, has previously been reported to be nearer 1:1 (Tosteson and Hoffman, 1960).

To provide additional evidence that the pump transport of Na and K were coupled in this system, simultaneous isotopic determinations of the dependence of $^{\circ}M_{Na}^P$ and $^iM_K^P$ on $[K]_o$ in high sodium HK cells were obtained. The results are shown in Fig. 10. The experiment was performed in magnesium medium

TABLE VI
PUMP FLUXES AND ATP HYDROLYSIS IN HIGH Na AND
IN NORMAL HK SHEEP RED CELLS

[Na] _o = 0 mM		[K] _o = 10mM			
(K) _i	$^iM_K^P$	$^{\circ}M_{Na}^P$	M_{ATP}^P	M_{ATP}^{ouab}	M_{ATP}^P
mmole/liter cells		mmole/liter cells·hr			
<2	2.0	2.9	0.67	0.20	0.47
84	0.51	—	0.32	0.26	0.06

HK 180. Cells were prepared in the appropriate PCMBs solutions according to the standard procedure (see Materials and Methods). The DTT reversal solution contained 11 mM glucose. The cells were washed and resuspended in a magnesium medium containing 15 mM Tris-Cl (pH 7.4, 37°C), $\pm 10^{-4}$ M ouabain, but no glucose. After 30 min of incubation, duplicate samples were taken for analysis, and 10 mM K was added to the flasks. Three additional samples (in duplicate) were taken at 30-min intervals (high sodium cells) or at 1-hr intervals (cells with normal cations). K influx in normal cell was taken from Exp. 62 (Fig. 2). High sodium cell K influx was measured by net changes in cell K. Na efflux was measured by accumulation of Na in the supernatant. ATP hydrolysis was estimated by taking the least square slope of the line determined by the experimental points (see Materials and Methods for analytic procedure). Initial ATP content was 1.2 and 1.0 mmole/liter cells for the normal and high Na cells, respectively (Exp. 51).

to avoid Na exchange diffusion (Tosteson and Hoffman, 1960). The curves drawn through the two sets of points are Michaelis-Menten curves which differ only in that the pump maximum for Na was assumed to be 1.5 times that for K. The mean ratio and standard deviation of the corresponding Na and K pump determinations was 1.54 ± 0.16 (SD).

The maximum ouabain-sensitive fluxes in both HK and LK cells occurred when the electrochemical potential of K (Tosteson and Hoffman, 1960) was greater outside than inside the cell, and when the potential of sodium was either greater inside the cell (in Na-free media) or approximately equal in the two compartments. Since under these conditions increased fluxes do not necessarily represent an increased energy expenditure, it was possible that the rate of ATP hydrolysis was unaffected by the increased pump activity. Table VI indicates that increases in ouabain-sensitive fluxes in HK cells in sodium-free

media were accompanied by an increased rate of ATP hydrolysis, even though these fluxes represent no thermodynamic work. It should be noted that although no external energy-supplying substrate was provided in this experiment, some ATP synthesis may have continued at the expense of intracellular compounds. One experiment in which adenosine diphosphate (ADP) was estimated indicated that loss of ATP was not accompanied by accumulation of ADP. This suggests that considerable ATP may have been produced by the adenylate kinase reaction. As a result, the ouabain-sensitive ATP hydrolysis indicated in Table VI is a minimal estimate.

Because LK cells have a considerably greater ouabain-insensitive K influx, and since, even when stimulated by decreasing K_i, their ouabain-sensitive fluxes are relatively small, acceptable measurements of net fluxes, ouabain-sensitive Na efflux, and ATP hydrolysis were not made in these cells.

DISCUSSION

In this discussion, we will first review the data indicating differences in the pump kinetic characteristics of HK and LK sheep red cells and compare these properties with those of the human red cell pump. Second, these observations will be interpreted in terms of the turnover and kinetic properties of individual pump sites in these cells. Third, those features which are apparently common to both sheep and human red cell pumps will be discussed. Fourth, the theoretical implications of these experiments for a more adequate model of alkali metal transport will be developed.

