

# Sodium Fluxes in the Erythrocytes of Swine, Ox, and Dog

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**ABSTRACT** Sodium fluxes were measured in erythrocytes from three species of mammals. Unidirectional fluxes were slowest in swine RBCs (low sodium cells), fastest in dog RBCs (high sodium cells), and between these extremes in ox cells (intermediate level of internal sodium). In addition, efflux and influx in swine cells both correlated positively with intracellular sodium concentration between 12 to 42  $\mu\text{eq/ml}$ . Tracer effluxes in swine and beef cells were separated into three components: active transport, diffusion, and exchange diffusion. The last two also contributed to influx. Transport was greater in swine cells than in beef, while the leak was similar in both. Pump to leak ratios were about 21 for swine and 3 for beef, a difference that probably accounts for the higher cell sodium in the latter. Exchange diffusion was faster in beef cells than in swine resulting in a larger tracer movement in beef. The exchange mechanism was temperature-sensitive, but was not inhibited by strophanthin. The unidirectional fluxes in canine cells were inhibited by low temperature, but they were sensibly unaffected by strophanthin. When placed in magnesium Ringer's solution (inhibits exchange diffusion in beef and swine cells) dog RBCs lost more than half of their internal sodium at a rate approximating the isotope flux in plasma or normal Ringer's solution. It was, however, not possible to separate the total tracer movement into pump, leak, and exchange.

## INTRODUCTION

Work in this laboratory on the chemical mechanism underlying active ion transport required a knowledge of some of the parameters of sodium movement in mammalian erythrocytes. Sodium and potassium movements in human red blood cells (RBCs) have been studied in great detail, but data on RBCs of other mammalian species are less plentiful. It is well known that the ability of these cells to regulate their ionic content varies. Most of them are able to maintain the low Na, high K interior typical of animal cells. Erythrocytes from dog and cat blood are almost totally lacking in this capacity; they

are virtually at equilibrium with plasma. And a few groups (*e.g.* some sheep and cattle) are intermediate between the two extremes (Kerr, 1937, Bernstein, 1954). These differences might reflect quantitative variation in transport mechanisms; they might simply be due to differences in "leakiness" of the membranes, or a combination of these factors might be involved. In order to examine these factors the work reported below was undertaken. While it was in progress an elegant analysis of the same problem was published by Tosteson and Hoffman (1960).<sup>1</sup> Their work was limited to sheep RBCs, but was otherwise carried out in much greater analytical and experimental detail than was ours. A brief resumé of some of their results will provide useful background for the work described below. Erythrocytes from two strains of sheep were used. One maintained a fairly low Na and high K; the other was virtually in ionic equilibrium with plasma. Measurement of sodium movement revealed that tracer efflux consisted of three distinct components:

1. A fraction of the efflux was inhibited by strophanthin (Schatzman, 1953) and by reduction of the extracellular potassium concentration (Glynn, 1956). The effects were not additive, and hence are presumably on the same component; this was taken to represent active extrusion by a coupled sodium-potassium transport "pump."

2. A much larger proportion of the efflux was insensitive to strophanthin and external K, but was abolished when extracellular sodium was replaced by magnesium or quaternary ammonium ions. This fraction was ascribed to an unknown mechanism that forced an exchange of sodium ions from one side of the membrane for sodium ions from the other side. Where coupling of identical ions is obligatory the exchange will be abolished by their removal from either side. Such a mechanism, termed "exchange diffusion," was suggested by Levi and Ussing (1948) to rationalize the high energy input apparently required for sodium extrusion from skeletal muscle. However, the demonstration has never been as direct as in sheep erythrocytes.

3. The last component of the efflux was diffusion ("leak") which is a function of the electrochemical activity of the ion in the intracellular compartment and membrane structure. Experimentally it is the residual efflux from cells that have been exposed to strophanthin while suspended in a sodium-free solution.

Sodium influx consisted solely of exchange diffusion and leak. Measurement of potassium movements showed that influx consisted of pump and leak fluxes, while efflux was pure leak; no exchange diffusion was noted. Three parameters were used for a complete description of ionic and osmotic steady states, but one of these, the ratio of transport to diffusion, provides a reasonable measure

<sup>1</sup> We would like to thank Dr. Tosteson and Dr. Hoffman for sending us a manuscript copy of their paper prior to its publication. It will be obvious that the design of some of our experiments, especially on ox cells, is based on their work.

of the ability of a cell to maintain the distribution of an ion in a steady state away from simple thermodynamic equilibrium.

We have examined sodium fluxes in one representative of each of the three cell types mentioned above. Pig RBCs were the low Na-high K system; dog provided the high Na-low K cells, and beef erythrocytes were intermediate with regard to both ions. Total sodium fluxes were examined, and the contributions of pump, leak, and exchange diffusion were evaluated for swine and beef. An unexpected variation in the pattern for dog erythrocytes prevented us from making a complete analysis of the flux, but the data permitted some inferences to be drawn in this case too. Potassium movement was not investigated although a complete description of the ionic steady state would require that it be characterized.

