J. Physiol. (1956) 134, 278–310

SODIUM AND POTASSIUM MOVEMENTS IN HUMAN RED CELLS

By I. M. GLYNN

From the Physiological Laboratory, University of Cambridge

(*Received* 16 *April* 1956)

Human red cells are generally believed to maintain their ionic composition by pumping potassium in and sodium out, using energy derived from glycolysis. In the present experiments ^{24}Na and ^{42}K have been used to study the movements of sodium and potassium across the red cell membrane under a variety of experimental conditions. The experiments fall into two groups. Those of the first group were designed to investigate the existence of a link between the active uptake of potassium and the active extrusion of sodium. Such a link is suggested by the complementary nature of the movements of potassium and sodium during incubation after cold-storage, and by observations on sodium movements at low external potassium concentrations (Harris & Maizels, 1951; Shaw, 1954). The aim of the experiments of the second group was to see whether the passive fluxes of sodium and potassium could be explained by free diffusion of the ions, in aqueous solution, through holes in the membrane. A preliminary account of this work has already been published (Glynn, 1954).

METHODS

Procedure. Human red cells were used in all experiments. The blood was usually freshly drawn but in a few experiments was stored at 5° C for up to 6 hr. Clotting was prevented by the addition of heparin (Pularin, Evans Medical Supplies) to give a concentration of 250,000 u./100 ml. Immediately before use the blood was centrifuged, and the plasma and buffy coat removed by suction; the cells were then washed twice in Ringer's solution by alternate resuspension and spinning. White cells were almost completely eliminated (see Table 1) by removal of the topmost layer of cells, with the supernatant, after each wash.

For influx experiments 20 ml. lots of the radioactive suspending media were placed in 50 ml. Erlenmeyer flasks and shaken in a water-bath until they had reached a steady temperature. At a known time a small volume, say 1 ml., of packed red cells prepared as above, was added to each of the flasks. Since the exact volume of cells did not matter it was possible to add the cells to twelve flasks within about 1 min. The flasks were shaken for a known time and the contents then transferred to 5 in. Pyrex test-tubes and centrifuged for 3 min. It took about 1 min to transfer the flask contents to tubes, and this was done in the same order as that in which blood was added to the flasks. Systematic errors due to inaccuracies in timing were avoided by staggering the test solutions. After spinning, the supernatants were sucked off and the cells washed three times in

Ringer's solution free from radioactive ions. The washed cells were then lysed, and the activities of the haemolysates determined.

Two possible sources of error must be considered. The first concerns the adequacy of the washing procedure. If the volume of the trapped fluid was 0·5 ml. and the volume of washing fluid 10 ml., each wash led to a twentyfold dilution of external activity. The three washes therefore reduced the activity of the trapped fluid by 8000. Even if the volume of trapped fluid was equal to the volume of the cells, the additional activity of the cell phase due to inadequate washing was only about 1/16,000 of the original activity of the outside solution. In all experiments this additional activity was less than 1% of the activity in the cells.

TABLE 1. Estimate of white cell contamination

Sample	White cell count
Original blood	7,900 ± 600
Packed red cells washed twice and resuspended for counting in equal vol. plasma	33 ± 12

The second possible source of error is loss of radioactive material from the cells during washing. By centrifuging for only three minutes after each wash, it was possible to complete the washings in about half an hour. At room temperature cells lose about 0·2% of their potassium in this time, so for potassium this error may be neglected. If cell sodium is taken to be 10 m-mole/l., the expected loss of sodium into Ringer's solution at room temperature is about 3% in half an hour. For this reason ice-cold potassium-free Ringer's solution was used as a washing fluid in sodium influx experiments, giving an estimated loss of a little under 1%.

In the experiments designed to measure tracer efflux, cells were first incubated for several hours with a solution containing radioactive sodium or potassium, and were then washed five times with equal volumes of a non-radioactive Ringer's solution. A small volume of cells, say about 1 ml., was added to each of the test solutions, previously warmed, and the suspensions were shaken in the water-bath. At the end of the period of incubation the flasks were well shaken and about two-thirds of the contents of each was transferred to a 5 in. Pyrex test-tube. After spinning for 3 min the supernatants were transferred to clean tubes and spun again to get rid of the few cells which, occasionally, were stirred up. The supernatants from the second spinning were put aside for determination of activity. The remainder of the contents of each flask was shaken thoroughly, and 1 ml. carefully removed with a tuberculin syringe and added to a known volume of water. From the colour of the solutions obtained in this way the quantity of red cells in the suspensions could be calculated.

It is necessary to consider how much activity is likely to have been added in the fluid trapped between the cells, as this will have appeared in the supernatants. If the volume of trapped fluid was 10% of the cell volume, fivefold washing with equal volumes of wash fluid should have led to a 100,000-fold reduction in the activity of the trapped fluid. If no activity leaked out of the cells during washing, the amount of activity likely to have been added in the trapped fluid is a small fraction of 1% of the activity leaving the cells during the experiment. Much more serious than activity carried over from the original incubating solution is activity leaking from the cells in the interval of, perhaps, 10 min between the final wash and the start of the experiment. To minimize changes in the amount and activity of the cell sodium, sodium efflux was measured over periods of about 20 min, so that the 10 min interval is equal to about half the duration of the experiment. If the Q_{10} for sodium efflux is 2·2 (see p. 299), the expected loss before the experiment begins is about 10% of the loss during the experiment. In order to correct for this error, immediately after adding the cells to the flasks containing the test solutions, a sample of cells was added to a flask of cold Ringer's solution which was then thoroughly stirred. Most of this suspension was at once centrifuged, and the rest was used to determine the quantity of cells present. From the activity of the supernatant and the quantity of cells present, the size of the error could be calculated and a correction made.

Temperature control. Two thermostatically controlled water-baths were used; in each, the temperature did not vary by more than 0·1° C from time to time or from one part of the bath to another. The incubating solutions were always allowed to stand in the bath for 20 min before the start of the experiment; this time was ample for them to reach bath temperature. Since only small volumes of cells were added to large volumes of solution, it may be assumed that the cells reached bath temperature within a period of time negligibly short compared with the duration of the experiment.

Estimation of radioactive isotopes. A conventional skirted liquid counter (20th-Century Electronics, type M6 M), fitted with a drainage tube and constant level device, was employed. To estimate the activity of cells, the cells were first lysed with about ten volumes of distilled water, and the haemolysate diluted till its activity was convenient for counting. The haemoglobin content (in arbitrary units) of the diluted haemolysate was subsequently determined by colorimetry. At the end of the experiment the activities per quantity of haemoglobin were converted into activities per volume of cells, using the optical density of a solution prepared by lysing a known weight of cells with a known volume of water. The chief advantage of this method of estimating activity is that small changes in cell volume during the course of the experiment do not introduce errors.