Differences in the (Na + K) Pump in Sheep and Human Red Cells

Table VII provides a summary of some of the kinetic characteristics of LK and HK sheep red cell pumps as well as the available corresponding data obtained by other investigators using human red cells. The difference in ($'M_K^P$)_{K_o}^{max} in HK and LK sheep red cells has been observed previously (Tosteson and Hoffman, 1960; Dunham and Hoffman, 1969). Once this difference in absolute magnitude is considered, however, the shapes of the HK and LK curves relating $'M_K^P$ to [K]_o in Na medium are indistinguishable (Fig. 1). This result is consistent with the hypothesis that the greater pump rate observed in HK cells is due only to the presence of a larger number of pump sites in the membranes of HK than of LK cells. However, two lines of evidence have been presented to show that this is not the case.

First, we have demonstrated that in Na-free media the apparent affinity of the pump for [K]_o in the two types of cells, as measured by the K_{1/2}, is different (0.20 ± 0.05 [SD] mM and 0.6 ± 0.1 mM in LK and HK cells, respectively) when measured in magnesium medium. By also demonstrating a similar difference in K_{1/2} for [K]_o in two other types of Na-free media, evidence was

provided that this finding reflects a difference in pump-K interactions, rather than in pump-Mg interactions in the two cell types. Moreover, since the addition of Na to the medium obliterates this distinction between HK and LK

TABLE VII
A SUMMARY OF KINETIC CHARACTERISTICS OF THE K PUMP OF HK,
LK, AND HUMAN RED CELLS

Characteristic	LK	HK	Human
Cells with normal internal cation composition			
Na _i , %	~88	~15	~8
(iM_K^E) _{K_o} ^{max} , mmole/(liter cells·hr)	0.24	0.84	1.66*
Shape of [K] _o activation curve in Na medium	Sigmoid	Sigmoid	Sigmoid*
K _{1/2} for [K] _o in Na medium, mM	~3	~3	~2*
Shape of [K] _o activation curve in Mg medium	M-M	M-M	M-M†
K _{1/2} for [K] _o in Mg medium, mM	0.2	0.6	0.1‡
Ratio (iM_K^E) _{K_o} ^{max} in Na and Mg media	~2.2	~1.8	~2.0‡
Na/K pump ratio	~1.1§	~1.2§	~1.3
Response to changes in Na _i			
Shape of Na _i activation curve	See Fig. 6	See Fig. 6	~M-M¶
Na _i at half activation, %	~93	~55	~15¶
Minimum Na _i for detectable pump, %	~70	~0	~0¶
High Na cells			
Na _i , %	>99	>97	~90
(iM_K^E) _{K_o} ^{max} , mmole/(liter cells·hr)	0.38+	2.8	7.2¶
Shape of [K] _o activation curve in Na medium	Sigmoid	Sigmoid	Sigmoid**
K _{1/2} for [K] _o in Na medium, mM	~3	~3	~3¶
Shape of [K] _o activation curve in Mg medium	M-M	M-M	?
K _{1/2} for [K] _o in Mg medium, mM	0.2	0.6	?
Na/K pump ratio	?	1.5	1.5¶

The data shown in this table were taken from experiments repeated in this paper except where indicated by a footnote.

* Sachs and Welt, 1967.

† Sachs, 1967.

§ Tosteson and Hoffman, 1960.

|| Garrahan and Glynn, 1967 *b*.

¶ Post et al., 1960.

** Maizels, 1968.

pumps, it can be concluded that the relative affinities of the HK and LK pumps for [Na]_o are also different.

Second, the data present in Fig. 6 indicate a striking difference in the curves relating iM_K^E to Na_i. It will be recalled that, as the shape of neither the in-

ternal nor the external activation curve was altered by changes in ion concentration on the opposite side of the membrane, these findings are independent of the large difference in internal composition found in fresh HK and LK cells.

In human red cells, the $K_{1/2}$ of the pump for $[K]_o$ in magnesium medium is 0.10 ± 0.01 mM (Sachs, 1967), and the curve relating ${}^iM_K^p$ to Na_i is reasonably well described by a Michaelis-Menten curve with half maximal stimulation being reached at $Na_i = 15\%$ (Post et al., 1960). All three cell types, therefore, show pump kinetics which differ from each other not only in maximum pump rate, but also in their apparent affinities for external and internal Na and K. While variation in numbers of independent pump sites among different types of cells can account for differences in rates, they clearly cannot account for differences in apparent ion affinities.

