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Regulation of Cell Volume by Active Cation Transport in High and Low Potassium Sheep Red Cells

D. C. TOSTESON and J. F. HOFFMAN

From the Laboratory of Kidney and Electrolyte Metabolism, National Heart Institute, Bethesda. Dr. Tosteson's present address is Department of Physiology, Washington University School of Medicine, St. Louis

ABSTRACT A model cell which controls its cation composition and volume by the action of a K-Na exchange pump and leaks for both ions working in parallel is presented. Equations are formulated which describe the behavior of this model in terms of three membrane parameters. From these equations and the steady state concentrations of Na, K, and Cl, values for these parameters in high potassium (HK) and low potassium (LK) sheep red cells are calculated. Kinetic experiments designed to measure the membrane parameters directly in the two types of sheep red cells are also reported. The values of the parameters obtained in these experiments agreed well with those calculated from the steady state concentrations of ions and the theoretical equations. It is concluded that both HK and LK sheep red cells control their cation composition and volume in a manner consistent with the model cell. Both have a cation pump which exchanges one sodium ion from inside the cell with one potassium ion from outside the cell but the pump is working approximately four times faster in the HK cell. The characteristics of the cation leak in the two cell types are also very different since the HK cells are relatively more leaky to sodium as compared with potassium than is the case in the LK cells. Both cell types show appreciable sodium exchange diffusion but this process is more rapid in the LK than in the HK cells.

INTRODUCTION

One of the remarkable properties of living cells is their capacity to maintain a relatively constant volume throughout life. Since cells generally contain a large number of charged macromolecules which cannot pass through the plasma membrane, osmotic forces produce a constant tendency to swell. In general, such systems can avoid swelling only if the hydrostatic pressure is sufficiently higher inside than outside the cell, or if the cell surface is im-

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permeable to a large fraction of the solutes in the external solution. In some bacterial and plant cells the former mechanism appears to be responsible for volume stability (Rothstein (1959)). For many years, it was believed that animal cells in general and red cells in particular (which lack a tough wall to support a pressure gradient) maintained a stable volume because they were impermeable to cations, or at least to the major extracellular cation, sodium (Van Slyke *et al.* (1923)). Jacobs (1931), Wilbrandt (1941), and Hoffman (1958) have pointed out that loss of cation impermeability would lead to "colloid-osmotic" swelling and hemolysis. With the use of isotopic tracers, it became clear that the surface of red cells (and animal cells in general) is permeable to sodium and potassium. It became necessary, therefore, to discard the idea that red cells maintain a stable volume because they are impermeable to cations and also to find another explanation for the fact that they do not swell and hemolyze.

A new approach to the regulation of cell volume began with the experiments of Harris (1941) and Danowski (1941) who showed that maintenance of normal cation composition in human red cells depends on glycolysis. Since that time many experiments have been reported which characterize the mechanism of active sodium and potassium transport in red cells (for reviews see Tosteson (1955 *a*); Harris (1956); and Glynn (1957)). From this work the idea has emerged that active cation transport occurs through the operation of a "pump" which extrudes one or more Na ions in exchange for an external K. It has also been recognized in a general way that the K-Na pump working against diffusion leaks for both ions controls the steady state cation composition and thereby the volume of the cell. However, an explicit formulation of the relation between these processes has not yet been put forward.

The purpose of this paper is to describe such a formulation of the relation between cation transport and volume regulation and to evaluate experimentally its applicability to two types of sheep red cells. Three membrane parameters, α, β, and N, which express relations between an ion exchange pump and passive diffusion leaks working in parallel are shown to define the steady state concentrations of Na and K and the cell volume when the ionic composition of the external fluid and the amount of "fixed" anion in the cell are known. A third transport mechanism, exchange diffusion, is included in the model but does not allow net ionic movement and is therefore not relevant to the control of cell composition. Conversely, the equations describing the model can be used to calculate the membrane parameters when the ionic composition of both cells and external fluid is known. The experiments to be reported in this paper describe direct measurements of these parameters in high (HK) and low (LK) potassium sheep red cells. Since red cell K is about 85 mM/liter in sheep with HK red cells, but only about 12

mM/liter in animals with LK red cells, it is possible to make a fairly stringent test of the range of applicability of the model cell by measuring the membrane parameters in these two cell types. The values observed experimentally conform quite well to those calculated from the ionic composition of HK and LK cells and the equations describing the model. This suggests that both HK and LK sheep red cells have a qualitatively similar cation transport apparatus; *i.e.*, both control their cation composition and volume through the operation of an exchange pump working in parallel with diffusion leaks as postulated in the model. However, there are marked quantitative differences in both pump and leaks between the two cell types. These differences lead to a great change in the relations between these processes (as expressed in the membrane parameters) and thus produce the striking differences in K and Na composition of HK and LK sheep red cells. Since the difference in cation composition between the two cell types apparently depends on one gene (Evans *et al.* (1956)), it is suggested that the molecular mechanism of pump and leak may be closely related.

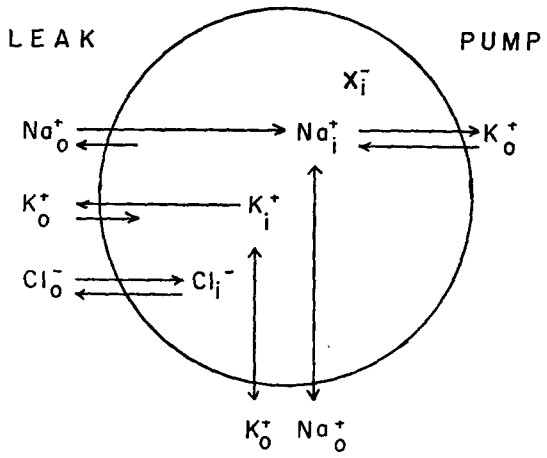

FIGURE 1. Model cell.

THEORETICAL SECTION

SYMBOLS

K_i, Na_i, Cl_i, X_i = amounts of K, Na, Cl, X in cell. X is non-diffusible solute
$(K)_i$, $(Na)_i$, $(Cl)_i$, $(X)_i$ = concentration of components in cell water
V_w = volume of cell water
Z_x = valence of X
M = net flux in mM/cell = ${}^iM - {}^oM$
${}^iM$ = influx M^L = leak flux M^e = exchange diffusion flux
${}^oM$ = outflux M^P = pump flux

$$ {}^{i}k_{K}^{L} = \frac{{}^{i}M_{K}^{L}}{(K)_o} \qquad {}^{i}k_{Na}^{L} = \frac{{}^{i}M_{Na}^{L}}{(Na)_o} \qquad {}^{o}k_{K}^{L} = \frac{{}^{o}M_{K}^{L}}{(K)_i} \qquad {}^{o}k_{Na}^{L} = \frac{{}^{o}M_{Na}^{L}}{(Na)_i} $$

$$ \alpha = \frac{{}^{i}k_{Na}^{L}}{{}^{i}k_{K}^{L}} \qquad \beta = \frac{{}^{i}M_{K}^{P}}{{}^{i}M_{K}^{L}} $$

ASSUMPTIONS

(1) There is a constant ratio between the pump influx of K and outflux of Na defined by the relation,

$$ \frac{{}^{o}M_{Na}^{P}}{{}^{i}M_{K}^{P}} = N. $$ The pump influx of Na and outflux of K are zero.

(2) The leak fluxes of K and Na conform to the flux ratio equation (Ussing (1949)).

(3) The specified pump and leaks are the only pathways through which a net flux of K and Na can occur; *i.e.*,

$$ {}^{o}M_{Na}^{e} = {}^{i}M_{Na}^{e} \qquad \text{and} \qquad {}^{o}M_{K}^{e} = {}^{i}M_{K}^{e} $$

(4) Penetrating anions are at thermodynamic equilibrium in the system.

(5) Water is at thermodynamic equilibrium in the system.