Counting errors. By counting at rates near 1000/min whenever possible, the background correction was made small and the correction for paralysis negligible. (Paralysis time for the scaler was 300 μsec.) Unless the samples were very weak, counting was continued for a time long enough to include about 10,000 counts, so that the standard error of counting was usually about 1%.

Sodium and potassium estimations were made with an 'EEL' flame photometer. The samples were diluted with distilled water to give concentrations within 20% of the standards. Measurements were generally made in quintuplicate, alternating standard and unknown, and results obtained in this way gave a mean with a standard error of between 1 and 2%. Cells were lysed, but not ashed, before analysis.

Haemoglobin was estimated as oxyhaemoglobin by measuring the optical density with a Hilger 'Spekker' colorimeter using an Ilford narrow-band yellow-green filter. Solutions were diluted to give an optical density of less than 0·4. Under the conditions used, solutions were stable and obeyed Beer's law. Preliminary investigation showed that results were reproducible to within 1%.

Correction for haemolysis. Lysis was estimated by measuring the optical density of the suspension medium at the end of the experiment, and comparing it with a standard solution of lysed red cells. The standard solution was prepared by lysing a weighed quantity of packed cells and making the solution up to a known volume. The density of the packed cells was assumed to be 1·09. In the sodium efflux experiments, a correction of 1 or 2% was made for sodium released by lysed cells; in potassium efflux experiments the correction was between 5 and 10%. Presumably there are always a few cells, leaky to sodium and potassium, which do not lose their haemoglobin, but there is no way of estimating the error produced by these cells.

Cell packing and haematocrit determinations. The cells used for preparing standard haemoglobin solutions were centrifuged at 1500 g for 1 hr—a period long enough to give maximal packing with this force. Shohl & Hunter (1941), using Evans Blue to determine plasma volume, showed that the volume of fluid trapped between cells packed under similar conditions is not greater than 4% of the total volume.

The cells used for influx and efflux experiments were packed for 10 min at 1500 *g*. The volume of trapped fluid is here about 10 or 12% of the cell volume. Since the cells were usually added to 10–30 times their own volume of suspension medium, dilution of the medium by the trapped fluid may be neglected. Increase in the concentration of individual constituents of the suspension medium, due to their addition in the trapped fluid, was avoided by judicious selection of the final washing fluid.

Haematocrits were determined by spinning for 10 min at 1500 *g* in tubes of the Wintrobe type. With haematocrits near 50%, results were reproducible to within 1%, but with suspensions

containing only 10% of cells the error was probably between 5 and 10%. Since the flux measurements did not depend on estimates of cell swelling or shrinking, haematocrits were measured in only a few of the experiments, to exclude large changes in cell volume. For this purpose the method was adequate.

Preparation of the isotopes. The isotopes were supplied by the Atomic Energy Research Establishment, Harwell, and were prepared by methods worked out by Keynes (1954). ^{24}Na was supplied as irradiated sodium bicarbonate. The salt was dissolved in a slight excess of 2N-HCl, and the resulting solution dried at 100° C over solid NaOH. ^{42}K was supplied as irradiated potassium bicarbonate and prepared as described by Hodgkin & Keynes (1953).

The amount of irradiation received by the cells during the experiments was usually less than 2·5 r/hr with ^{24}Na, and less than 1·5 r/hr with ^{42}K. Sheppard & Beyl (1951), using potassium loss as an index of cell damage, found that doses of less than 1000 r did not appear to harm the cells.

The suspending media. In most of the experiments the cells were suspended in media prepared by mixing calculated volumes of the following two solutions:

(i) 'Na Ringer's' solution—Na^+, 151 mM; Mg^{2+}, 1 mM; Ca^{2+}, 2·2 mM; Cl^-, 149 mM; phosphate, 2·5 mM: pH, measured with a glass electrode, $7{\cdot}3 \pm 0{\cdot}1$.

(ii) 'K Ringer's' solution—K^+, 146 mM; Na^+, 4·7 mM; Mg^{2+}, 1 mM; Ca^{2+}, 2·2 mM; Cl^-, 149 mM; phosphate, 2·5 mM: pH, measured with a glass electrode, $7{\cdot}3 \pm 0{\cdot}1$. The 'K Ringer's' solution was buffered with *sodium* phosphates so that the same stock buffer solution could be used to make up both 'Ringer's' solutions.

In a few of the experiments some or all of the sodium in the suspending medium was replaced by choline. Solutions of choline chloride were prepared by dissolving the solid, supplied by Messrs Roche, in distilled water. They were always made up within 48 hr of being used, and were stored at 5° C. Glucose, when required, was added as a 20% solution to give a final concentration of 100 or 200 mg/100 ml. The 20% solution was always freshly prepared on the day of use.

Washing solutions. For washing it was sometimes necessary to have balanced solutions free from sodium or potassium or both. The 'Na Ringer's' solution, described above, provided a convenient potassium-free solution. A sodium-free solution was prepared by making up the 'K Ringer's' solution as described but with potassium phosphate buffer. When a fluid free from both sodium and potassium was required, isotonic choline chloride was used, or a solution containing 151 mM choline, 1 mM-Mg^{2+}, 2·2 mM-Ca^{2+} and 154 mM-Cl^-.

RESULTS

The active fluxes

If the active efflux of sodium is linked to the active influx of potassium, a factor which affects one of these fluxes should also affect the other. Such an effect, if observed, would be evidence for the existence of a linkage, provided that the factor were one that was not, otherwise, likely to affect both of the fluxes. For example, both potassium influx and sodium efflux are known to depend on energy from glycolysis, so it is no evidence of linkage that they are both reduced by depriving the cells of glucose. On the other hand, if removal of potassium from the outside medium could be shown to abolish or reduce sodium efflux, this would be suggestive of a linkage; and if, as the outside potassium was removed, reduction in sodium efflux were found to parallel the falling off of potassium influx, the evidence would be much stronger. The first experiments were therefore designed to investigate the relationship between potassium influx and external potassium concentration.

Potassium influx

Effect of external potassium concentration on potassium influx. Cells, washed with potassium-free Ringer's solution, were added to a series of flasks containing glucose-Ringer's solutions in which different amounts of sodium had been replaced with ^{42}K-labelled potassium. The flasks were incubated at 37·5° C, and samples were removed from time to time over about 2·5 hr. After centrifuging, the cells were washed and analysed for ^{42}K. A graph of activity against time was drawn for each flask and from the slopes of these graphs the influx at each concentration was calculated.