Interpretation of Differences in K-Na Transport in HK and LK Sheep Red Cells in Terms of Turnover and Kinetic Properties of Individual Pump Sites

For more than a decade the technique of ouabain binding has been investigated as a means of estimating the number of pump sites on individual cells (Glynn, 1957; Dunham and Hoffman, 1969; Hoffman, 1969; Baker and Willis, 1970). Some of the recent developments in this technique are given by Hoffman (1969) and will not be discussed here. The fundamental assumption made in all estimates of the number of pump sites by the ouabain-binding method is that each site can bind one and only one ouabain molecule and that such binding constitutes a necessary and sufficient condition for inhibition of that site. To our knowledge, there is no evidence against the possibility that pumps in different cells bind different numbers of ouabain molecules. Moreover, the possibility that the number of ouabain molecules bound per site is a function of the internal ion composition of various cells has not been rigorously excluded.

The most frequently cited indirect evidence for the validity of the hypothesis that one and only one ouabain molecule reacts with and inhibits one pump site is that the ratio of pump activity to ouabain binding is similar in a wide variety of cells (Baker and Willis, 1970). This result would be expected if the 1:1 assumption is correct and the turnover numbers of the sites in the cells compared are the same. The pump activity used in this ratio is generally obtained by measuring the pump rate in fresh cells suspended in media containing a near-saturating concentration of K. Under these conditions, Dunham and Hoffman found the HK/LK ratio of pump rates to be 8.4 as compared to the HK/LK ouabain-binding ratio of 6.3, thus indicating the HK and LK cells have approximately the same turnover number (K ions pumped per site per minute).

Two objections can be raised to this and similar correlations. First, while

care is usually taken to assure that the cells are compared in similar suspending media, no consideration is taken of the fact that the Na_i in different types of cells varies considerably. Since pump rate is known to be a function of internal as well as external ions, one criterion for an adequate comparison of pump rates is that the ratio should be determined at the same internal ionic composition.

Second, similarity of internal composition alone is inadequate to insure a valid comparison of pump rates in different types of cell. cursory examination of the HK and LK curves in Fig. 6 shows that the HK/LK pump ratio depends strongly on which common value of Na_i is chosen for the comparison. Perhaps the "most valid" comparison can be made at the Na_i at which both HK and LK pumps are maximally activated, that is, when K_i is reduced to very low levels. Under these conditions, we have obtained HK/LK pump ratios between 5:1 and 9:1 which are quite similar to the HK/LK ratio of ouabain-binding sites determined by Dunham and Hoffman and which are consistent with the conclusion that turnover numbers are the same, i.e. approximately $2.4 \times 10^4/\text{min}$ for both HK and LK sites (calculated with site estimate of Dunham and Hoffman, the maximum pump rate in high sodium cells from Table VII, and assuming a mean cell volume of $35 \mu^3$). This is similar to the $3 \times 10^4/\text{min}$ value which can be calculated for high sodium human red cells (using the maximum pump rate in Table VII) a site per cell estimate of 200 (Hoffman and Ingram, 1969; Keynes and Ellory, 1969), and a volume of $82 \mu^3/\text{cell}$. However, these estimates of turnover numbers depend on measurements of ouabain binding carried out on HK and LK cells with normal internal ion composition and must be considered tentative.

We conclude that the data presented here demonstrate unequivocally that the kinetic characteristics of individual pump sites are different in HK, LK, and human red cells. However, in view of the considerations set out above, the question of whether the differences in maximal pump rates among the cells are due to differences in the number of pump sites per unit membrane or in the turnover numbers of each site cannot be answered with certainty until the number of sites can be measured directly.

Similarities Among the (Na + K) Pumps of Sheep and Human Red Cells

In the preceding sections some of the differences in HK and LK sheep red cells have been considered. If nothing else, the uncertainty involved in extrapolating results obtained in one tissue to other tissues of the same, or a different, species has been demonstrated. The recognition of differences, however, implies that there exists a substrate of similarity against which the differences become apparent. It is not particularly surprising that the (K + Na) pump, in HK and LK sheep red cells are quite similar. Of more interest perhaps is

the question of what characteristics common to both HK and LK pumps are also common to the pump of human red cells.