(6) There is no hydrostatic pressure difference between inside and outside of the cell.

(7) Electroneutrality obtains both inside and outside the cell.

I. ANALYSIS

The unknown quantities $(K)_i$, $(Na)_i$, $(Cl)_i$, and V_w are to be calculated from the known quantities $(K)_o$, $(Na)_o$, $(Cl)_o$, X_i, Z_x, and the membrane parameters α, β, and N. The four unknown quantities are related by the following four simultaneous equations.

(1) $$ (Cl)_i(\alpha'(Na)_i + (K)_i) = (Cl)_o(\alpha'(Na)_o + (K)_o) $$

where $\alpha' = \alpha/N$

(2) $$ \frac{(K)_i}{(Na)_i} = Q\frac{(K)_o}{(Na)_o} $$

where $Q = f(\alpha, \beta, N)$

(3) $$ (K)_i + (Na)_i + (Cl)_i + \frac{X_i}{V_w} = (K)_o + (Na)_o + (Cl)_o $$

(4) $$ (K)_i + (Na)_i - (Cl)_i + \frac{Z_xX_i}{V_w} = 0 $$

The first equation expresses the equality between the diffusion potential due to K and Na leaks and the equilibrium potential for Cl. The second equation expresses the effect of the membrane parameters α, β, and N in determining the ratio of cell K to Na concentration. Both these equations are derived below. The third and fourth equations are statements of osmotic equilibrium between cell and environment and electroneutrality within the cell.

II. DERIVATION OF EQUATION 1

(5) From Assumption 2,

$$(a)\ \frac{{}^{i}M_{K}^{L}}{{}^{o}M_{K}^{L}} = \frac{(K)_o}{(K)_i} \exp\left[-\frac{FE}{RT}\right]$$

$$(b)\ \frac{{}^{i}M_{Na}^{L}}{{}^{o}M_{Na}^{L}} = \frac{(Na)_o}{(Na)_i} \exp\left[-\frac{FE}{RT}\right]$$

(6) From assumption 4,

$$E = -\frac{RT}{F} \ln \frac{(Cl)_o}{(Cl)_i}$$

(7) By definition,

$$(a)\ M_{K}^{L} = {}^{i}M_{K}^{L} - {}^{o}M_{K}^{L}$$

$$(b)\ M_{Na}^{L} = {}^{i}M_{Na}^{L} - {}^{o}M_{Na}^{L}$$

(8) From assumptions 1 and 3 in the steady state,

$$M_{Na}^{L} = -NM_{K}^{L}$$

(9) Substituting 5, 6, and 7 into 8 and rearranging,

$$\frac{{}^{i}M_{Na}^{L}}{{}^{i}M_{K}^{L}} = N\left[\frac{(K)_i(Cl)_i - (K)_o(Cl)_o}{(Na)_o(Cl)_o - (Na)_i(Cl)_i}\right]\frac{(Na)_o}{(K)_o}$$

(10) From the definition of α,

$$\alpha = N\left[\frac{(K)_i(Cl)_i - (K)_o(Cl)_o}{(Na)_o(Cl)_o - (Na)_i(Cl)_i}\right]$$

(11) Defining $\alpha' = \alpha/N$ and rearranging,

$$(Cl)_i(\alpha'(Na)_i + (K)_i) = (Cl)_o(\alpha'(Na)_o + (K)_o), \text{ Equation 1.}$$

III. DERIVATION OF EQUATION 2

(12) From 5,

$$\frac{(K)_i}{(Na)_i} = \left[\frac{{}^oM_K^L\,{}^iM_{Na}^L}{{}^iM_K^L\,{}^oM_{Na}^L}\right]\frac{(K)_o}{(Na)_o}$$

(13) From assumption 3, in the steady state,

(*a*) ${}^oM_K^L = {}^iM_K^L + {}^iM_K^P$

(*b*) ${}^oM_{Na}^L = {}^iM_{Na}^L - {}^oM_{Na}^P$

(14) From the definitions of the rate constants, α, β, N, and α',

(*a*) ${}^iM_K^P = \beta\,{}^iM_K^L$

(*b*) ${}^oM_{Na}^P = \frac{N\beta(K)_o\,{}^iM_{Na}^L}{\alpha(Na)_o} = \frac{\beta(K)_o\,{}^iM_{Na}^L}{\alpha'(Na)_o}$

(15) Substituting 13 *a*, *b* and 14 *a*, *b* into 12

$$\frac{(K)_i}{(Na)_i} = \left[\frac{1+\beta}{1 - \frac{(K)_o\beta}{\alpha'(Na)_o}}\right]\frac{(K)_o}{(Na)_o}$$

(16) Setting

$$Q = \frac{1+\beta}{1 - \frac{\beta\,(K)_o}{\alpha'(Na)_o}}$$

we obtain

$$\frac{(K)_i}{(Na)_i} = Q\frac{(K)_o}{(Na)_o}, \qquad \text{Equation 2.}$$

IV. SOLUTION OF EQUATIONS 1, 2, 3, AND 4 SIMULTANEOUSLY

(17) $$(Na)_i = \frac{-b + \sqrt{b^2 - 4ac}}{2a}$$

where

$$a = (1 - Z_x)\left(1 + Q\frac{(K)_o}{(Na)_o}\right)\left(\alpha' + Q\frac{(K)_o}{(Na)_o}\right)$$

$$b = Z_x((\mathrm{Na})_o + (\mathrm{K})_o + (\mathrm{Cl})_o)\left(\alpha' + Q\frac{(\mathrm{K})_o}{(\mathrm{Na})_o}\right)$$

$$c = -(\mathrm{Cl})_o(\alpha'(\mathrm{Na})_o + (\mathrm{K})_o)(1 + Z_x)$$

$$(\mathrm{K})_i = (\mathrm{Na})_i\left[Q\frac{(\mathrm{K})_o}{(\mathrm{Na})_o}\right] \tag{18}$$

$$(\mathrm{Cl})_i = \frac{(\mathrm{Cl})_o(\alpha'(\mathrm{Na})_o + (\mathrm{K})_o)}{(\mathrm{Na})_i\left(\alpha' + Q\frac{(\mathrm{K})_o}{(\mathrm{Na})_o}\right)} \tag{19}$$

$$V_w = \frac{X_i}{((\mathrm{Na})_o + (\mathrm{K})_o + (\mathrm{Cl})_o) - ((\mathrm{Na})_i + (\mathrm{K})_i + (\mathrm{Cl})_i)} \tag{20}$$

METHODS

Experimental Procedure The experiments were performed on sheep blood drawn freshly into heparin on the day of the experiment. Blood from four different sheep, two of the high potassium (HK) and two of the low potassium (LK) genetic type was studied. For the sake of brevity, most of the results included in this paper are from experiments on the blood of only two of these animals. The results obtained with the other two sheep were in agreement with those shown here. The red cells were washed three times with 0.17 M NaCl and the buffy coat discarded prior to use. These washed cells were suspended in approximately 30 volumes of an isosmotic medium and incubated at 37°C. The composition of the medium in the standard system was as follows: 165 mM Na, 5.0 mM K, 150 mM Cl, 9.35 mM HPO_4, 1.65 mM H_2PO_4 (pH = 7.4), glucose 200 mg. per cent, chloromycetin 1 mg. per cent. Alterations in the composition of this standard medium were made in particular experiments and will be mentioned below. The timing of additions of tracer amounts of either $K^{42}Cl$ or $Na^{24}Cl$ to the incubating cell suspension and subsequent removal of samples depended on whether influx or outflux was to be measured.