#### METHODS

Blood was collected by exsanguination or venipuncture and transported to the laboratory immediately after addition of heparin (2 IU/ml). Plasma was then separated from a large volume of blood and stored at  $-30^{\circ}\text{C}$  or, more usually at  $0^{\circ}\text{C}$ , for subsequent use as a suspending medium. The cells from this fraction were discarded. Whole blood was stored at  $0^{\circ}\text{C}$  after gassing with carbogen (95 per cent  $\text{O}_2$ :5 per cent  $\text{CO}_2$ ) and addition of adenosine (1 mg/ml). Adenosine maintains the ability of swine cells to extrude sodium even after prolonged storage (Kirschner and Harding, 1958); its effect on beef and dog erythrocytes is not known, but it was added to them too. Blood for use in tracer efflux experiments was stored after the RBCs were loaded with isotope. The cells were labeled by incubation with  $\text{Na}^{22}$  (1.6  $\mu\text{c}/\text{ml}$ ) at  $38^{\circ}\text{C}$  or at room temperature.

One of the important relationships examined in swine erythrocytes was the dependence of Na efflux on cell Na concentration ( $[\text{Na}]_i$ ). It was possible to produce cells with a wide range of  $[\text{Na}]_i$  by storing them in the cold for different lengths of time. However, the concentration increased at the rate of about 2.5  $\mu\text{eq}/\text{ml}$  packed cells (day), and nearly 3 weeks were required to produce cell sodiums of 40  $\mu\text{eq}/\text{ml}$ . This procedure has the disadvantage that the real independent variable is time at  $0^{\circ}\text{C}$ , and it is possible that parameters other than  $[\text{Na}]_i$  vary during this time. However, the presence of a strophanthin-sensitive efflux indicated that both swine and beef cells were viable after such prolonged storage. Dog cells, on the other hand, proved to be unstable when stored for longer than a week. Substantial hemolysis occurred even at low temperature, and this became unmanageable on incubation at  $37^{\circ}\text{C}$ . Therefore, dog cells were used either when fresh or during the first 5 days after collections.

Before an experiment the stored cells were washed several times with isotonic  $\text{MgCl}_2$  at  $0^{\circ}\text{C}$  and resuspended to make a 10 to 15 per cent suspension. The upper layers of cells were discarded at each wash, to remove leucocytes. The resuspending medium was either stored plasma or a Ringer's solution having either sodium (NaR) or magnesium (MgR) as the predominant cation. The compositions of these salines are given below. The pH was adjusted to 7.4 by gassing with carbogen before use. All

cell suspensions contained adenosine (1 mg/ml) and glucose (1 mg/ml). Heparin was added to the plasma suspensions.

COMPOSITION OF RINGER'S SOLUTIONS\*

	Na	K	Ca	Mg	Cl	Tris†
NaR	140	5.6	4.1	—	150	10
MgR	—	5.6	4.1	90	190	10

\* All values are in millimoles per liter.

† Tris(hydroxymethyl)aminomethane.

The suspensions were decanted into flasks which were suspended in a constant temperature bath (37°C) and shaken at 120/min. During the first 5 minutes of shaking the flasks were equilibrated with the gas mixture following which they were closed with glass stoppers. Each time the flask was opened for sampling the gas phase was reequilibrated with O<sub>2</sub>:CO<sub>2</sub>.

Since there were several methodological differences in handling the three types of cells a detailed procedure will be given first for experiments with swine RBCs. Procedural variations used with the other cells will then be described.

*Swine Erythrocytes* With one exception the experiments were run in plasma. Each cell suspension was divided into 3 aliquots, one of which was placed in a bath at 0°C. The other two were incubated at 37°C. Strophanthin-K (5 µg/ml) was added to one of the latter; the second served as a control. After 5 minutes for gasing and thermal equilibration carrier-free Na<sup>22</sup> was added. A pair of "zero time" samples was immediately withdrawn from each flask for determination of initial cell sodium concentration. Subsequently duplicate hourly samples were removed from each flask. Each sample (ca. 10 ml) was diluted to 40 ml with isotonic choline chloride at room temperature and centrifuged for 3 minutes at 22,000 times gravity. The supernatant was discarded and the cells were resuspended and centrifuged again. A test with C<sup>14</sup>-labeled inulin showed that less than 0.02 per cent of the original extracellular medium was trapped by the packed cells after the second supernatant was removed. The packed cells were hemolyzed and 1 aliquot of the hemolysate was taken for determination of hemoglobin by the acid hematin method of Cohen and Smith (1919). Another aliquot was deproteinized with trichloroacetic acid (TCA). Total sodium was determined by flame photometry and radioactivity by measuring the beta emission of Na<sup>22</sup> either with a conventional end window GM tube, or with a gas flow tube with an ultrathin window.

Influx was computed from tracer entry into the cells, essentially by the unsteady state method of Sheppard and Martin (1950). Efflux was computed from the tracer flux and net change in cell sodium. In the control experiments hourly changes in [Na]<sub>i</sub> were substantial, and even with the limited precision of our flame photometry (see below) estimation of net fluxes was possible. Hence each 1 hour experimental period was treated as an entity. The strophanthin- and cold-treated cells, on the other hand, were nearly in a steady state. Both gained Na, but so slowly that hourly

changes in cell sodium were too small for reliable estimation. Instead, the average net uptake was computed from sodium concentrations determined at the beginning and end of the experiment (2 or 3 hours).