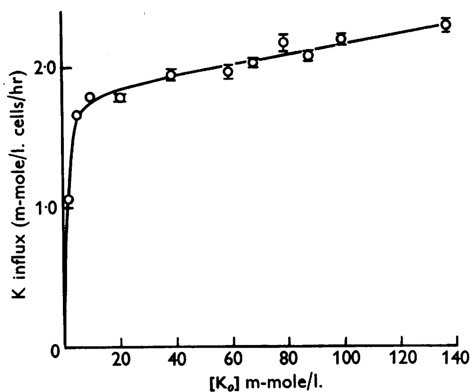

Fig. 1

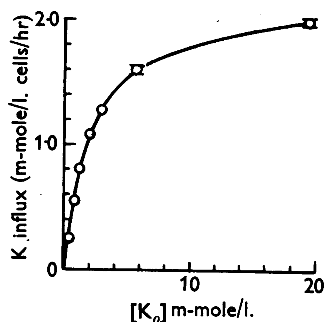

Fig. 2

Fig. 1. Expt. no. R.N. 1. The effect of external potassium concentration on potassium influx. Glucose concn. 100 mg/100 ml.

Fig. 2. Expt. no. W.A.H. 1. The effect of external potassium concentration on potassium influx. Glucose concn. 100 mg/100 ml.

The results of two experiments are given in Figs. 1 and 2. The first covers the range of potassium concentrations from 2 to 137 mM (sodium varying from 149 to 14 mM), and the second covers, rather more closely, the range from 0·39 to 20 mM (sodium varying from 150 to 131 mM). Four further experiments—one covering the whole range up to 140 mM-K, one devoted mainly to the linear part of the curve above 20 mM-K, and two restricted to the range below 10 mM-K—gave results in line with those shown, and will not be discussed further.

The errors likely to have been introduced by the washing procedure have been considered in the section on Methods and are very small. Another possible source of error is increase in the external potassium concentration caused by leakage of potassium from the cells during the experiment. This error was made negligible, even at very low potassium concentrations, by the use of very dilute cell suspensions—about 5%. The potassium concentration in each suspension medium at the end of the experiment was determined by

flame photometry. The changes were not very great, and since the potassium leaking from the cells was practically inactive, while potassium influx at very low concentrations is almost proportional to the concentration, the increased influx must have been almost exactly offset by decreased specific activity in the medium.

Fig. 1 shows that potassium influx increases rapidly with the external potassium concentration at very low concentrations, but levels off as the potassium concentration is increased. The curve never becomes horizontal but has a linear portion with an appreciable slope. Before the interpretation of this curve is discussed, it is necessary to compare these results with those of other workers, since although these findings are qualitatively very similar to those obtained by Shaw (1955) with horse cells, they are in contradiction to accepted ideas about human cells.

It is usually said that with human cells potassium influx is independent of external potassium concentration over a range from 2 to 74 mM. This belief is based on the work of Raker, Taylor, Weller & Hastings (1950), Sheppard, Martin & Beyl (1951), and Solomon (1952). The finding of Raker *et al.* that potassium influx drops from 1·67 to 1·33 m-mole/l. cells/hr when the external concentration is raised from 4 to 38·5 mM-K, and then rises to 1·58 when the potassium concentration is further increased to 7·44 mM, points to the existence of a large experimental error. Sheppard *et al.* measured influx at external potassium concentrations of 19·5, 31·0 and 57·0 mM, and obtained about the same value at the three concentrations. They state, however, that there is an 8% uncertainty in their figures. Solomon measured potassium influx at many different concentrations between 4·75 and 16·75 mM, and stated that influx was independent of concentration over this range; but his results show considerable scatter and in a later paper (Streeten & Solomon, 1954) he has fitted to them a curve of Langmuir or Michaelis type with a half saturation value of about 2·1 mM. This curve is in good agreement with the present findings apart from the absence of a linear portion—not a serious discrepancy since the highest potassium concentration used by Solomon was 16·75 mM, and he justifies the approach to horizontal of his theoretical curves by quoting the results of Raker *et al.* for 74·4 mM. Flynn & Maizels (1949) found that when cold-stored cells were incubated with glucose, the net uptake of potassium was greater with 25 mM-K outside than with 5 mM-K outside (although the results were complicated by changes in cell volume). Davidsen & Kjerulf-Jensen (1950) also report increased potassium influx at high external potassium concentrations.

It is difficult to think of any single mechanism which would give an influx against concentration curve of the form of Fig. 1. The most likely hypothesis —originally suggested by Shaw (1955) to explain the results he obtained with horse cells—seems to be that the influx is made up of two parts. It is supposed that one part depends on a mechanism which becomes saturated at moderately high potassium concentrations, and the other on a mechanism which resembles free diffusion in that influx is proportional to concentration. Following the procedure of Shaw, the influx against concentration curve has therefore been fitted to an equation of the form:

$$m_i = \frac{\alpha[K_o]}{\beta + [K_o]} + \gamma[K_o],$$

where m_i is the potassium influx in m-mole/l. cells/hr; $[K_o]$ is the external potassium concentration; α, β, and γ, are constants. In the expression on the right-hand side, the first term represents the hyperbolic part of the curve, and the second the linear component. Without the linear component the equation is formally identical with the Michaelis equation of enzyme kinetics, and β is analogous to the half-saturation or Michaelis constant. If the interpretation of the influx curve is correct, the two components of influx would have to be of a quite different nature, and one might expect that only one of these components would be driven by glycolysis. The dual nature of the influx could therefore be tested by depriving the cells of glucose and seeing whether the whole curve or only one component of it was changed.

The effect of glucose on potassium influx. Red cells, prepared in the usual way, were washed five times with glucose-free Ringer's solution. A volume of Ringer's solution rather greater than the volume of cells was used each time,

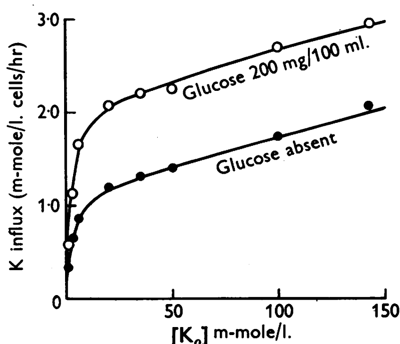

Fig. 3. Expt. no. I.M.G. 2. The effect of glucose on the potassium influx against $[K_o]$ curve. The continuous lines represent the equation

$$y = \frac{\alpha[K_o]}{\beta + [K_o]} + \gamma[K_o].$$

For the upper curve $\alpha = 2{\cdot}12$, $\beta = 2{\cdot}2$, $\gamma = 0{\cdot}006$; for the lower curve $\alpha = 1{\cdot}16$, $\beta = 2{\cdot}2$, $\gamma = 0{\cdot}006$.

and, after mixing, the cells were allowed to stand for 2 min (losing glucose into the washing fluid) before centrifuging. Since the half-time for glucose exchange is about 25 sec (Wilbrandt, 1938) this procedure presumably removed most of the glucose from the cells; the final washings certainly gave very little reduction with Benedict's solution. The washed cells were incubated for 5–8 hr in glucose-free Ringer's solution, the solution being changed once during the incubation. The aim of this long incubation was to render the cells 'energy-deficient' by depleting any stores of available energy they might have possessed. The cells were washed twice with potassium-free Ringer's solution, and small samples were then added to two series of flasks. Both series covered a range of potassium concentrations; one series contained 200 mg glucose per

100 ml., while the other had none. At known intervals, samples of cells from each of the flasks were centrifuged, washed and analysed for activity. Influx was calculated from the activity-time curves.