Influx Experiments The tracer was added to the cell suspension after 10 to 15 minutes of incubation to allow temperature equilibration. Two procedures for sampling were used: (*a*) Aliquots removed from the incubating cell suspension were centrifuged for 5 minutes at 20,000 × *g*. The supernatant fluid was removed for analysis. An aliquot of the packed cells was then pipetted for analysis. Analytical values obtained from these packed cells were corrected for medium trapped between the cells during centrifugation. The amount of trapped medium was estimated by taking a sample immediately after addition of tracer K^{42} and Na^{24}, centrifuging, and measuring the radioactivity of the supernatant and the packed cells. The fractional volumes of distribution of both ions in the packed cells are shown in Table I. These values of 0.04 to 0.05 are probably in excess of the fractional volume of distribution of I^{131}-labeled albumin which is usually 0.01 to 0.02 in human red cells (Toste-

son *et al.* (1955*b*); Maizels (1959)) and may include part of a small rapidly exchanging cation compartment located at the surface of the sheep red cells, similar to that noted in the recent observations of Maizels on human red cells and Joyce and Weatherall (1958) on sheep red cells. (*b*) A known volume (*e.g.*, 10 ml.) of cell suspension was removed from the incubation flask. The volume of cells in this volume was calculated from measurements of the hemoglobin concentration in the packed cells used in making up the incubation suspension and the hemoglobin concentration in the suspension itself. The aliquot was centrifuged, a sample of the supernatant removed for analysis, and the packed cells washed three times with ice cold 0.12 M $MgCl_2$ taking care to avoid loss of any cells. The washed cells were then quantitatively transferred to a volumetric flask for analysis. When inadvertent loss of cells occurred during washing, it was detected and corrected for by measuring the hemoglobin concentration in all dilutions of red cells prior to analysis and normalizing all

TABLE I

FRACTION OF SHEEP RED CELL VOLUME WHICH IS TRAPPED MEDIUM

Cell type	HK Sheep No. 15				LK Sheep No. 17			
Tracer	K^{42}		Na^{24}		K^{42}		Na^{24}	
Strophanthidin	+	−	+	−	+	−	+	−
Mean	0.048	0.060	0.044	0.045	0.051	0.051	0.041	0.040
Range	(0.047–0.048)	(0.054–0.065)	(0.043–0.045)	(0.045–0.046)	(0.052–0.050)	(0.042–0.053)	(0.044–0.038)	(0.042–0.039)
No. of measurements	2	6	2	2	2	6	2	2

The values in the table are from measurements made on the red cells of one HK and one LK sheep.

analytical values to the same hemoglobin concentration. Measurements of influx obtained by procedures (*a*) and (*b*) agreed, but procedure *b* was more reproducible and accurate when measuring the very slow K influx which occurred in the presence of strophanthidin. The strophanthidin used in these experiments was obtained from the Bios Company. It was dissolved in ethanol. An amount of ethanol equal to that containing strophanthidin was added to all control flasks.

Outflux Experiments Packed washed cells were incubated overnight at 37°C. in 3 to 4 volumes of standard medium containing either Na^{24} or K^{42}. The following morning the cells were washed three times in ice cold aliquots of the medium to be used in the subsequent measurement of outflux. The cells were then suspended in about 30 volumes of medium and incubated at 37°C. for several hours. Sampling was carried out by either procedure (*a*) or (*b*) described above for influx experiments with similar results.

Analytical Procedures K^{42} and Na^{24} were obtained as the carbonate from the Brookhaven National Laboratory and converted to the chloride by titration with

HCl before use. Both isotopes were measured in a well-type scintillation counter. Total K and Na were measured with a barrier-layer-cell internal standard flame photometer. Hemoglobin concentrations were estimated by measuring the optical density at 540 millimicrons with a quartz prism spectrophotometer. pH was measured with a glass electrode. Water content of the red cells was measured by weighing the packed cells before and after drying at 105°C. in an oven for 24 hours. Chloride concentrations were measured with a Cotlove automatic chloride titrator (Cotlove (1958)). We are indebted to Dr. E. Cotlove for performing the chloride analyses.

Calculations from the Data Fluxes were calculated by the unsteady state procedure of Sheppard and Martin (1950) or by the method previously described (Tosteson *et al.* (1955 *b*)). In calculating the net ion movement in the experiments directed toward estimating the leak permeabilities of K and Na, the cell content of each ion expressed as millimoles per that number of cells which initially occupied 1 liter was calculated from measurements of cell concentrations of K, Na, and water at the beginning of the experiment and at the time of each sample by the following relations.

$$V_s = \frac{1}{1 + \frac{1.35 W_c}{1 - W_c}} \tag{21}$$

where V_s is the milliliters of solid per milliliter of red cells, W_c is the grams of water per gram of red cells, and 1.35 is the density of red cell solids.

$$A^t = \frac{V_s^o}{V_s^t} C^t \tag{22}$$

where A^t is the amount of an ion at time, t, in that number of the cells which occupied 1 liter at the start of the experiment, C^t is the concentration of the ion in millimoles per liter of cells at time t, V_s^o is V_s at the start of the experiment, and V_s^t is V_s at time, t. In these experiments, α was calculated by the relation,

$$\alpha = \frac{{}^{i}k_{Na}^{L}}{{}^{i}k_{K}^{L}} = \frac{M_{Na}}{M_K}\left[\frac{(K)_o(Cl)_o - (K)_i(Cl)_i}{(Na)_o(Cl)_o - (Na)_i(Cl)_i}\right]$$

where M_K and M_{Na} are the net fluxes of K and Na respectively expressed in millimoles/(original liter R.B.C.) × (hour).

RESULTS

1. *Ionic Composition and Calculated Membrane Parameters in HK and LK Sheep Red Cells* Table II shows the ionic composition of the sheep red cells used in these experiments. They represented extremes in the flock or twenty-five sheep whose red cells were analyzed. From the concentration of K, Na, and Cl in these cells and in sheep plasma, and the equations set down in

the theoretical section, values for α' and β also shown in Table II were calculated. These quantities are kinetic parameters which describe the movement of K and Na across the plasma membranes of the cells. We have attempted to measure these parameters directly by appropriate kinetic experiments. In the rest of this paper we will describe these experiments and compare the directly measured values of α' and β with those calculated from the theory and the steady state concentrations of K, Na, and Cl. Since the theory predicts that α' and β will be very different in the low K as compared with the high K sheep red cells, the theory will be put to a fairly stringent test.

TABLE II

IONIC COMPOSITION AND CALCULATED MEMBRANE PARAMETERS OF SHEEP RED CELLS

Cell type		$(K)_o$	$(Na)_o$	$(K)_i$	$(Na)_i$	$(Cl)_i/(Cl)_o$	V_w	α'	β
			mM/liter H_2O				*ml./ml.*		
HK	Mean	4.91	139	121	36.8	0.67	0.695	0.67	16
Sheep No. 15	Range	4.86–4.96	138–140	118–127	31.4–41.4	0.65–0.70	0.669–0.725		
	No. of measurements	4	4	48	50	5	22		
LK	Range	5.00	139	17.4	137	0.70	0.695	0.17	1.4
Sheep No. 17	Mean	4.96–5.04	139–140	15.5–21.7	129–144	0.67–0.72	0.668–0.723		
	No. of measurements	4	4	57	57	5	23		

The values in the table are from nine experiments in which the red cell cations and water were measured and three experiments in which the chloride concentration ratio was measured in one HK (No. 15) and one LK (No. 17) sheep. The plasma cation values were measured on two HK and two LK sheep.