*Beef Erythrocytes* Net changes were generally too small for reliable estimation (on the order of 1 per cent per hour). For this reason influx and efflux were determined by simultaneous measurement of tracer movement on two samples of the same cell population suspended in plasma. Influx was followed on one sample for three 1 hour periods. The aliquots for analysis were suspended in isotonic  $MgCl_2$  (pH 7.4, 0°C) and washed twice. After discarding the supernatant the packed cells were hemolyzed, and aliquots of the hemolysate were used directly (without deproteinizing) for measurement of cell sodium and radioactivity. Efflux was determined on the other sample of cells (they had been previously loaded with  $Na^{22}$ ). In two experiments efflux was calculated from the decrease in intracellular isotope; in seven experiments from the appearance of tracer in the plasma. Appropriate corrections for hemolysis were made in the latter.

Several experiments were run with cells suspended in NaR or MgR. The pH of the Ringer's solutions, originally 9.2–9.3, was decreased to 7.3–7.6 by gassing with carbonogen, and the washed cells were suspended after equilibration with the gas. Measurements made at the beginning and end of each run showed that the pH usually (but not always) decreased by 0.1–0.3 pH unit during the 4 hour incubation.

Efflux was determined from the appearance of tracer in the Ringer's solution; influx was not determined. Samples were taken at 0, 2, and 4 hours after incubation at 37°C (instead of hourly as in the plasma experiments).

Flame photometric measurement of cellular sodium showed that these cells were very nearly in a steady state. The necessary flux computations were made by the steady state procedure of Sheppard *et al.* (1951). Since influx and efflux were both measured isotopically the values obtained (see section on results) provided an independent check on the validity of the assumption.

*Dog Erythrocytes* Estimations of fluxes in plasma and in NaR were made exactly as described for beef cells. In MgR the net loss of sodium from the cells was so large that periodic determination of intracellular sodium sufficed for estimation of extrusion.

*Analytical Reliability* The chief limitation in accuracy derived from measurement of intracellular sodium concentrations. These were determined by a direct reading flame photometer with no internal standard. The standard deviation in a series of readings on one sample was about  $\pm 1$  per cent, but precision was probably not this good in replicate samples worked up independently through the entire preparative procedure. Errors due to interfering materials in the hemolysates were not assessed. However, our values for cell sodium in beef and dog cells agree with those published previously (Kerr, 1937, Bernstein, 1954), and hence it is unlikely that any appreciable systematic error was involved. Since a deproteinized TCA extract was used in the work with pig cells the chance of systematic interference was even smaller (ion standards were made up in the appropriate TCA concentration). However, random variation was appreciable. Part of this was due to the hemoglobin

determination which gave a coefficient of variability of 0.014 between members of twelve pairs in one determination. Part was undoubtedly due to imprecision in the photometry. The over-all coefficient of variability between members of twenty pairs of combined hemoglobin and sodium estimations in two experiments was 0.033. This is very large for the type of information sought in the swine experiments. For example, RBCs with cell sodiums of 20  $\mu\text{eq/ml}$  have an expected net extrusion of 3  $\mu\text{eq/ml}$  (hr.). Due to the variability of the method any value between 1.7 and 4.3  $\mu\text{eq/ml}$  (hr.) might obtain in a single experiment if analyses are not replicated. This will affect the efflux because the net change is used in its calculation. Of course, the range of fluxes noted is extreme; it was obtained by assuming that the two measurements deviated in opposite directions from the real values. All our analyses were done in duplicate, and hence variability should be minimized. In order to approach estimates that are reliable most of the data are summarized in terms of means or regression coefficients instead of appearing in the form of single "typical" experiments.

TABLE I  
SODIUM FLUXES IN STORED MAMMALIAN ERYTHROCYTES

These cells had been stored for about 3 days in all cases. The choice of a 3 to 4 day storage period was arbitrary but permitted comparisons to be made with cells for which storage time was constant. The instability of canine erythrocytes on cold storage (*cf.* Methods) required that the duration of storage be brief.

Animal	[Na] <sub>i</sub>	Unidirectional fluxes			N*
		Efflux‡	Influx‡	Net	
	$\mu\text{eq/ml}$	$\mu\text{eq/ml (hr.)}$	$\mu\text{eq/ml (hr.)}$	$\mu\text{eq/ml (hr.)}$	
Swine	13.4	2.01±0.33	1.06±0.27	-1.0	4
Ox	78.9	10.6±0.9	8.8±1.1	-1.8	5
Dog	124	24.4±0.5	22.9±4.9	-1.6	4

Units for concentrations and fluxes refer to volume of packed cells.

\* N = No. of experiments.

‡ Mean value ± standard deviation.

Influxes in swine cells and all values in bovine and canine RBCs are much less sensitive to this variability. Flame values for sodium were used only to compute specific activities, and net changes were not used in computing unidirectional fluxes. The significant variable here was radioactivity for which the coefficient of variability (combined with hemoglobin determinations) was less than 0.02. Changes in tracer concentration were large from one measurement to the next, and hence the uncertainty was of the same order of size as the coefficient.