Fig. 3 shows the results of an experiment of this sort. It is obvious, from inspection, that glucose deprivation has no significant effect on the linear component of influx but reduces the Michaelis component by about 50%, ($\alpha = 2{\cdot}12$; $\alpha' = 1{\cdot}16$) thus tending to confirm the dual nature of the influx. A further point that emerges is that, although the maximum size of the Michaelis component is reduced, the half saturation value, given by β, remains about the same.

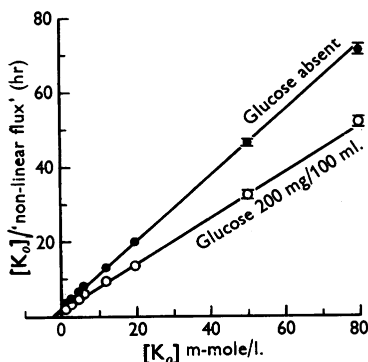

Fig. 4

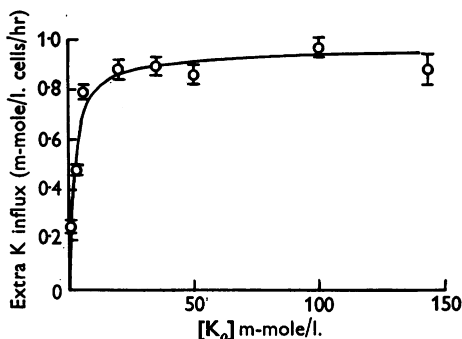

Fig. 5

Fig. 4. Expt. no. W.A.H. 3. The effect of glucose on potassium influx. A factor equal to $\gamma[K_o]$ has been subtracted from the total influx at each external potassium concentration. The residual 'non-linear flux' has then been divided into $[K_o]$, and the quotient $[K_o]$/'non-linear flux' plotted against $[K_o]$. The intersection of both curves with the $[K_o]$ axis at the same point demonstrates that β is unchanged.

Fig. 5. Expt. no. I.M.G. 2. The effect of external potassium concn. on the extra potassium influx which occurs in the presence of glucose. The continuous line represents the equation

$$y = \frac{(2{\cdot}12 - 1{\cdot}16)[K_o]}{2{\cdot}2 + [K_o]}.$$

This point is brought out in Fig. 4 in which the results of another, similar, experiment are plotted in a different way. A factor equal to γ $[K_o]$ has been subtracted from the total influx at each external potassium concentration. The residual 'non-linear flux' has then been divided into $[K_o]$, and the quotient $[K_o]$/'non-linear flux' is shown plotted against $[K_o]$. The linearity of the curves obtained in this way shows that a Michaelis equation does adequately describe this part of the influx, while the intersection of both curves with the $[K_o]$ axis at the same point demonstrates that β is unchanged.

There is, of course, no reason for believing that the glucose-depleted cells were completely 'energy-deficient' and one cannot, therefore, assume that the influx into these cells was entirely passive. But the additional influx in the

cells provided with glucose must have been caused by the glucose, since the cells were otherwise identical. The extra flux may therefore be taken to be entirely active even though it did not necessarily, or even probably, represent the whole of the active influx. The way in which the active influx depends on the external potassium concentration can be seen by plotting the extra flux against $[K_o]$, as has been done in Fig. 5. The points in Fig. 5 are derived directly from the experimental results given in Fig. 3 by finding the difference between cells with and without glucose at each concentration. The continuous line is obtained by plotting the difference between the theoretical curves for cells with and without glucose, i.e.

$$\left(\frac{\alpha[K_o]}{\beta+[K_o]}+\gamma[K_o]\right)-\left(\frac{\alpha'[K_o]}{\beta+[K_o]}+\gamma[K_o]\right)=\frac{(\alpha-\alpha')[K_o]}{\beta+[K_o]}.$$

Fig. 5 shows that the relation between active potassium influx and the external potassium concentration may be described by a Michaelis type curve with a half-saturation constant of about 2·2 mM-K. Fig. 4, from a different experiment, gives a half-saturation value of about 2·5 mM-K. Since the potassium influx seems to be half maximal at the same external potassium concentration whether or not glucose is present, further estimates of the half-saturation constant of the active component of potassium influx can be obtained from curves showing influx in the presence of glucose at different external potassium concentrations. The available estimates are collected in Table 2.

TABLE 2. Estimates of the half-saturation constant (β) of the Michaelis component of potassium influx

Expt. no.	Type of expt.	β (mM)
I.M.G. 2	± glucose	2·2
W.A.H. 3	± glucose	2·5
W.A.H. 1	+ glucose	1·8
R.N. 1	+ glucose	c. 1·7
D.M.C. 1	+ glucose	2·0

Sodium efflux

Effect of external potassium concentration on sodium efflux. The next step was to find the effect of the external potassium concentration on the outward flux of sodium. Cells were incubated for 3–4 hr with ^{24}Na-labelled glucose-Ringer's solution, washed five times, and added to a range of flasks containing glucose-Ringer's solutions with different potassium contents. After a period of incubation, the suspension media were removed from the cells and the activities of the media were measured. The second period of incubation was usually about 20 min, and was kept short to minimize changes in cell sodium content and specific activity.

The combined results of four experiments are shown in Fig. 6, and the data from which the figure is constructed are given in Table 3. The line drawn

through the points in Fig. 6 has no theoretical basis and is simply drawn by eye. Before discussing the interpretation of these results it is necessary to consider their reliability.