2. *Measurement of Pump and Leak Influxes of K. Measurements of the Membrane Parameter β* That component of the total flux which is sensitive to strophanthidin is taken to be a measure of the pump flux. Previous work to some extent justifies this assumption. Schatzmann (1953) first showed that compounds of this type blocked active transport of K and Na in red cells. Since that time a large number of workers have shown that cardiac glycosides and aglycones block active transport of K and Na in many different animal tissues (Hajdu and Leonard (1959)). The effect of strophanthidin on K influx and outflux is shown in Tables III *a* and III *b*. In these experiments the cells were suspended in the standard medium (see Methods), the cation composition of which simulated blood plasma. Strophanthidin had a negligible effect on K outflux in both cell types (Table III *a*). This result with sheep red cells differs from that of Glynn (1957) on human red cells since he found

TABLE III*a*

EFFECT OF STROPHANTHIDIN ON K OUTFLUX IN SHEEP RED CELLS

Cell type	Experiment No.	$(K)_o$	$(Na)_o$	${}^oM_K$	${}^oM_K{}^{stroph}$
		mM/liter		*mM/(liters R.B.C.) × (hr.)*	
HK					
Sheep No. 15	23	5.0	165	0.61	0.64
	30	5.0	165	0.80	0.71
LK					
Sheep No. 17	23	5.0	165	1.23	1.18
	30	5.0	165	1.95	1.88

TABLE III*b*

EFFECT OF STROPHANTHIDIN ON K INFLUX IN SHEEP RED CELLS MEASUREMENT OF β

Cell type	Experiment No.	$(K)_o$	$(Na)_o$	${}^iM_K$	${}^iM_K^{stroph}$	${}^iM_K^P$	β Measured	β Calculated
		mM/liter		*mM/(liters R.B.C.) × (hr.)*				
HK								
Sheep No. 15	25*a*	5.0	165	0.66	0.043	0.62	14	16
	25*b*	5.0	165	0.61	0.038	0.57	15	16
LK								
Sheep No. 17	25	5.0	165	0.30	0.14	0.16	1.1	1.4
	4	5.0	165	0.26	0.10	0.16	1.6	1.4

The fluxes recorded above were obtained in two experiments each on the washed red cells of one HK and one LK sheep. They are representative of a total of nine experiments on the cells of two HK and two LK sheep. The number in the experiment column indicates the separate measurements of influx and outflux since these were not made simultaneously. The fluxes were measured by procedure (*b*) described under Methods, except for influx in Experiment 4 in which procedure (*a*) was used. The fluxes in the presence of strophanthidin, M^{stroph}, were measured in solutions containing 5×10^{-5} M/liter of this compound. The strophanthidin-insensitive influxes were corrected to a $(K)_o = 5.0$ mM/liter by making use of the fact that this flux is linearly dependent on $(K)_o$ in the range between 0 to 20 mM/liter. ${}^iM_K^P$ is the difference between ${}^iM_K$ and ${}^iM_K{}^{stroph}$.

that digoxin produced some inhibition of K outflux. It will be noted that the magnitude of K outflux in LK cells in these experiments substantially exceeds the values obtained for K influx. This may indicate some increase in the K leak in these washed LK cells above that which obtains when they are suspended in plasma. In any case, because of the absence of an appreciable effect of strophanthidin on K outflux and because preliminary data indicate the absence of significant K exchange diffusion in cells suspended in the plasma-like standard medium, we identify the strophanthidin-insensitive K

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influx with the leak influx. Strophanthidin inhibits K influx in both HK and LK cells but the magnitude of the strophanthidin-sensitive flux is about four times greater in the HK as compared with the LK type (Table III *b*). The concentration of strophanthidin used, 5×10^{-5} M, was shown to be sufficient to produce maximal inhibition of K influx. Recalling that β is the ratio of K influx through the pump to K influx through the leak (see Theoretical Section), we can directly calculate this parameter for HK and LK cells from the data shown in Table III *b*. These values are shown in the

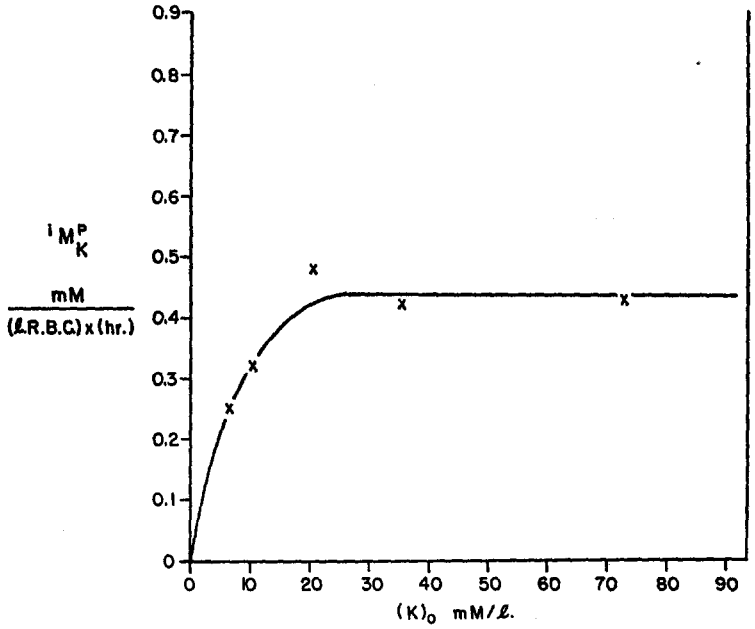

FIGURE 2. Effect of external K on pump influx of K in HK sheep red cells.

table and can be seen to agree quite well with the values calculated from steady state concentrations and theory shown in Table II. The slightly low value of the measured as compared with the theoretical value for β in one of the experiments with LK cells may be due to the increased leak mentioned above.

Figs. 2 and 3 show the effects of external K on pump and leak influx of K respectively. These data were obtained by substituting K for tetraethyl ammonium ions (TEA) in the external solution. No sodium ions were present in the medium. The data from HK cells show the usual saturable pump and approximately linear leak curves and are similar to those reported by Shaw (1955) for sheep red cells. The LK cells became abnormally leaky in TEA and therefore, it was not possible to obtain a good pump curve. The leak

curve for LK cells is included to show the approximately linear relation between leak influx and $(K)_o$ in these cells.

3. *Measurements of Exchange Diffusion of Na* Table IV shows the effect of strophanthidin on the influx and outflux Na. It will be noted that the drug produces no significant effect on Na influx in either cell type. Na outflux is slightly inhibited by strophanthidin in HK cells but apparently stimulated by the drug in LK cells. The latter result is probably due to the failure to correct for slight hemolysis in computing the outflux. This correction was

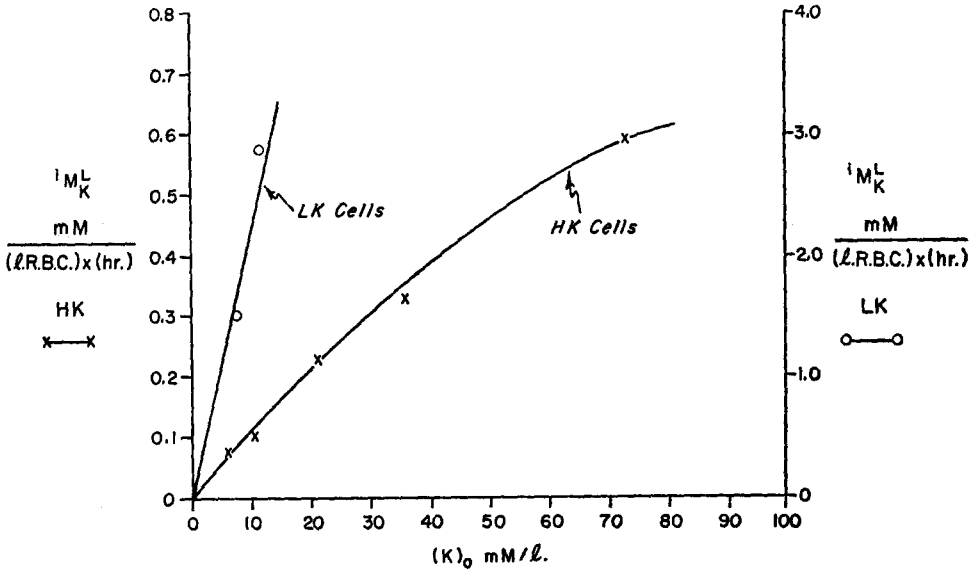

FIGURE 3. Effect of external K on leak influx of K in sheep red cells.

made in later experiments on these cells suspended in Na-free media. The absolute magnitude of the strophanthidin-sensitive Na outflux in HK cells is about the same as the magnitude of strophanthidin-sensitive K influx, but the high steady state flux of Na makes the percentage change in Na outflux produced by the drug small in both cell types, but particularly in the LK variety. We suspected that this effect might be due to the presence of considerable Na exchange diffusion in the system (Levi and Ussing (1948)) and, therefore, undertook some experiments to examine this possibility.