As was shown in Fig. 1 for both HK and LK cells, the activation of the pump by $[K]_o$ in a sodium medium cannot be described by the Michaelis-Menten equation. The curve presented in the Eadie plot indicates that the maximum value of $^iM_K^p/[K]_o$ occurs not as $^iM_K^p$ and $[K]_o$ approach zero, but rather at some intermediate point. This characteristic of the Eadie plot is equivalent to finding that a Lineweaver-Burk plot of the data is convex, or that the direct plot of $^iM_K^p$ versus $[K]_o$ is sigmoid. It has been shown by Sachs and Welt (1967) and by Garrahan and Glynn (1967 *a*) that the $[K]_o$ activation curve is sigmoid for the human red cell pump.

The $K_{1/2}$ for $[K]_o$ for the HK pump in sodium media is about 3 mM. The range of values obtained for normal HK cells under these conditions was 2.5–3.8 mM, using the method of fitting a Lineweaver-Burk plot of the data to a second-order polynomial by least square regression. The variation in $K_{1/2}$ was largely due to uncertainty in $(^iM_K^p)_{K_o}^{max}$. The range of values obtained for the $K_{1/2}$ of the LK pump was even greater due to the large ouabain-insensitive influx of K, and the relative smallness of the pump rate. However, as shown in Fig. 1, the HK and LK data are equally well approximated by a single curve, once the difference in absolute pump rates is taken into account. It should be emphasized that the assigning to both pumps of a $K_{1/2}$ of approximately 3 mM $[K]_o$ (Table II), was meant to indicate that no difference in the $K_{1/2}$'s had been demonstrated, not that they were necessarily identical.³ Sachs and Welt (1967) used the same method of fitting curves to Lineweaver-Burk plots of their human red cell data. From the constants which they derived from their plots, the $K_{1/2}$ for human cells in the presence of high $[Na]_o$ can be calculated to be approximately 1.9 mM $[K]_o$. Garrahan and Glynn (1967 *a*) found $K_{1/2}$ to be 1.3–1.5 mM $[K]_o$ for human cells under similar conditions. These data would seem to indicate that the $K_{1/2}$ for $[K]_o$ in sodium media is somewhat lower in human than in sheep red cells. Considering the wide variation in data available for both species, however, it seems premature to conclude that such a difference has been proven.

When the pump activation by $[K]_o$ is studied in sodium-free media, the data for both HK and LK cells are adequately fitted to a linear Eadie plot. Sachs (1967) has also found this to be the case for the human red cell pump in a magnesium medium. Garrahan and Glynn (1967 *a*) used a choline medium in similar experiments and agreed that the activation curve was well approximated by the Michaelis-Menten equation, but suggested that a "slight inflexion" may occur at 0.015 mM $[K]_o$. In magnesium medium, therefore, all

³ P. Dunham has recently determined the $K_{1/2}$ for both HK and LK cells in Na media to be 3 mM $[K]_o$ (personal communication).

three cell types show pump kinetics which are described by Michaelis-Menten kinetics. The $K_{1/2}$'s, however, are different for all three cell types, as was mentioned in the preceding section.

The depression of $({}^iM_K^P)_{K_o}^{\max}$ observed in sheep cells in magnesium medium as opposed to sodium medium is also evident in Sachs's data on human cells, although it was not commented upon. The reductions calculated from Table III for LK and HK sheep cells and from Sachs's data on human cells are 54%, 43%, and 50%, respectively.

Models of the (Na + K) Pump

Of more fundamental importance than any of the preceding similarities, we believe, is the demonstration that the shapes of the internal and external activation curves are independent in both types of sheep cells under all of the conditions tested thus far. Obviously, further investigations are necessary to evaluate whether or not the independence of the activation of the pump by ions on the *cis* and *trans* sides of the membrane is characteristic of sheep (and human, etc.) cells under all conditions. Nonetheless, on the basis of the evidence presented here, it seems worthwhile to consider the implications of the rigorous independence of *cis* and *trans* activation curves as a criterion for models of the (Na + K) pump.