If the results are taken at their face value, and the internal sodium concentration is assumed to have been about 10 mM, then in 20 min the cells in the potassium-rich solutions must have lost about 10% of their ^{24}Na. Since efflux was calculated on the basis of the initial specific activity

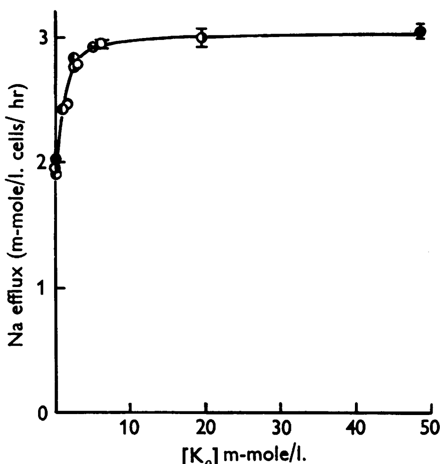

Fig. 6. Effect of external potassium concentration on sodium efflux. Glucose present. Combined data from the results shown in Table 3. ●, R.B.K. 1: mean of four estimations; ◐, R.E.T. 1, ◑, M.S. 1, ○, R.D.K. 1: mean of three estimations. ⊗, the efflux at 4·9 mM-K outside has been set arbitrarily at 2·93 m-mole/l. cells/hr, and the fluxes at other values of [K$_o$] have been scaled accordingly.

TABLE 3. Effect of external potassium concentration on sodium efflux in the presence of glucose

Expt. no.	[K$_o$] (mM)	Na efflux (m-mole/l. cells/hr)	Duration (min)	Final [K$_0$] in 'K free' flasks (mM)
R.B.K. 1	0	2·01 ± 0·02		
	4·9	2·93 ± 0·04	18	0·024 ± 0·001
	48·7	3·06 ± 0·05		
R.E.T. 1	0	1·97 ± 0·03		
	0·96	2·47 ± 0·02	21	0·049 ± 0·003
	2·40	2·87 ± 0·01		
	4·79	3·01 ± 0·02		
M.S. 1	0	1·68 ± 0·01		
	2·4	2·38 ± 0·05	23	0·034 ± 0·005
	4·9	2·52 ± 0·03		
	19·5	2·58 ± 0·07		
R.D.K. 1	0	2·07 ± 0·05		
	1·39	2·62 ± 0·03	24	0·029 ± 0·002
	2·88	2·96 ± 0·02		
	5·75	3·13 ± 0·04		

Each of the figures for sodium efflux and for final [K$_o$] is the mean of three, except in Expt. R.B.K. 1, where the figures are the mean of four. The S.E. of the means are given.

of the intracellular sodium, the figures for the sodium efflux at high potassium concentrations should be something like 5% higher. In the absence of external potassium the correction will be about 3 or 4%. With lower internal sodium concentrations the errors will be correspondingly greater.

Another possible source of error is that, as will be shown later, sodium influx is increased at very low external potassium concentrations. The effect this will have had on the results depends on the way sodium efflux varies with the internal sodium concentration. This relationship is difficult to establish with any certainty, because there is no way of altering the sodium content and yet leaving the cells identical in every other respect. Harris & Maizels (1952) followed sodium efflux from cold-stored cells, and found that over successive periods of 6, 5 and 13 hr the quotient obtained by dividing the average efflux by the average sodium content remained the same. If their conclusion, that efflux is proportional to $[Na_i]$, is correct, then in the present experiments sodium influx will not have affected the results, since increased sodium content will have just offset decreased specific activity.

A final source of uncertainty is Solomon's slowly exchanging fraction. The figures for specific activity used in the present experiments were derived by dividing the total activity in the cells by the total cell sodium. If 17% of the cell sodium exchanges as slowly as Solomon (1952) suggests, this fraction will contain very little activity after incubation for only a few hours, and the activity figure will be too high by something like 20%. The whole curve should therefore be shifted down by about this amount.

Despite all the uncertainties, the results shown in Fig. 6 indicate that removing potassium from the external medium reduces sodium efflux. If Fig. 6 is compared with the figure showing the effect of external potassium concentration on potassium influx, there is one striking difference. Even in the absence of external potassium there appears to be a considerable sodium efflux while there is and can be no potassium influx. Of course, the external potassium concentration, even in a 20 min experiment, is never quite zero, but the final potassium concentration in the suspension media from the initially potassium-free flasks was measured and found to be very small (see Table 3). The potassium influx at such low concentrations is nowhere near comparable with the sodium efflux. There seemed to be three ways of explaining the results.

When cells are suspended in a potassium-free solution there is a continuous loss of potassium from the cells. Although the concentration of potassium in the bulk of the suspension medium remains low if the suspension is dilute, there is no way of knowing the effective potassium concentration at the surface of the cells. It seemed possible that the sodium efflux which remained when the external potassium concentration approached zero was the result of an exchange of sodium with potassium from the surface layer. A similar explanation has been put forward by Hodgkin & Keynes (1955*a*) to explain the residual sodium efflux found in nerves soaked in potassium-free solutions. However, the residual efflux is relatively greater in red cells, and these cells lack the neurilemma which in nerves provides the suggested barrier to potassium diffusion.

The second possibility was that the active fluxes were only loosely linked,

so that abolition of potassium influx reduced, but did not completely eliminate, sodium efflux.

The third possibility was that the active fluxes were tightly linked, and that the sodium efflux which occurred in the absence of potassium was not active. It proved possible to choose between these theories since the effects of glucose deprivation predicted by them are different.

Effect of glucose on the sodium efflux against $[K_o]$ *curve.* Cells were washed repeatedly to deprive them of glucose, and were then incubated with ^{24}Na-labelled glucose-free Ringer's solution for about 4 hr to load them with ^{24}Na.

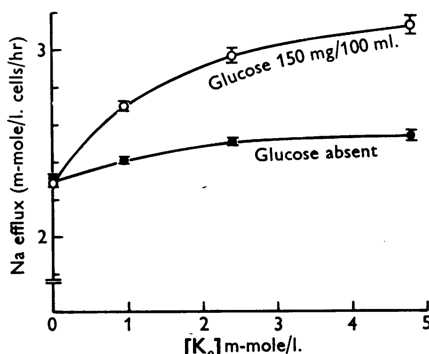

Fig. 7. Expt. no. T.V. 1. The effect of glucose on sodium efflux at different external potassium concentrations. Each point is the mean of three estimations.

TABLE 4. The effect of glucose on sodium efflux at zero external potassium concentration

Expt. no.	Na efflux (m-mole/l. cells/hr) +G	Na efflux (m-mole/l. cells/hr) −G	Final $[K_o]$ (mM) +G	Final $[K_o]$ (mM) −G	Duration (min)
T.V. 1	2·29±0·02	2·32±0·02	0·033±0·001	0·030±0·003	22
R.D.K. 1	2·07±0·05	2·13±0·02	0·029+0·002	0·037+0·001	24
M.T.S-K. 1	1·73±0·08	1·64±0·09	0·081±0·004	0·098±0·017	23

The figures for Expts. T.V. 1 and R.D.K. 1 are the means of three estimations. The figures for Expt. M.T.S-K. 1 are the means of two estimations. The S.E. of the means are given.