That portion of the outflux of an ion which depends on the presence of the ion in the external solution is taken to be a measure of the exchange diffusion component of the total steady state flux. Table V shows the results of experiments in which Na outflux was measured in HK and LK cells suspended in media containing 0.16 M Na, 0.16 M choline, 0.17 M tris(hydroxymethyl) aminomethane (Tris), or 0.12 M Mg as the major cation. All these salts were

present as the chloride. All the solutions were free of potassium in order to eliminate pump outflux. All the solutions were buffered with 10 mM Tris adjusted to pH 7.4. It is clear that Na outflux is reduced by removing external

TABLE IV

EFFECT OF STROPHANTHIDIN ON Na FLUXES IN SHEEP RED CELLS

Cell type	Experiment No.	$(K)_o$	$(Na)_o$	${}^iM_{Na}$	${}^iM^{stroph}_{Na}$	${}^oM_{Na}$	${}^oM^{stroph}_{Na}$	${}^oM^{P}_{Na}$
		mM/liter		*mM/(liters R.B.C.) × (hr.)*				
HK								
Sheep No. 15	5,5	5.0	165	2.3	2.5	3.0	2.4	0.6
	6,7	5.0	165	2.8	3.2	3.6	2.9	0.7
LK								
Sheep No. 17	5,5	5.0	165	3.6	3.6	3.4	4.4	—
	6,7	5.0	165	4.1	4.2	4.8	5.7	—

The flux values in this table were obtained in two experiments on the red cells of one HK and one LK sheep. They are representative of a total of four experiments on the cells from two HK and two LK sheep. The two numbers in the experiment column indicate the separate measurements of influx and outflux since these were not made simultaneously. The fluxes were measured by procedure (*a*) described under Methods. M^{stroph} was measured in the presence of 5×10^{-5} M/liter of strophanthidin.

TABLE V

Na OUTFLUX FROM SHEEP RED CELLS INTO K-FREE SOLUTIONS. EVIDENCE FOR Na EXCHANGE DIFFUSION

External cation	Na outflux	
	HK cells Sheep No. 15	LK cells Sheep No. 17
	mM/(liters R.B.C.) × (hr.)	
Na	3.5	4.9
Choline	0.40	1.4
Tris		1.3
TEA	0.44	2.0
Mg	0.16	0.59

The fluxes in this table were obtained in four experiments on the red cells of one HK and one LK sheep. The fluxes were measured by procedure (*b*) described under Methods.

Na from both HK and LK cells. The effect is most marked when Na is replaced by Mg. The magnitude of the Na exchange diffusion flux is rather greater in the LK than in the HK cells. The effect of varying the external Na concentration on Na outflux is shown in Fig. 4. In this experiment Na was substituted for Mg in the absence of external K. In all the cases shown in Table V, addition of K to the medium stimulated Na outflux. This stimulation was inhibited by the addition of strophanthidin, whereas strophanthidin

had no effect on Na outflux in the absence of external K. This point will be considered in greater detail below.

4. *Measurements of Pump Outflux of Na. Measurement of the Membrane Parameter N* As noted previously, the high Na exchange diffusion flux shown above for both HK and LK cells suspended in Na-rich solutions, made it difficult to obtain a reliable figure for the strophanthidin-sensitive or pump outflux of Na under these experimental conditions. This uncertainty makes it impossible to evaluate N, the coupling ratio of the pump when the cells are

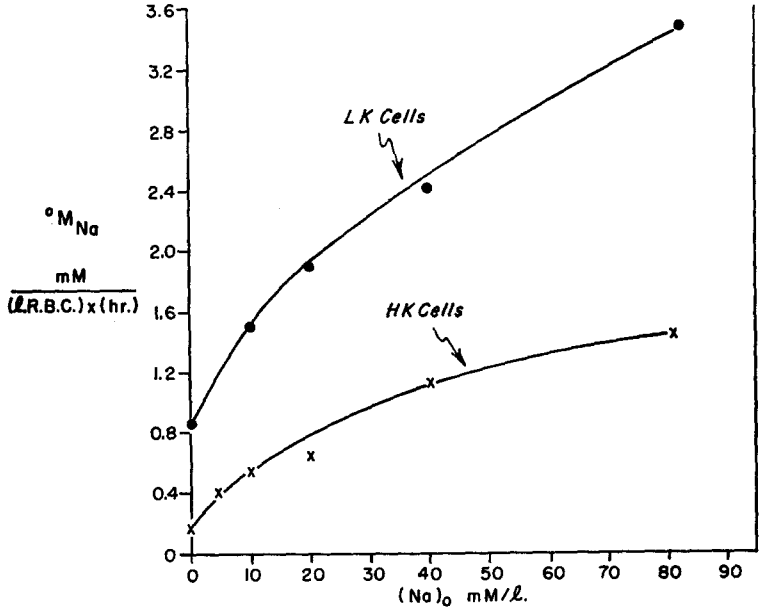

FIGURE 4. Effect of external Na on Na outflux in sheep red cells.

suspended in a high Na medium. We have attempted to measure N when the cells are suspended in Na-free media. The results of these experiments are shown in Table VI. Measurements of Na outflux and K influx in the presence and absence of strophanthidin were made on cells suspended in each medium. Data obtained on HK and LK cells suspended in media containing only K, Mg (and 10 mM Tris as buffer) as cations are shown in the table. Note that the strophanthidin-sensitive K influx approximately equals the strophanthidin-sensitive Na outflux in both cell types. That is, N, the coupling ratio of the pump, is approximately unity for both HK and LK sheep red cells. The absolute magnitude of the pump flux is four to five times greater in HK than in LK cells. Removal of external K reduced Na outflux to about the same extent as addition of strophanthidin in the presence

of external K. The Na outflux is insensitive to strophanthidin when no K is present outside the cells.

Fig. 5 shows the dependence of ${}^{o}M_{Na}^{P}$ on $(K)_o$ in HK cells. This pump flux also shows the saturable character noted in the curve relating ${}^{i}M_{K}^{P}$ to $(K)_o$

TABLE VI

PUMP FLUXES IN SHEEP RED CELLS IN Mg SOLUTIONS MEASUREMENTS OF N

Cell type	Experiment No.	$(K)_o$	$(Stroph)_o$	${}^{i}M_K$	${}^{i}M_K^P$	${}^{o}M_{Na}$	${}^{o}M_{Na}^P$	N
		mM/liter			*mM/(liters R.B.C.) × (hr.)*			
HK Sheep No. 15	25, 27	21	0	0.68	0.54	0.78	0.62	1.1
		21	5×10^{-5}	0.14		0.16		
		0.0	0			0.16		
		0.0	5×10^{-5}			0.10		
HK Sheep No. 1	29, 30	10	0	0.57	0.46	0.70	0.57	1.2
		10	5×10^{-5}	0.11		0.13		
		0.1	0			0.15		
		0.1	5×10^{-5}			0.12		
LK Sheep No. 17	31, 27	6.6	0	0.66	0.14	0.98	0.13	0.9
		6.6	5×10^{-5}	0.52		0.85		
		0.1	0			0.82		
		0.1	5×10^{-5}			0.78		
	24	19	0			0.73	0.18	1.3
		19	5×10^{-5}			0.55		
		0.2	0			0.56		
		0.2	5×10^{-5}			0.50		
LK Sheep No. 22	29, 30	10	0	0.77	0.12	1.27	0.14	1.2
		10	5×10^{-5}	0.65		1.13		
		0.0	0			0.94		
		0.0	5×10^{-5}			0.94		

The fluxes in this table are from experiments on the red cells from two HK and two LK sheep. The two numbers in the experiment column indicate the separate measurements of K and Na fluxes since these were not made simultaneously. The fluxes were measured by procedure (*b*) described under Methods.