Independence of activation curves can be defined explicitly as follows. If the ratio of the fluxes (M) measured at any two different external ion concentrations (O_j and O_k), but at the same internal ion concentration, is independent of the internal ion concentration (I_j or I_k) at which the ratio is determined, then the external activation curve is independent of the internal ion concentrations. In mathematical form,

$$\frac{M(O_1, I_1)}{M(O_2, I_1)} = \frac{M(O_1, I_2)}{M(O_2, I_2)} \quad (2)$$

Rearrangement of equation 2 to yield

$$\frac{M(O_1, I_1)}{M(O_1, I_2)} = \frac{M(O_2, I_1)}{M(O_2, I_2)} \quad (3)$$

indicates the tautology that if the external activation curve is independent of the internal ions, then the internal activation curve is independent of the external ions. Stated another way, if the independence condition defined in equation 2 applied to K-pump fluxes in sheep red cells, then

$${}^iM_K^P = f(O) \times g(I) \quad (4)$$

where $f(O)$ is a function of external (and *not* internal) Na and K, and $g(I)$ is a function of internal (and not external) Na and K concentration. Equation 4

may be more easily related to the experimental results if we define $(iM_K^P)_{I,O}^{\max}$ as the pump rate observed when $f(O) = f(O)^{\max}$ (e. g. saturating $[K]_o$ in high $[Na]_o$ medium) and $g(I) = g(I)^{\max}$ (e. g. $Na_i = 100\%$). In addition if $F(O) = f(O)/f(O)^{\max}$ and $G(I) = g(I)/g(I)^{\max}$ for all values of (O) and (I) , then equation 4 can be rewritten as

$$iM_K^P = (iM_K^P)_{I,O}^{\max} \times F(O) \times G(I). \quad (5)$$

Given this formulation, $G(I)$ is described for HK and LK cells by the curves given in Fig. 6, except that the vertical scale, instead of being expressed in millimoles per liter of cells per hour, is in fraction of maximum activation, with a maximum value of 1.0 when $Na_i = 100\%$. Similarly, $F(O)$ is described

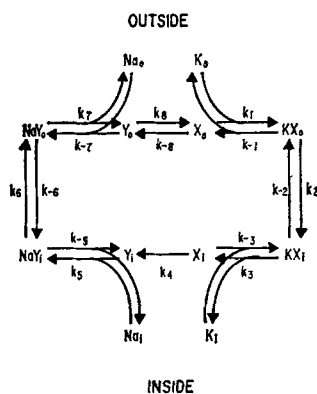


FIG. 11 a. Scheme of the Shaw model, modified from Glynn (1956). See text for discussion.

by curves like those in Figs. 1, 2, 4, and 5 with a similar change in the vertical scale. Thus, if the dependence of iM_K^P on the external Na and K is known for any one combination of internal Na and K, and if the dependence of iM_K^P on internal Na and K are known for any one combination of external Na and K, then the independence condition as stated in equation 5 makes it possible to predict iM_K^P for any arbitrary combination of internal and external Na and K.

Of equal interest is the fact that pumps which demonstrate kinetics described by equations 4 and 5 appear to be incompatible with many of the previously proposed models for the $(Na + K)$ -transport system. Most commonly encountered models of the $(Na + K)$ pump are based on the scheme which Shaw (1954) developed to account for the coupling of the transport of Na and K, and the permeability of the process with respect to $[K]_o$ (Fig. 11 a). While most of these models are of the carrier-mediated transport type, it is important to realize that, kinetically, they are indistinguishable from any number of models which postulate a sequential reaction of the pump mecha-

nism with first one of the substrate cations and then the other. For example, a model which postulated that the pump consists in part of a channel through the membrane which first allows diffusion of K inward and then Na outward with an obligatory change in state after the net passage of a stoichiometric number of each ion variety, yields kinetic equations of similar mathematical form (see Patlak, 1957). We will, therefore, define sequential models as those which postulate that the pump exists in at least two states: one which is capable of transporting K and another which is capable of transporting Na. Coupling of the sequential pump model is achieved by requiring that an obligatory change of state occurs with the net transport of a stoichiometric number of ions of one type. Defined in this manner, sequential models include the semi-physical models of Post et al. (1969), Jardetzky (1966), and Opit and Charnock (1965), as well as the mathematical models of Shaw and Glynn (see Glynn and Lew, 1969).