The energy-deficient ^{24}Na-loaded cells were washed five times in potassium-free Ringer's solution and added to two series of flasks. Each series covered the same range of potassium concentrations; one series contained 200 mg glucose/100 ml. Efflux was calculated from the activities of the suspension media at the end of a measured period of incubation.

Fig. 7 shows the results of an experiment of this type. The range of potassium concentrations studied was restricted to 0–5 mM as this was sufficient to give the interesting part of the curve. The apparent difference in slope between the rising part of the curve in Fig. 6 and the upper curve in Fig. 7 is due to a change in scale.

The most striking thing about the results of this experiment is that glucose appears to have no effect on sodium efflux in the absence of potassium (see also Table 4). If the efflux of sodium into potassium-free solution were active but not linked to potassium influx, or active and linked to potassium influx from the surface layer, one would expect glucose to have some effect. The complete absence of any effect suggests very strongly, first, that this residual efflux is not active, and secondly, that the active efflux is tightly linked to potassium influx and is therefore absent when there is no potassium outside. A corollary of the second conclusion is that removal of glucose should greatly decrease the effect of external potassium on sodium efflux. Inspection of Fig. 7 shows that this is so. The extra sodium efflux produced by increasing the external potassium concentration from 0 to 5 mM is 75% abolished by removal of glucose. Since there is no reason to believe that the glucose-free cells are entirely 'energy-deficient', the small residual effect of potassium is not surprising.

An alternative way of explaining these results is to suppose that glucose cannot enter the cells in the absence of potassium. This hypothesis can be rejected since several workers—see, for example, Klinghoffer (1935), Le Fevre (1948)—have measured glucose uptake from solutions containing only glucose and sodium chloride.

For comparison with Fig. 5 it is interesting to derive the curve relating the active sodium efflux to external potassium concentration. The extra efflux produced by glucose has been calculated from the difference between the flasks with and without glucose, at each potassium concentration, and the quotient obtained by dividing this extra flux into $[K_o]$ has been plotted against $[K_o]$—see Fig. 8. Fig. 9 shows the extra flux plotted against $[K_o]$; the continuous line is the Michaelis curve with constants derived from the slope and negative intercept of the curve in Fig. 8. Considering the errors of the method and the fact that measurements were made at only four potassium concentrations, the half-saturation value of 1·8 agrees well enough with the figures obtained in experiments on potassium influx (see Table 2). The height of the curve depends to some extent on how deficient in energy the glucose-free cells were, and therefore gives only a minimum value for active sodium efflux. Nevertheless, the fact that removal of glucose decreases active potassium uptake and active sodium extrusion by roughly the same amount suggests that the linked system involves a one-to-one exchange of sodium for potassium.

Effect of $[K_o]$ *on sodium efflux; another method.* The crux of the evidence for linkage of the active fluxes is the demonstration that sodium efflux is reduced in the absence of external potassium. The estimations of sodium efflux so far described were obtained by incubating cells previously loaded with tracer, and measuring the activity appearing in the suspension medium. This method is subject to uncertainty because, to calculate the specific activity of the

outgoing sodium, it is necessary to assume that the cells and suspension medium behave as a two-compartment system. Harris & Prankerd (1953), and also Solomon & Gold (1955), found that the loss of ^{24}Na from previously loaded cells could be described by a single rate constant. Gold & Solomon (1955), however, found that a small fraction of cell sodium failed to exchange in 24 hr; and theoretical analysis of their influx curves, assuming that there were two homogeneous intracellular compartments, led them to suggest that about 17 % of the cell sodium exchanged with a very much lower rate constant.

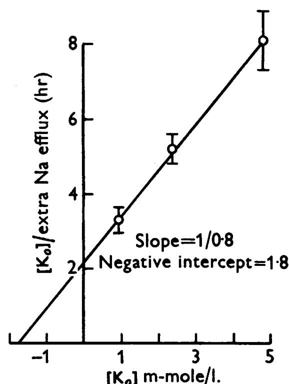

Fig. 8

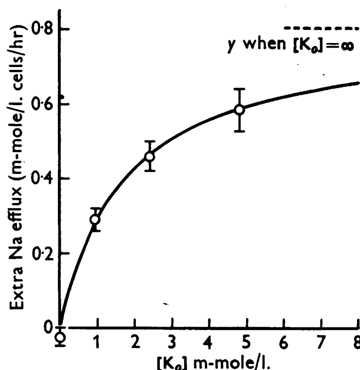

Fig. 9

Fig. 8. Expt. no. T.V. 1. The extra sodium efflux which occurs in the presence of glucose has been calculated from the differences between the flasks with and without glucose. The quotient obtained by dividing the extra flux into $[K_o]$ is plotted against $[K_o]$.

Fig. 9. Expt. no. T.V. 1. The effect of external potassium concn. on the extra sodium efflux which occurs in the presence of glucose. The continuous line represents the equation

$$y=\frac{0{\cdot}8[K_o]}{1{\cdot}8+[K_o]}.$$

In the present experiments, the absolute value of the efflux is of less interest than its dependence on external conditions; and the existence of a small compartment of very slowly exchanging sodium will not matter unless the fraction of sodium in this compartment is altered by the change in the external conditions. Nevertheless, it seemed desirable to check the results by an independent method. Sodium efflux was therefore estimated from simultaneous measurements of net movements and of ^{24}Na influx.

Red cells were washed three times in potassium-free glucose-Ringer's solution and divided into two lots. The first lot was incubated in a glucose-Ringer's solution labelled with ^{24}Na and containing 8 mM-K, and the second in a solution identical except that it lacked the potassium. At known times samples of cells were withdrawn from each flask, washed as quickly as possible —twice with an ice-cold Ringer's solution containing choline instead of

sodium, and once with isotonic choline chloride—and then lysed. The haemolysates were analysed for activity and total sodium. Fig. 10 shows the results obtained.

From the initial slopes of the activity-time curves, the influx of sodium in the presence and absence of potassium can be calculated. By comparing these figures with the figures showing net sodium movements, an estimate of efflux is obtained which is independent of any assumptions regarding the state of internal sodium. The values for efflux obtained in this way—2·5 m-mole/l. cells/hr in the absence of potassium and 3·5 m-mole/l. cells/hr with 8 mM of external potassium—are slightly higher than those obtained earlier by the direct method, but are of the same order of magnitude and show unequivocally the rise due to potassium.