(Fig. 2). These data were obtained by incubating HK cells in TEA-K solutions which contained no Na. Therefore, the strophanthidin-insensitive Na outflux represents a good approximation to ${}^{o}M_{Na}^{L}$ under these conditions. This leak outflux of Na is independent of $(K)_o$ between 5 and 150 mM/liter but increases somewhat in K-free solutions. LK cells suspended in TEA-K solutions were too leaky to Na to allow estimation of the pump outflux for the ion in this system.

5. *Measurements of Net Fluxes of K and Na with Pump Blocked. Measurements of the Membrane Parameter* α From the steady state concentrations of ions in cells and plasma and the equations describing the model it can be computed that α'_{HK} should be much greater than α'_{LK} (Table II). It will be recalled from the Theoretical Section that $\alpha' = \alpha/N$. Thus, this difference in α' in the two cell types could be due either to a difference between α_{HK} and α_{LK} or to a difference between N_{LK} and N_{HK}. We have eliminated the latter possibility by showing that N_{LK} approximately equals N_{HK}. That is, the LK cell does not maintain volume stability along with a low K concentration by the operation of an "uncoupled" cation pump which ex-

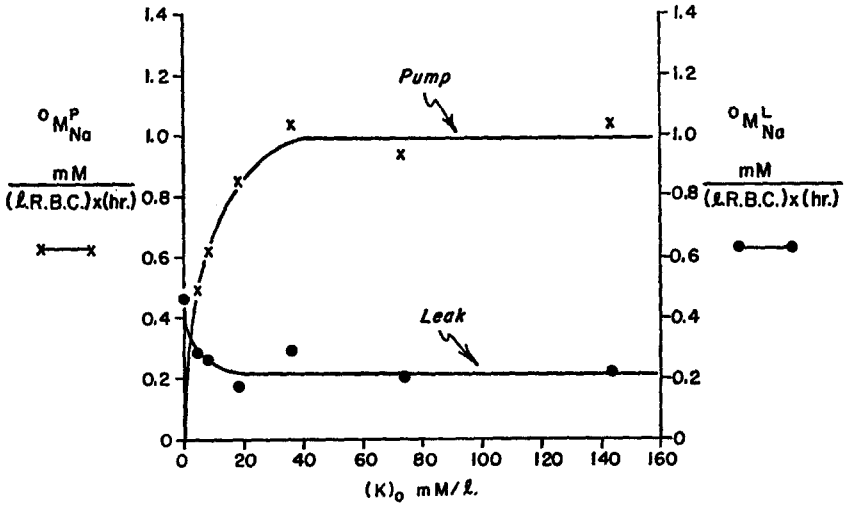

FIGURE 5. Effect of external K on outflux of Na in HK sheep red cells.

trudes more Na in exchange for an external K ion than is the case in the HK cell. Rather, both cell types possess a pump with the same coupling ratio (about unity). If the theoretical model is applicable to these cells, we must conclude that α_{HK} must be much greater than α_{LK}. Recalling that

$$\alpha = {}^{i}k^{L}_{Na}/{}^{i}k^{L}_{K}$$

it is evident that the theory predicts that the LK cell will be less leaky to Na relative to K than is the case in HK cells. In order to test this prediction, α_{HK} and α_{LK} were measured experimentally as described below.

In the case of K, ${}^{i}k^{L}_{K}$ can be evaluated from the influx of K in the presence of strophanthidin. As will be noted in Table III*b*, the influx of K into strophanthidin-poisoned LK cells exceeds that into HK cells in qualitative conformance with the theory. Calculation of ${}^{o}k^{L}_{K}$ assuming the entire outflux to occur via the leak yields a similar qualitative result. The problem of meas-

uring ${}^{i}k^{L}_{Na}$ is made difficult by the presence of considerable Na exchange diffusion in both cell types. To circumvent this problem we attempted to measure both ${}^{i}k^{L}_{K}$ and ${}^{i}k^{L}_{Na}$ from net movements of the ions in strophanthidin-poisoned cells placed in media designed to create large electrochemical gradients of K and Na between cells and environment. Thus, HK cells were incubated in a medium containing little K and much Na while LK cells were placed in a high K-low Na solution. Aliquots removed from time to time were analyzed for cell and medium concentrations of K, Na, and Cl, and cell water. From these data the net fluxes of K and Na and the electrochemical potential gradients for both ions were calculated assuming the membrane potential to equal the chloride equilibrium potential (assumption 4 in the Theoretical Section). From these quantities, ${}^{i}k^{L}_{K}$ and ${}^{i}k^{L}_{Na}$ were calculated. The results of a typical experiment are shown in Table VII. It is clear

TABLE VII

NET K AND Na FLUXES IN SHEEP RED CELLS POISONED WITH STROPHANTHIDIN MEASUREMENT OF α

Cell type	${}^{i}k^{L}_{K}$	${}^{i}k^{L}_{Na}$	α_{meas}	α'_{meas}	α'_{calc}
	$V_w/hr.$	$V_w/hr.$			
HK					
Sheep No. 15	0.0065	0.00343	0.57	0.52	0.67
LK					
Sheep No. 17	0.0110	0.00233	0.21	0.19	0.17

The data in the table were obtained in one experiment representative of four performed on the cells of one HK and one LK sheep.

that ${}^{i}k^{L}_{K}$ is greater for LK than for HK cells while the converse obtains for ${}^{i}k^{L}_{Na}$. The measured values of α'_{HK} and α'_{LK} are in fairly good agreement with those predicted from the model. Thus, the markedly different K and Na composition in the two cell types is not due to the action of intrinsically different kinds of cation pumps, *i.e.* pumps with different coupling ratios, but rather to a striking difference in the characteristics of the cation leaks.

6. *Effect of Blocking Pump on Cell Volume* An important property of the model described in the Theoretical Section is that it allows for control of cell volume by regulation of the cell cation content through the operation of pump and leak working in parallel. It follows that blocking the cation pump should result in an increase in cell volume which is proportional to the increase in cell cation content. Specifically, it can be shown that

$$\frac{V^t}{V^{t=0}} - 1 = h\left[\frac{(K_i + Na_i)^t}{(K_i + Na_i)^{t=0}} - 1\right] \tag{23}$$

where h is the ratio of cell to medium total cation concentration at the start of the experiment (see Appendix for derivation). Fig. 6 shows a plot of these quantities from an experiment designed to measure α in HK and in LK cells. Clearly the swelling is proportional to the increase in cell cations. The measured slope of the line relating the two quantities was 0.57 in both cell types. The slope predicted by Equation 23 was 0.67 for HK and 0.65 for LK cells.

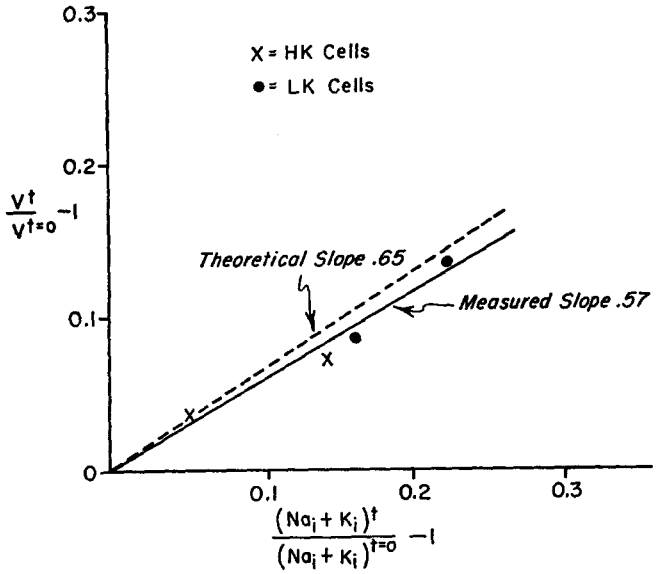

FIGURE 6. Swelling of sheep red cells with cation pump blocked by strophanthidin.