## RESULTS

Raw flux data for the three genera are presented in Table I. Two facts are apparent on examination of the table. First, there is a striking difference in the unidirectional fluxes among the three groups. Our values for ox RBCs agree with those published by Sheppard *et al.* (1951), but our fluxes for the dog were

higher by a factor of 1.7. On the other hand, Harris and Pranker (1957) obtained values of about 21  $\mu\text{eq/ml (hr.)}$  for canine cells. Sodium fluxes in swine erythrocytes have not been published before. It should be noted that the values are for cells that had been stored for 3 days before the measurements were made, and the cell sodium was therefore higher than it would have been in fresh cells. The fluxes were also probably somewhat higher since there is a correlation between tracer movement and cell sodium (see below). However, it is obvious that sodium movement in these cells approximates that in human erythrocytes, and is far slower than in beef or dog. The other point to note is that the tracer flux seems to vary directly with the cell sodium concentration.

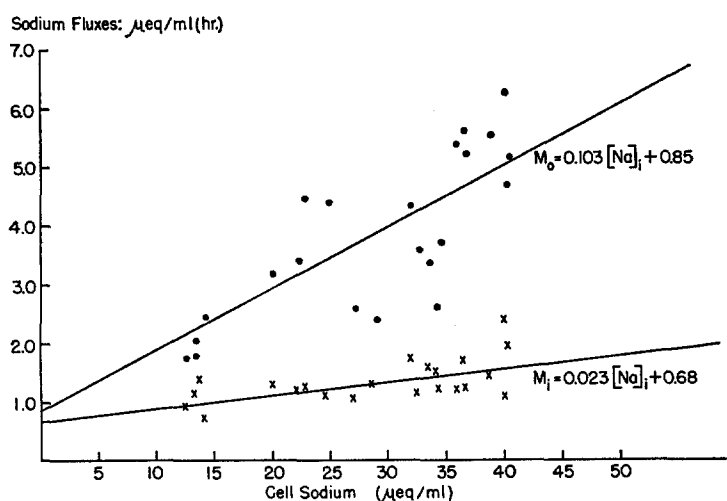


FIGURE 1. Sodium fluxes as a function of  $[Na]_i$  in swine erythrocytes. Influxes (×) and effluxes (○) at different cell sodium concentrations. The points represent twenty-two experimental periods (60 to 90 minutes each) in nine experiments. The equations were obtained by the method of least squares and the lines constructed from them.

More will be said about this correlation in the Discussion. It is perhaps worth pointing out here that the efflux, determined by tracer movement, is obviously not a valid measure of *active* sodium extrusion in erythrocytes.

*Fluxes in Swine Erythrocytes* Fig. 1 shows the dependence of influx and efflux on intracellular sodium concentration. The data are described by the equations under each line; neither logarithmic nor hyperbolic equations provided a better fit. However, the relationship obviously must become non-linear for efflux at lower concentrations. An over-all regression coefficient ( $k_e$ ) is obtained from the slope of the line for efflux, and another ( $k_i$ ) from the slope for influx. When the cells were exposed to strophanthin there was little change in influx at any concentration; neither slope nor intercept is significantly different

from those for untreated cells (Fig. 2 A). In the lower half of Fig. 2 efflux is plotted as a function of concentration and it can be seen that strophanthin caused a sharp drop in  $k_e$ . In fact  $k_e$  is essentially equal to  $k_i$  in the presence of this inhibitor.

The existence of a correlation of *influx* with intracellular sodium concentration was unexpected. On the simplest hypothesis  $k_i$  is simply a measure of sodium diffusion through the membrane, but this quantity should not be affected by the intracellular concentration. However, if exchange diffusion were occurring, its rate might well be limited by the low intracellular concen-

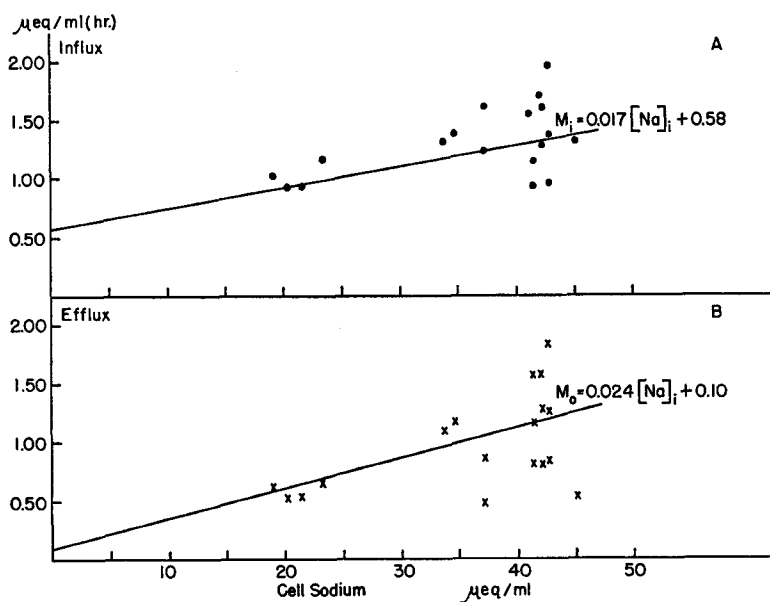


FIGURE 2. Sodium fluxes in strophanthin-poisoned swine erythrocytes. A, sodium influx; B, sodium efflux. Strophanthin ( $5 \mu\text{gm/ml}$  suspension) was added at the beginning of the experiments.

tration, rising as the latter increased. Exchange diffusion can be abolished by suspending erythrocytes in a sodium-free medium, and hence the efflux of tracer should be smaller for cells suspended in MgR than for cells in NaR. Table II shows the results of two such experiments in which the efflux of  $\text{Na}^{22}$  was measured for two successive 1 hour periods. It is apparent that the tracer movement from cells in normal Ringer's solution was larger during both periods, and hence these data also indicate the presence of a small exchange component in the total unidirectional tracer fluxes. This, in turn, explains two points in Figs. 1 and 2; and the coincidence in regression coefficients for  $k_i$  (control) and both  $k_i$  and  $k_e$  in strophanthin-treated cells. In all cases, the



variable depending on cell sodium is the exchange component, and this is insensitive to strophanthin.