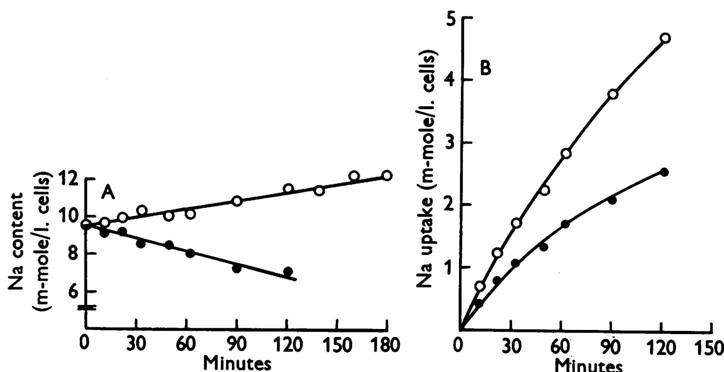

Fig. 10. Expt. no A.A.S. 1. Simultaneous measurements of *Na uptake and net Na movements. Cells stored at 2° C for 6 hr before use. ○, K absent from suspension medium; ●, 10 mM-K in suspension medium. A: net gain of Na in absence of K, 0·9 m-mole/l. cells/hr; net loss of Na in presence of K, 1·3 m-mole/l. cells/hr. B: continuous lines represent equation

$$y=\frac{m_i}{k}(1-e^{-kt}).$$

In absence of K: $m_i=3{\cdot}4$ m-mole/l. cells/hr, $k=0{\cdot}4$ hr^{-1}; in presence of K: $m_i=2{\cdot}2$ m-mole/l. cells/hr, $k=0{\cdot}6$ hr^{-1}.

Another estimate of the relative magnitudes of the sodium efflux with and without potassium can be obtained from the influx curves if it is assumed that efflux is proportional to the internal sodium concentration. This was roughly true in the experiment of Harris & Maizels (1952) referred to on page 288, although the conditions there were rather different in that the cell sodium was higher than in the present experiment and potassium was always present. However, if proportionality between sodium efflux and [Na_i] is assumed, then the activity-time curves should be of the form

$$\text{activity}=\frac{m_i}{k}(1-e^{-kt}),$$

where m_i is the influx and k is proportional to efflux. The continuous lines in Fig. 10B are curves of this form fitted to the experimental points, and the ratio of the values of k for the two curves is a measure of the effect of potassium on sodium efflux. The value of 1·5 obtained for this ratio agrees well with the figure of 1·4 obtained by taking the ratio of the effluxes found by the more reliable method.

The passive fluxes

So far, potassium influx and sodium efflux have been considered. It has been shown that potassium influx consists of a small component, which is proportional to the external potassium concentration and which does not require glucose, and a larger component, which becomes saturated at about 10 mM-K and which is much reduced in the absence of glucose. Sodium efflux has been divided into an active component, dependent on glucose and tightly linked to potassium influx, and a component which persists in the absence of potassium, and which is unaffected by glucose. It is now proposed to consider in turn the linear component of potassium influx, potassium efflux, the sodium efflux in the absence of external potassium, and sodium influx.

The linear component of potassium influx

It seemed possible that the linear component of potassium influx was caused by free diffusion through a leak. Such a leak might be present in all the cells or in just a few, perhaps only those with slightly damaged membranes. If free diffusion were responsible, this component of influx should have a low temperature coefficient—equal to that for diffusion of potassium in solutions of the same ionic strength as Ringer's solution, plus a small correction for the slight electrical potential barrier across the red-cell membrane. The temperature coefficients of the linear and non-linear components of potassium influx were therefore determined, measurements being made of potassium influx at three different potassium concentrations at each of two temperatures. Temperatures of 29·0 and 37·7° C were chosen so as not to be too far from the physiological range. The procedure used was the same as in the potassium influx experiments already described.

Fig. 11 shows the total potassium influx at each concentration and temperature. The curves are linear between 30 and 100 mM-K, and the slopes of the regression lines give the magnitude of the linear component of influx at each temperature. From the ratio of the slopes the apparent energy of activation and effective Q_{10} can be calculated. Similarly, the ratio of the intercepts of the regression lines on the vertical axis gives the apparent energy of activation and Q_{10} for the Michaelis component of the influx. The results, given in Table 5, are not particularly accurate, but the conclusions are clear enough.

The Q_{10} and apparent activation energy of the total potassium influx agree well with figures obtained by previous workers. Thus Raker *et al.* (1950) give

a figure of 14,500 ± 1300 cal/mole for the apparent energy of activation, while Sheppard & Martin (1950) found a value of 15,800 and Solomon (1952) of 12,300 ± 1300 cal/mole. What is more remarkable is that the linear component has a temperature coefficient of about the same magnitude as the Michaelis component and very much higher than the Q_{10} for free diffusion. From data in the International Critical Tables, the Q_{10} for diffusion of potassium into a medium containing 100 mM, for the temperature range 28–38° C, should be 1·21, equivalent to an apparent activation energy of 3540 cal/mole. To this must be added a correction for the electrical potential. It is impossible to predict this precisely, but the contribution to the activation energy if each ion had to surmount the electrical barrier at a single step can be calculated. Unless the potential has a maximum in the thickness of the membrane, it is

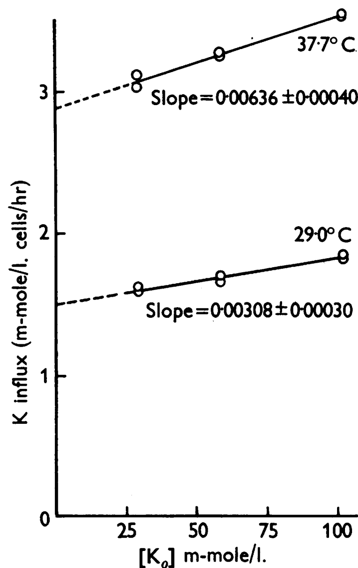

Fig. 11. Expt. no. R.H.A. 1. The effect of temperature on potassium influx.

TABLE 5. The effect of temperature on potassium influx at different external potassium concentrations; glucose present

Temp. (° C)	Linear component (m-mole/ l. cells/ hr/mM-K)	Michaelis component (m-mole/l. cells/hr)	
29·0	0·00308 ± 0·00030	1·50 ± 0·04	
37·7	0·00636 ± 0·00040	2·88 ± 0·09	
	Linear component	Michaelis component	Free diffusion: from data in International Critical Tables
Q_{10}	2·32 ± 0·27	2·14 ± 0·09	1·21
Apparent E_A, (cal/mole)	15,600 ± 2200	14,100 ± 890	3540

Expt. no. R.H.A. 1, see also Fig. 11.

difficult to see how this calculated figure could be anything but a maximum value. If the ratio of Cl_o/Cl_i is taken to be 1·4 (Harris & Maizels, 1952) the potential given by the equation

$$E=\frac{RT}{zF}\log_e\frac{Cl_o}{Cl_i}$$

is 9·0 mV. This is equivalent to an energy barrier of height 208 cal/(mole of univalent cations), which is almost negligibly small. It follows that, unless a rise in temperature is assumed to increase the number of holes, the linear component of potassium influx cannot be explained by free diffusion in aqueous solution.