The mathematics of sequential bisubstrate reaction models of enzyme reactions was investigated by Cleland (1963), who found that a scheme identical with the Shaw model (the Tetra-Iso-Ping-Pong-Bi-Bi-mechanism) yielded activation curves which were highly interdependent, the $K_{1/2}$ for one substrate becoming independent of the second substrate concentration only when the latter was present in saturating concentrations. Baker and Stone (1966) discussed theoretical kinetic features of various models. They pointed out that, in general, sequential models are incompatible with equation 4. Moreover, schemes which they present as being compatible with independence of *cis* and *trans* pump activation curves require that one of the substrates can never be saturating, i.e. $(M_K^P)_{i,0}^{\max}$ is infinite.

While space does not permit presentation of our general kinetic analysis of sequential models, we will show briefly how the example shown in Fig. 11 *a* is compatible with the independence criterion only under certain very special conditions. To do this the equations describing the model shown in Figs. 6 and 11 *a* are solved by the steady-state method of King and Altman (1956). The small *k*'s represent rate coefficients which are not functions of Na or K concentrations but may be functions of other parameters (i.e., ATP) which will be considered constant. Positive and negative subscripts designate forward (clockwise) and backward (counterclockwise) coefficients, respectively, while the numerical value of the subscript indicates the reaction step (Fig. 11 *a*). Two assumptions are made to simplify the mathematics:

- (a) $[K]_i$ and $[Na]_o$ are constant;
- (b) step 4 is irreversible (e.g., because ATP is a reactant).

These assumptions do not affect the conclusions which will be drawn, and they greatly reduce the complexity of the equations.

Given the above limitations, the model in Fig. 11 *a* yields an equation for the net transport of K or Na (M^p) of

$$M^p = \frac{A[\text{K}]_o[\text{Na}]_i}{B[\text{K}]_o[\text{Na}]_i + C[\text{K}]_o + D[\text{Na}]_i + E} \quad (6)$$

where

$$A = k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8,$$

$$B = k_1 k_6 (k_2 k_3 k_4 [k_{-7}[\text{Na}]_o (k_{-6} + k_6) + k_{-6} k_8 + k_7 k_8 + k_6 k_8 + k_6 k_7] + k_6 k_7 k_8 [k_{-3}[\text{K}]_i (k_{-2} + k_2) + k_{-2} k_4 + k_3 k_4 + k_2 k_4 + k_2 k_3]),$$

$$C = k_1 k_2 k_3 k_4 (k_{-5} k_{-6} k_{-7} [\text{Na}]_o + k_{-5} k_{-6} k_8 + k_{-5} k_7 k_8 + k_6 k_7 k_8),$$

$$D = k_6 (k_{-7} [k_{-6} k_{-8} + k_6 k_{-8} [\text{Na}]_o] + k_6 k_7 k_8 + k_6 k_7 k_{-8}) (k_{-1} k_{-2} k_{-3} [\text{K}]_i + k_{-1} k_{-2} k_4 + k_{-1} k_3 k_4 + k_2 k_3 k_4),$$

$$E = k_{-5} k_{-6} k_{-7} k_{-8} [\text{Na}]_o (k_{-1} k_{-2} k_{-3} [\text{K}]_i + k_{-1} k_{-2} k_4 + k_{-1} k_3 k_4 + k_2 k_3 k_4).$$

If the equation 6 is to be compatible with equations 4 and 5, the denominator of the equation 6 must be of the form $f'(O)g'(I)$.

Since there are no higher order terms in the denominator, the general solution is that

$$f'(O) = a[\text{K}]_o + b \quad \text{and} \quad g'(I) = c[\text{Na}]_i + d,$$

and

$$M^p = A \left(\frac{[\text{K}]_o}{a[\text{K}]_o + b} \right) \left(\frac{[\text{Na}]_i}{c[\text{Na}]_i + d} \right). \quad (7)$$