If this explanation is correct it is possible to partition the total fluxes quantitatively into their components. Tracer influx, according to the model described above, consists of leak and exchange fluxes. The former should be independent of cellular sodium concentration, but cell sodium is rate-limiting for the latter, and causes influx to increase as it rises. The regression coefficient ( $k_x$ ) for influx ( $M_{in}$ ) vs concentration ( $[Na]_{cell}$ ) is therefore a measure of exchange diffusion. The average for all influx data in Figs. 1 and 2 *A* is 0.021 hr.<sup>-1</sup>. A coefficient for the leak pathway ( $k_d$ ) can be computed by subtracting  $k_x$  from the regression coefficient ( $k_e$ ) for the efflux concentration curve in strophanthin-treated cells (Fig. 2 *B*) which gives a value of 0.003 hr.<sup>-1</sup>. An

TABLE II  
SODIUM EFFLUX IN NaR AND MgR: SWINE RBCs

Cells were loaded with Na<sup>22</sup>, washed, and divided into two groups. One was resuspended in NaR, the other in MgR. Both suspensions contained adenosine (1 mg/ml) and glucose (2 mg/ml) They were brought to 38°C and the extrusion of Na was computed from the appearance of Na<sup>22</sup> in the extracellular medium after 1 and 2 hours. The fluxes in NaR are somewhat higher than those noted for cells in plasma at similar cell sodium concentrations. The reason for this difference was not investigated.

Experiment	Solution	Cell Sodium $\mu\text{eq/ml}$	Efflux	
			0-1 hr. $\mu\text{eq/ml (hr.)}$	1-2 hrs. $\mu\text{eq/ml (hr.)}$
1	NaR	15.9	4.24	3.22
	MgR		3.03	2.98
2	NaR	17.0	5.06	4.59
	MgR		3.26	3.26

alternative method is to extrapolate the line for influx back to zero cell sodium where the residual flux should be pure diffusive movement. The extrapolated values from Figs. 1 and 2 *A* and the plasma sodium concentration (155  $\mu\text{eq/ml}$ ) give a value of 0.004 hr.<sup>-1</sup>. The agreement with the first method should not be overemphasized for the extrapolation requires that the influx regression line remain linear at low cell sodium concentrations. There is no evidence for this, and it is, in fact, unlikely to be true if the dependence of influx on concentration is due to a chemical system that shows saturation kinetics.

The coefficients  $k_x$  and  $k_d$  characterize the influx of sodium for swine RBCs, and for the concentration range studied here we can write the function  $M_{in} = k_x [Na]_{cell} + k_d [Na]_{plasma}$ . Efflux, on the other hand, consists of an additional component, also dependent on intracellular concentration, namely flux through the pump. The pump flux, like exchange diffusion, is unlikely to be a

linear function of concentration. However, over this range of concentrations total efflux is essentially linear as shown in Fig. 1. Since  $k_x$  and  $k_d$  behave like linear coefficients in this range the slope of the efflux line in Fig. 1 ( $k_e$ ) can be written ( $k_p + k_x + k_d$ ) where  $k_p$  is a pump coefficient. The data give  $k_p = 0.079 \text{ hr.}^{-1}$ . Thus the pump is responsible for 75 to 80 per cent of the efflux, exchange for nearly 20 per cent, and leak diffusion for only 3 per cent.

The influx was markedly slowed when the experiments were conducted at  $0^\circ\text{C}$ . Calculation of a "rate coefficient" from the flux data gave a value of  $0.003 \text{ hr.}^{-1}$  and the quantitative correspondence with  $k_d$  suggests that the residual flux may be due to passive leak. If this is true, the temperature-sensitive component is (according to this model) exchange diffusion. There was a net gain in  $[\text{Na}]_i$  in nearly all the experiments (eight out of nine). However, the change was too small to be estimated reliably in 2 or 3 hours, and hence effluxes were not computed.