Another prediction of the model is that the rate of swelling of cells in which the pump is blocked should depend on α and the composition of the external solution bathing the cells. Quantitatively, it can be shown that V, the volume, depends on these quantities as follows:

$$\frac{V^t}{V^{t=0}} - 1 = A(B + (K)_o + \alpha(Na)_o)t \tag{24}$$

where A and B are constants independent of α and extracellular cation concentrations. Thus, if α is approximately unity, as is the case in HK cells, the strophanthidin-poisoned cell should swell at about the same rate in Na as in K. However, if α is low as in the LK cell, the pump-blocked cell should swell more rapidly in a K solution than in an Na solution. Figs. 7 *a* and 7 *b* show a plot of Equation 24 compiled from data obtained in an experiment similar to that described for Table VII. The results conform to the prediction from the theory. It is clear that interference with the cation pump results in

cell swelling which has characteristics consistent with the model proposed in the Theoretical Section.

DISCUSSION

It is of interest to compare the measurements of K and Na transport in sheep red cells described here with similar observations reported previously by other workers (Sheppard *et al.* (1951); Joyce and Weatherall (1958)). Both

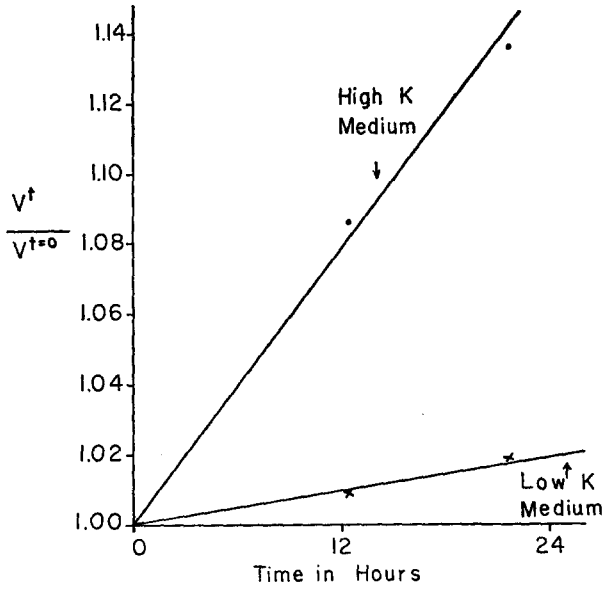

FIGURE 7*a*. Effect of cation composition of medium on rate of swelling of LK sheep R.B.C. with K-Na pump blocked.

groups have pointed out that sheep red cell K and Na are kinetically heterogeneous, small fractions (0.2 to 10 per cent) of the cell contents of each ion exchanging with the medium at a much higher rate than the remaining large fractions. As Joyce and Weatherall point out, the fast fractions will not be measured if the cells are washed between removal from the medium and counting. Since this was done in many of our experiments, our observations refer only to the major, slower moving fractions of cell K and Na. The influxes of K and Na in the sheep which we studied fall well within the range of fluxes observed by Joyce and Weatherall (0.2 to 0.9 mM K/(liter R.B.C.) × (hour) and 2.2 to 4.6 mM Na/(liter R.B.C.) × (hour)) for the slow fractions. One final point raised by Joyce and Weatherall requires comment. They state "whether the differences between animals (HK and LK sheep) depends on the presence of different proportions of two kinds of cell, one rich in sodium and one rich in potassium, or whether all the cells of a given

animal have about the same ratio of sodium to potassium, characteristic of that animal, remains to be established." We favor the latter alternative for the following reasons. First, in the extreme HK and LK types no appreciable number of cells of the opposite cation composition (*e.g.*, HK cells in the LK population) can be present and still allow for the observed total cell population composition. Also, we have separated the red cell population from a sheep centrifugally into five fractions and found all fractions to have essentially the same cation composition (30 mM K/liter R.B.C.).

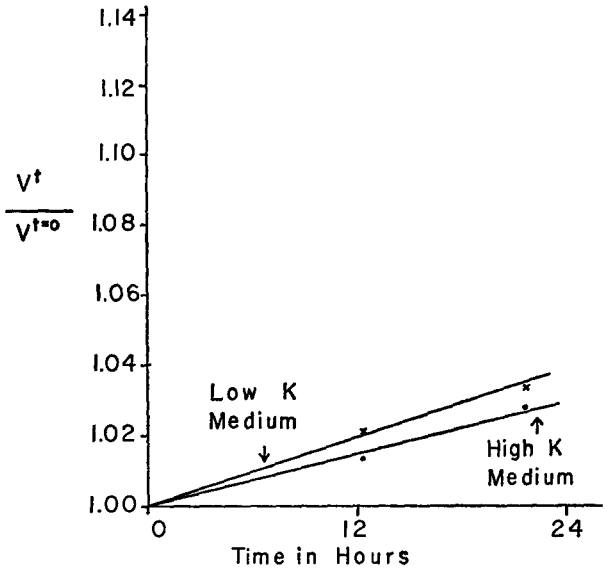

FIGURE 7*b*. Effect of cation composition of medium on rate of swelling of HK sheep R.B.C. with K-Na pump blocked.

Table VIII shows the results of our comparisons of membrane parameters predicted by the steady state concentrations of sodium, potassium, and chloride in HK and LK sheep red cells and in the blood plasma and the equations describing the model cell, with values for these parameters measured directly in appropriate kinetic experiments. The relatively good agreement supports the conclusion that both types of cells control their cation composition and volume in a manner consistent with the model. One important similarity and two significant differences between the two cell types are evident. The similarity is that both HK and LK cells appear to have a cation pump which exchanges one potassium from the external medium with one sodium from the cytoplasm (*i.e.*, N is about unity in both cell types). The differences are that the pump to leak ratio, β, and the ratio of Na to K leak rate constant, α, are both much higher in HK than in LK cells.

The significance of these findings can perhaps be made clearer by a com-

parison of sheep red cells with nerve and muscle cells. In both excitable cells α is 0.01 to 0.03 in the resting state (Hodgkin (1951)); that is, sodium diffuses through the leak much less readily than does potassium. This also is the case in LK sheep red cells. However, the cation composition of the LK cell differs markedly from that of the excitable cell because of the relatively weak pump in the LK sheep red cells. Analysis of the equations describing the model shows that the cation composition of a cell is very sensitive to the value of β when α is low. The results of such an analysis are shown in Fig. 8 in which the ratio of the cellular potassium to sodium concentration is plotted on the ordinate as a function of β on the abscissa for different values of α. In making the calculation it was assumed that the cell was suspended in blood plasma and had a cellular chloride concentration equal to that found in sheep red cells. We have not measured β in nerve and muscle cells but it is clear that small changes in this parameter could easily convert a low K to a high K cell and

TABLE VIII

COMPARISON OF THEORETICAL WITH MEASURED VALUES FOR MEMBRANE CATION PARAMETERS IN SHEEP RED CELLS

Cell type	β_{meas}	β_{calc}	α_{meas}	N_{meas}	α'_{meas}	α'_{calc}
HK						
Sheep No. 15	15	16	0.57	1.1	0.52	0.67
LK						
Sheep No. 17	1.4	1.4	0.21	1.1	0.19	0.17

vice versa. Indeed, this fact could be related to the ease with which potassium is mobilized from muscle under some circumstances in the intact animal. Although the HK sheep red cells differ markedly from nerve and muscle cells with respect to the value of α, the cation composition of these cells simulates closely that of the excitable tissues. The probable reason for this in terms of the model is that β is much higher in HK red cells than it is in nerve and muscle cells.