The conditions which allows equation 6 to be written in the form of equation 7 are that $B = ac$, $C = ad$, $D = bc$, and $E = bd$. Thus the necessary and sufficient condition for equation 6 to be compatible with equations 4 and 5 for all values of $[\text{Na}]_i$ and $[\text{K}]_o$ is that

$$BE = CD = abcd, \quad (8 a)$$

or, in expanded terms, that

$$\begin{aligned} & (k_{-5} k_{-6} k_{-7} k_{-8} [\text{Na}]_o) (k_{-3} (k_{-2} + k_2) [\text{K}]_i + k_{-2} k_4 + k_2 k_3 + k_2 k_4 + k_3 k_4) \\ & = k_2 k_3 k_4 (k_{-7} (k_{-5} k_{-6} + k_{-5} k_{-8} + k_{-6} k_{-8} + k_6 k_{-8}) [\text{Na}]_o + k_{-5} k_{-6} k_8 \\ & \quad + k_{-5} k_{-6} k_{-8} + k_{-5} k_7 k_8 + k_{-5} k_7 k_{-8} + k_6 k_7 k_{-8} + k_6 k_7 k_8). \end{aligned} \quad (8 b)$$

Substituting the constants F , G , H , I , and J for the combinations of the k_i 's in 8 *b*, we obtain

$$F[\text{Na}]_o (G[\text{K}]_i + H) = I[\text{Na}]_o + J. \quad (8 c)$$

The constant J must be positive definite because no values of $k_{\pm i}$ can be negative and no value of k_{+i} can be zero and still permit forward operation of the cycle. Hence, the model in Fig. 11 *a* conforms to the independence criterion only if $[\text{Na}]_o$ is not zero. Moreover, equation 8 is satisfied only if a special relationship exists between $[\text{Na}]_o$ and $[\text{K}]_i$ and the rate coefficients.

Thus, our investigation of the kinetic characteristics of sequential models has found that mathematical solutions do exist which provide for independence of internal and external activation curves. These solutions require that physically unlikely, fortuitous relationships must obtain among the rate coefficients of the various reactions in the sequence, and the concentrations of

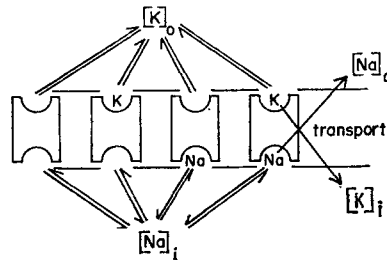


FIGURE 11 *b*. Four possible states of the rapidly equilibrating random Bi-Bi pump mechanism is represented in a patch of cell membrane. Each pump-ion complex is in equilibrium with the ions in bulk solution on each side of the membrane. Whether or not the *cis* ion site is occupied has no effect on the affinity of the *trans* site for its substrate ion. The fraction of the sites not in equilibrium with the ions in the *cis* and *trans* solutions (e.g. those in the process of transporting ions) must be negligible. The rate of transport is proportional to the number of pumps associated with the appropriate ions on both the internal and external surfaces.

external Na and internal K. Moreover, independence requires the presence of external Na. These theoretical requirements are not compatible with the experimental data on sheep red cells. Evidence provided above (e.g. Fig. 8) is consistent with the conclusion that the pump fluxes of Na and K were independent both in the presence and absence of external Na. The absence of significant K-K exchange was indicated by Tosteson and Hoffman (1960), and more recently by Ellory and Lew (1970). In the light of these considerations, we believe that it is unlikely that the (K + Na) pump in sheep red cell operates by a sequential mechanism.

Certain models which are not sequential can conform with the requirement for independence of activation of the pump by *cis* and *trans* ions (equation 4). A scheme of this type is the rapidly equilibrating random bi bi mechanism of Cleland (1963). This mechanism is illustrated in Fig. 11 *b*. It postulates that the pump mechanism includes separate receptor sites for $[\text{K}]_o$ and $[\text{Na}]_i$, and that the number of *cis* sites which are loaded is independent of the *trans* ion

concentration. The rate of the actual transport step is proportional to the fraction of pump sites which are loaded with appropriate ions on both the *cis* and *trans* surfaces. This model yields a rate equation of the form of equation 7 if the rate constant for the transport step is much smaller than the rate constants for the dissociation of the ions from the receptor sites.

Previously proposed models which are of this type are the induced electron transfer model of Skou (1957) and the rotator model of Hoffman (1961). It is easy to incorporate into these models such features as multiple sites for $[Na]_o$ and $[K]_o$, and competition between these ions and $[K]_i$ and $[Na]_i$. If such features are included, activation curves which are indistinguishable from those observed in HK and LK sheep cells as well as in human red cells may be obtained.

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