TABLE III  
SODIUM FLUXES IN PLASMA: OX ERYTHROCYTES

Experiment	Cell sodium	Fluxes			N	
		Efflux	Influx	Net		
	$\mu\text{eq/ml}$	$\mu\text{eq/ml (hr.)}$	$\mu\text{eq/ml (hr.)}$	$\mu\text{eq/ml (hr.)}$		
Strophanthin	Control	79.3	$10.6 \pm 0.9$	$8.8 \pm 1.1$	-1.8	5
	Experimental		$9.2 \pm 1.5$	$9.0 \pm 1.4$	-0.2	
Cold ( $0^\circ\text{C}$ )	Control	89.7	$17.0 \pm 2.1$	$13.8 \pm 1.6$	-3.2	4
	Experimental		$1.09 \pm 0.48$	$0.97 \pm 0.18$	-0.1	

*Fluxes in Beef Erythrocytes* The internal sodium in beef RBCs increases when they are stored at  $0^\circ\text{C}$ , but so slowly that after 4 weeks of storage  $[\text{Na}]_i$  has risen only from 70 to about  $90 \mu\text{eq/ml}$ . The range was too narrow to study the variation of fluxes with concentration, and the experimental approach developed by Tosteson and Hoffman was used for partitioning the total fluxes. First, however, the fluxes of cells suspended in plasma were measured under three sets of conditions; for untreated cells at  $37^\circ\text{C}$ , for cells exposed to strophanthin, and for untreated cells at  $0^\circ\text{C}$ . The design of the experiments, as described under Methods, was such that one set of untreated cells served as controls for the strophanthin runs and another as controls for the low temperature group. From a statistical point of view the design pairs every efflux with an influx value; each strophanthin and each low temperature treatment is paired with a control. Table III shows mean effluxes and mean influxes for cells exposed to strophanthin and low temperature. The same data are shown for the corresponding controls. An unbiased estimate of the standard deviation for each mean is also shown. Analysis of the data shows that efflux is signifi-

cantly higher than influx in the untreated cells ( $p < 0.01$  for a one tailed test with 8 degrees of freedom). One group of untreated cells had been stored for 3 weeks and had a higher cell sodium than the other group which had been stored for only a week. The former showed a greater net extrusion, but much of this was due to one run (net efflux of  $7.5 \mu\text{eq/ml (hr.)}$ ). We feel that much more extensive work would be required to confirm the significance of this apparent dependence on cell sodium (or storage time). There is little question, however, that a net extrusion of sodium occurs from stored beef erythrocytes. Further analysis shows that reduction of efflux by strophanthin is marginally significant ( $p < 0.05$  for a one tailed test), and data to be described below confirm this. Strophanthin has no effect on influx. Both efflux and influx are markedly temperature-dependent ( $p < 0.001$  for both fluxes).

The data in Table III show that  $\text{Na}^{22}$  movement is considerably faster in both directions than it was in pig RBCs. Lack of sensitivity to strophanthin suggested that most of the tracer movement is not mediated by the Na-K pump. On the other hand, the sensitivity to temperature is at least compatible

TABLE IV  
SODIUM EFFLUX IN NaR AND MgR: OX RBCs

Medium	$[\text{Na}]_i$	Efflux	<i>N</i>
		$\mu\text{eq/ml (hr.)}$	
NaR	79	$11.2 \pm 2.4$	5
MgR	75	$1.40 \pm 0.40$	8

with some chemical mechanism; again, exchange diffusion seemed to be a likely possibility. RBCs loaded with  $\text{Na}^{22}$  were suspended in MgR, and tracer efflux was measured. The data in Table IV show that although efflux in NaR is not appreciably different from that in plasma, cells in MgR can extrude sodium only 10 per cent as fast. A similar decrease in extrusion occurred when the cells were suspended in a sodium-free Ringer's solution based on choline or tetramethyl ammonium. The residual efflux is presumably due to active extrusion plus diffusion from the high sodium interior. Inhibition of the pump under these conditions (*i.e.* in MgR) should permit us to estimate its contribution, and the residual flux ought to represent the leak. The transport mechanism was inhibited in two ways; with strophanthin, and by inhibiting glycolysis with iodoacetamide. Data from a pair of experiments are shown in Table V. It can be seen that efflux in MgR is sharply reduced, and to about the same extent by both compounds. We have not computed coefficients for beef cells for we lack even the empirical basis provided by Figs. 1 and 2 for the assumption of linearity. The flux variations shown in Tables IV and V make it apparent that about 85 to 90 per cent of the total efflux is exchange diffusion

TABLE V  
INHIBITION OF EFFLUX IN MgR: OX RBCs

Treatment	Efflux		N
	0-2 hrs.	2-4 hrs.	
	<i>μeq/ml (hr.)</i>	<i>μeq/ml (hr.)</i>	
Control	1.31±0.44	1.48±0.40	4
Strophanthin (5 μg/ml)	0.32±0.03	0.45±0.13	2
Iodoacetamide (5 × 10 <sup>-4</sup> M)	0.27±0.05	0.40±0.14	2

TABLE VI  
SODIUM FLUXES IN PLASMA: DOG RBCs

Treatment	Fluxes			N
	Efflux	Influx	Net	
	<i>μeq/ml (hr.)</i>	<i>μeq/ml (hr.)</i>	<i>μeq/ml (hr.)</i>	
Control	24.4±0.5	22.9±4.9	-1.5	4
Strophanthin (5 μg/ml)	24.9±8.7	25.3±5.0	+0.3	4
Cold	1.04±0.66	2.65±1.89	+1.6	6