It should be emphasized that the formulation of the model cell presented here does not depend on any assumption regarding the detailed characteristics of the cation pump. In particular, it does not specify how the pump fluxes will vary in response to changes in the concentrations of K and Na. Nor is the treatment limited to any particular ratio between the number of sodium ions pumped out and potassium ions pumped into the cell, the only requirement being that there is some definite value for this ratio when the cell is in the steady state. The fact that this ratio does have a definite value does not, of course, *demand* that there must be an intimate coupling between outward pumping of Na and inward pumping of K. It *is* assumed that there is no downhill movement of an ion through the pump, *e.g.* inward transport of

Na, such a process being treated as exchange diffusion. Clearly, this classification is to some extent arbitrary, but it does allow us to restrict the use of the word pump to a form of transport which affects the cell content of an ion. We feel that the choice is to some extent justified by its success in making sense of the observations. When more is known about the detailed mechanism of the pump, it may well turn out that what we have classified as exchange

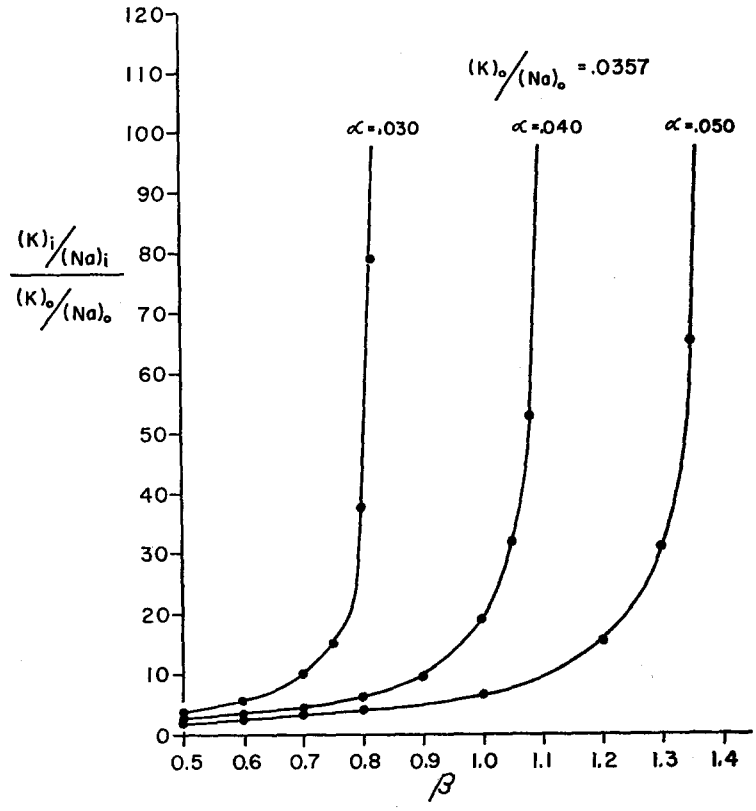

FIGURE 8. Effect of β on $(K)_i/(Na)_i$ for different values of α.

diffusion is, in fact, a characteristic of the cation pump. In summary, then, the treatment of the model cell does no more and no less than specify the quantitative relations between Na and K pumps and leaks *when the cell is in the steady state in any particular external solution.*

It is noteworthy that all three of the different mechanisms of cation transport defined conceptually in the model and operationally in the experimental portion of this paper; that is, pump, leak, and exchange diffusion, are quantitatively very different in the two types of sheep red cells. Evans *et al.* (1956) has recently presented evidence suggesting that the difference between these two phenotypes resides at a single genetic locus. Although it is

possible that a single gene can control the synthesis of three different types of macromolecules in the cell membrane, it seems more reasonable to suspect that the three different mechanisms of cation transport occur at the same or adjacent membrane sites.

APPENDIX

Derivation of Equation 23

(1°) $$V^t = V^{t=0} + \Delta V$$

where V^t is the volume of the cells at time t, $V^{t=0}$ is the volume of the cells at zero time, and ΔV is the increase in volume which occurs during time t.

(2°) $$\Delta V = \frac{\Delta Na_i + \Delta K_i}{(Na)_o + (K)_o}$$

where ΔNa_i is the increase in the amount of Na in that number of cells which at zero time had a volume of $V^{t=0}$, ΔK_i is the analogous increase in cell content of K, and $(Na)_o + (K)_o$ is the total extracellular cation concentration. This equation is a statement of the fact that cell swelling is assumed to occur by colloid osmosis of extracellular fluid.

(3°) $$V^t - V^{t=0} = \frac{\Delta Na_i + \Delta K_i}{(Na)_o + (K)_o}$$

(4°) By definition of ΔNa_i and ΔK_i,

$$V^t - V^{t=0} = \frac{(Na_i + K_i)^t}{(Na)_o + (K)_o} - \frac{(Na_i + K_i)^{t=0}}{(Na)_o + (K)_o}$$

where $(Na_i + K_i)^t$ is the cell content of Na and K at time t and $(Na_i + K_i)^{t=0}$ is the cell content of Na and K at time zero.

(5°) Dividing both sides of 4° by $V^{t=0}$

$$\frac{V^t}{V^{t=0}} - 1 = \frac{(Na_i + K_i)^t}{V^{t=0}[(Na)_o + (K)_o]} - \frac{(Na_i + K_i)^{t=0}}{V^{t=0}[(Na)_o + (K)_o]}$$

(6°) Setting $$h = \frac{(Na_i + K_i)^{t=0}}{[(Na)_o + (K)_o]V^{t=0}}$$

(7°) we obtain $$\frac{V^t}{V^{t=0}} - 1 = h\left[\frac{(Na_i + K_i)^t}{(Na_i + K_i)^{t=0}} - 1\right]$$

which is Equation 23 in the text.

Derivation of Equation 24

(8°) By the same reasoning used in Appendix Equation 2°,

$$\frac{\Delta V}{\Delta t} = \frac{M_{Na} + M_K}{(Na)_o + (K)_o}$$

(9°) $$M_K = {}^{i}k_K^L[(K)_o - r(K)_i]$$

where r is $(Cl)_i/(Cl)_o$ and the other symbols are as defined on page 171 in the text.

(10°) Similarly

$$M_{Na} = {}^{i}k_{Na}^L[(Na)_o - r(Na)_i]$$

(11°) Substituting 9° and 10° into 8° and noting that

$$\alpha = {}^{i}k_{Na}^L/{}^{i}k_K^L$$

we obtain,

$$\frac{\Delta V}{\Delta t} = \frac{{}^{i}k_K^L[(K)_o - r(K)_i] + \alpha {}^{i}k_K^L[(Na)_o - r(Na)_i]}{(Na)_o + (K)_o}$$

(12°) Rearranging,

$$\frac{\Delta V}{\Delta t} = \frac{{}^{i}k_K^L}{(Na)_o + (K)_o}[(K)_o + \alpha(Na)_o - r((K)_i + \alpha(Na)_i)]$$

(13°) Dividing by $V^{t=0}$

$$\frac{V^t}{V^{t=0}} = 1 + \frac{{}^{i}k_K^L \Delta t}{V^{t=0}[(Na)_o + (K)_o]}[(K)_o + \alpha(Na)_o - r((K)_i + \alpha(Na)_i)]$$

(14°) Setting

$$A = \frac{{}^{i}k_K^L}{V^{t=0}[(Na)_o + (K)_o]}$$

$$\text{and } B = -r((K)_i + \alpha(Na)_i)$$

(15°) $$\frac{V^t}{V^{t=0}} = 1 + A(B + (K)_o + \alpha(Na)_o)\Delta t$$

which is Equation 24 in the text.

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