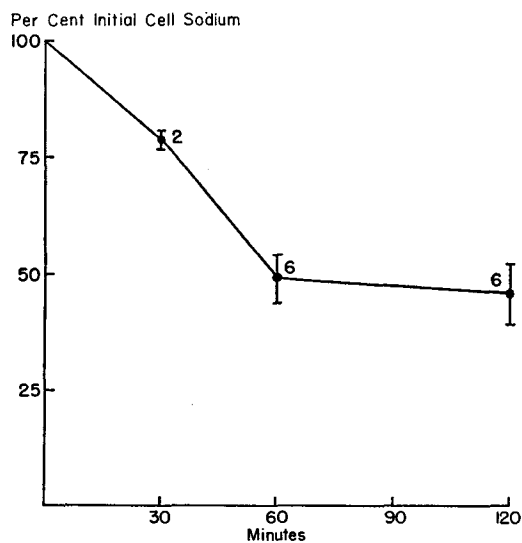


FIGURE 3. Sodium extrusion from dog erythrocytes in sodium-free Ringer's. The cells were suspended in magnesium Ringer's solution at time zero. Cell sodium remaining during the experiment is expressed as percentage of the initial concentration. The circle is an average of several experimental values, and standard deviations are shown by the vertical lines. The number of experiments run is indicated by the numbers next to the points.

under these conditions. The pump accounts for only about 10 per cent and leak diffusion for about 3 per cent of the total. Thus, the pump is much less active in beef cells than in swine.

*Dog Erythrocytes* Canine RBCs present a case apparently similar to beef. Tracer fluxes are very high, nearly three times those in the ox, and they are sensibly unaffected by strophanthin. However, they are much slower at low temperature. These data are shown in Table VI.

An attempt was made to separate the movement into components by suspending the cells in MgR. In this case there was no inhibition of the efflux; instead a large net extrusion of more than half of the cell sodium occurred during a 2 hour experiment. This phenomenon is shown in Fig. 3. It was also noted in two of these experiments that the net loss of sodium, like the tracer movement, is unchanged by strophanthin. This might suggest that the rapid tracer movement and net extrusion are mediated by the same system. The difference between this case, on the one hand, and that in swine and ox cells is apparent. It will be considered further below.

#### DISCUSSION

It is clear from the foregoing that our data have been interpreted in terms of a model in which the fluxes and distribution of sodium and potassium are controlled by the membrane. This is only one of two models currently in vogue; in the other high intracellular potassium is due to the affinity of intracellular anions for this ion. The membrane is merely a morphological boundary with no special functional characteristics apart from a high resistance to ionic diffusion (Kurella, 1961, Simon, 1961, Ling, 1960, Harris and Pranker, 1957). Adherents of both interpretations agree on one point; that the *other* model leaves unexplained certain observations, some of them important. The gap between them is otherwise broad as was made clear in a recent symposium (Kleinzeller and Kotyk, 1961). A detailed review of the controversy is clearly out of place in an experimental paper. Yet a definite point of view has been adopted here, and some reasons for our preference for the membrane model should be cited.

The heuristic value of the model has been enormous (Ussing *et al.*, 1960). It has allowed us to integrate into a consistent picture not merely the intracellular ionic pattern, ionic fluxes, and transmembrane resting potentials in many types of cells but with relatively minor modifications transepithelial potentials and ionic translocation across layers of cells. Thus, the same coupled sodium-potassium transport mechanism that has been proposed to account for ionic behavior in single cells also accounts for the movement of sodium chloride and for electrical potentials across the amphibian skin (Ussing, 1960) as well as the vertebrate nephron (Whittembury *et al.*, 1961). No variant of the "fixed

charge" hypothesis, on the other hand, has done more than account for the high intracellular potassium and low sodium, and for an apparent decrease in tracer flux with time. Quantitative correlation between potassium distribution and membrane potential (which is one of the important arguments for rejection of the membrane model) has been even less successful on the basis of the internal anion model, and it has even been proposed that the potential is an artifact introduced by the electrodes (Kurella, 1961). Actually, of course, it has been clear for some years that unless cells are in a steady state the membrane potential may be set by any combination of diffusible ions; the relationship depends only on penetrabilities and distributions (Hodgkin, 1951). Another objection to membrane-mediated movement is based on the observation that tracer equilibration cannot be described as a simple exponential function of time. This case has been treated quantitatively within the framework of a modified internal anion model (Harris and Pranker, 1957), but it has been pointed out that deviations from the behavior expected of a membrane model can equally well be explained by the geometry of the system (in muscle, *cf.* Hodgkin and Horowicz, 1959), or by heterogeneity in a population of cells (in erythrocytes, *cf.* Joyce, 1958). One datum from this paper would seem to support a role for the membrane in ion movement. The component of total sodium efflux termed exchange diffusion can hardly represent leak diffusion from an intracellular pool since the requirement for extracellular sodium in swine and beef cells is not characteristic of diffusive movement. This flux has a high temperature coefficient, and in sheep cells at least, seems to show saturation kinetics (Tosteson and Hoffman, 1960); both observations are commensurate with a chemical mechanism. On the other hand, it is insensitive to strophanthin, and hence differs from the active mechanism. The membrane hypothesis has no difficulty in accommodating this phenomenon, and a possible relationship between the exchange system and the pump will be discussed below.

The most serious limitation of the internal anion models is their inability to account for ionic fluxes and potential differences across epithelia. It is possible to insist that there need be no connection between cellular phenomena and those in epithelia (*e.g.* Troshin, 1961),<sup>2</sup> but this argument only emphasizes the heuristic character of the membrane model. Moreover, there are such marked experimental similarities between fluxes in both systems as to render the argument suspect. Thus, strophanthin inhibits not only sodium extrusion from cells (Schatzman, 1953), but sodium transport across epithelia such as the frog skin (Koefoed-Johnsen, 1957). Potassium-free solutions are inhibitory in both types of system (Hodgkin and Keynes, 1955, Huf and Wills, 1951). The

<sup>2</sup> The remark referred to occurs in a discussion on page 107 of the symposium cited in the Bibliography.

same is true for inhibitors of cholinesterase (Van der Kloot, 1956, Kirschner 1953). An ATPase requiring both sodium and potassium for activity has been ascribed a role in ion movement for reasons summarized by Post (1961). This enzyme, described first by Skou (1957) in nerve, has recently been found in mammalian kidney (Whittam and Wheeler, 1961). The existence of the enzyme argues for no particular mechanism, but its occurrence in both epithelia and populations of cells is at least worthy of note in the context of this argument.

Thus, while some flux data and the particular ion distribution found in animal (but not plant) cells can be explained by the fixed charge hypothesis, membrane-mediated transport accounts for an enormously wider range of phenomena and requires fewer *ad hoc* or untestable hypotheses.

It is quite clear that the Na-K pump is much less effective in beef RBCs than in swine, and it is probable that the mechanism in dog cells is the weakest in this series. Our data fail even to establish the existence of such a pump in the dog although one must exist in order to prevent osmotic hemolysis. As noted above membrane leakiness is similar in both swine and bovine cells, perhaps somewhat less in the former. There is also excellent agreement between  $k_i$  for swine cells and for the low sodium sheep erythrocytes studied by Tosteson and Hoffman. The values are only about one-third of that found by Harris and Maizels (1952) for human RBCs. This may indicate that the latter are leakier than pig and sheep cells, or it might simply indicate that there was significant exchange diffusion in human cells. Pump to leak ratios are about 25 for swine cells and 3 for beef. Corresponding values for low and high sodium sheep cells are about 7 and 1. High cell sodium in both instances is due to a much less active extrusion mechanism in membranes with leaks differing little from low sodium systems. It is worth noting that cell sodium concentration varies inversely with pump to leak ratios in the four cases cited. Unfortunately neither pump nor diffusion leak in canine RBCs can be assessed from our data.

It was interesting to find differences in exchange diffusion among the three genera of animals. The positive correlation of exchange flux with cell sodium seems to be a very general phenomenon. In addition to the data described above we found one Jersey cow with a cell sodium level lower than the other beef RBCs but higher than swine; it also showed tracer movements intermediate between the two (unpublished experiments). Although the difference was smaller the same correlation existed in sheep RBCs. It is possible that the relationship is fortuitous, but it is so general that a meaningful phenomenon might be involved. For example, the conversion of a sodium carrier to a potassium carrier might be the rate-limiting step in transport in these systems. Inefficient conversion would limit the activity of the pump, and at the same time provide a high concentration of the sodium form of the carrier which can shuttle back and forth. A low activity pump can explain the high cell sodium

and is in accord with the data on pump activity *vs.* sodium concentration; the high rate of exchange diffusion would then be due simply to a high concentration of the sodium form of the carrier at the outer edge of the membrane. Differences among RBCs in  $k_x$  would, on this hypothesis, reflect variations in permittivities to the sodium carrier. In this light the data of Tosteson *et al.* (1960) become very interesting. They were able to show that a strophanthinsensitive, sodium-activated ATPase is present at high levels in ghosts of low sodium sheep cells, but in very small quantities in the high sodium system. Assuming, as several investigators (Post, 1961, Skou, 1960) have done, that this enzyme is involved in some energetic step in transport these observations can all be rationalized if the energetic step is the conversion of a sodium-specific compound into one that is potassium-specific.

Evidence for exchange diffusion in canine erythrocytes is indirect. There is a very high tracer turnover, and this might, in the absence of other evidence, be due either to exchange or simply to a very leaky membrane. The marked inhibition of this flux by low temperature would seem to argue against simple diffusion through aqueous pores. On the other hand, the system differs from the exchanges in swine, beef, and sheep cells in that it is not rigidly coupled to external sodium but can apparently either lose NaCl or take up magnesium in exchange for cellular sodium. A recent report by Rogers (1961) showed that the uptake of  $Mg^{28}$  by dog cells is very rapid while uptake by beef cells is so slow that he was unable to measure it. This is exactly what one might deduce from our data. The exchange in canine cells is consistent with the picture presented some years ago by Davson for RBCs from the cat (Davson, 1940). When these erythrocytes were suspended in isotonic KCl there was a very rapid extrusion of Na, and part of this involved exchange with external K. Sodium extrusion was very rapid (on the order of the net changes noted in Fig. 3), and it was sensitive to external anion, to pH, and to temperature. For these reasons Davson suspected that some chemical process was involved.

One feature emerging from these data may be significant for investigation of the biochemical mechanisms underlying ion movement. It is obvious that marked quantitative differences exist in the underlying chemical systems in different mammalian RBCs. It follows that a comparative study may show useful correlations between, for example,  $k_p$  and the rate of turnover of a compound suspected of playing a role in active transport. And if our suggestion that a connection may exist between exchange diffusion and the pump can be substantiated we have another variable the biochemical correlates of which may afford an insight into the chemical reactions involved in non-diffusion ion movement.

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