Potassium efflux

Potassium efflux is a downhill flux and the simplest way of explaining it would be to suppose that the membrane was slightly leaky. A simple leak would allow potassium to pass indifferently in the two directions, and the relation between inward and outward flux through the leak would depend only on the relative concentrations (more strictly activities) inside and outside, and on the electrical potential across the membrane. Where ions move independently under the influence of a concentration gradient and an electric field, Ussing (1950) has shown that

$$\frac{m_o}{m_i}=\frac{f_i c_i}{f_o c_o}\,e^{zEF/RT},$$

where m_o and m_i denote the inward and outward fluxes, f_i and c_i, and f_o and c_o, denote the activity coefficient and concentration of the ion under consideration on the two sides of the membrane, z is the valency of the ion, E is the electrical potential, and R, T and F have their usual meanings. For red cells

$$E=\frac{RT}{F}\log_e\frac{[Cl_i]}{[Cl_o]},$$

and for a univalent ion whose activity coefficient is assumed to be the same on the two sides of the membrane Ussing's equation becomes (Shaw, 1955)

$$\frac{m_o}{m_i}=\frac{c_i}{c_o}\,\frac{[Cl_i]}{[Cl_o]}.$$

Since the chloride distribution ratio is about 0·71, and the concentration of potassium in the cell water about 150 mM, an efflux of magnitude m_o through the leak should be accompanied by an influx via the same route of

$$\frac{m_o}{150\times 0{\cdot}71}\ \text{m-mole/l. cells/hr per mM-K in the external fluid.}$$

The magnitude of potassium efflux into glucose-Ringer's solution has been determined in a number of experiments, both by observing the loss of ^{42}K

from previously loaded cells, and by simultaneous measurements of potassium influx and net movements. The latter method makes no assumptions about the homogeneity of the intracellular potassium. The results are summarized in Table 6. If 1·6 m-mole/l. cells/hr is taken as an average figure and substituted into Ussing's equation, the influx through the hypothetical leak works out as 0·015 m-mole/l. cells/hr per mM-K in the external fluid. The only part of the potassium influx that behaves at all as though it were freely diffusing—disregarding for the moment the high Q_{10}—is the linear part. The magnitude of

TABLE 6. The magnitude of potassium efflux into glucose-Ringer's solutions

Expt. no.	Technique	$[K_o]$ (mM)	K efflux (m-mole/l. cells/hr)
R.T. 1	Tracer loss	4·0	$1{\cdot}87 \pm 0{\cdot}10$
L.G.B. 2	Tracer loss	24·0	$1{\cdot}32 \pm 0{\cdot}04$
P.J.F. 1	Tracer loss	9·7	$1{\cdot}72 \pm 0{\cdot}09$
L.G.B. 5	Net movements*	0–0·029	$1{\cdot}83 \pm 0{\cdot}10$
I.M.G. 5	Net movements*	0–0·186	$1{\cdot}39 \pm 0{\cdot}06$
R.N. 1*b*	Tracer influx + net movements	4·9	$1{\cdot}66 \pm 0{\cdot}17$

* In experiments L.G.B. 5 and I.M.G. 5 small corrections for influx have been made. Influx was estimated from curves of K influx against external K concentration.

TABLE 7. The magnitude of the linear component of potassium influx

Expt. no.	Linear component of K influx (m-mole/l. cells/hr/m-mole $[K_o]$/l.)
I.M.G. 2	0·0060
W.A.H. 3	0·0070
R.H.A. 1	0·0064
R.N. 1*a*	0·0070
I.M.G. 6	0·0075
A.A.S. 2	0·0035

(All values ± say 10%)

this in each of the experiments in which it has been determined is given in Table 7. Clearly these values are much smaller than the predicted influx, and the idea of a simple leak as the sole cause of potassium efflux must be regarded as untenable. This seems to represent a difference between horse and human cells, since in horse cells Shaw (1955) found good agreement between the observed efflux and that calculated from the linear component of influx on the assumption that Ussing's relationship held.

As potassium efflux in human cells did not appear to be entirely the result of straightforward diffusion through a leak, it seemed worth while to investigate the relationships between influx and efflux by measuring efflux into different external potassium concentrations. Preliminary experiments showed that the loss of ^{42}K from cells previously loaded occurred at a practically uniform rate for about $1\frac{1}{2}$ hr. Efflux was therefore measured by incubating ^{42}K-loaded cells for an hour with the test solutions and determining the initial and final activities of the solutions. A correction, usually of between 5 and 10%, was necessary to allow for haemolysis. In the flasks containing potassium,

influx of ^{42}K could be neglected because the ^{42}K emerging from the cells was diluted with a relatively very large amount of ^{39}K outside. In the flasks with zero $[K_o]$ the efflux m_o was calculated by assuming

$$\frac{d[{}^*K_o]}{dt}=\frac{v_c}{v_s}m_o-k[{}^*K_o]\frac{v_c}{v_s},$$

where $[{}^*K_o]$ is the concentration of potassium (radioactive = total) in the external fluid, v_c and v_s are respectively the volumes of the cell and fluid compartments, and k is the slope of the initial part of the potassium influx curve which was taken as 0·85 m-mole/l. cells/hr. per mM-K in the outside fluid.

Integrating

$$t_2-t_1=\frac{v_s}{kv_c}\log_e\frac{m_o-k[{}^*K_{o1}]}{m_o-k[{}^*K_{o2}]}.$$

The results of an experiment designed to measure efflux of K into solutions containing between 0 and 146 mM-K are given in Table 8. Although there is considerable scatter in the results, it is possible to draw two conclusions.

TABLE 8. The effect of external potassium concentration on potassium efflux into glucose-Ringer's solutions

Expt. no. L.G.B. 2

$[K_o]$ (mM)	K efflux (m-mole/l. cells/hr)
0–0·33	1·10 ± 0·02
2·0	1·26 ± 0·05
24·0	1·32 ± 0·04
73·0	1·26 ± 0·04
146·0	1·20 ± 0·03

Each figure in the last column is the mean of three estimations ± S.E. of the mean.

(i) The absence of any great fall in potassium efflux as $[K_o]$ is raised excludes the possibility of a mechanism, such as Hodgkin & Keynes have suggested for squid nerve, in which ions are constrained to move through the membrane in single file along a narrow channel or a series of sites. (ii) At external potassium concentrations of just over 100 mM the electrochemical potential of potassium on the two sides of the membrane should be about the same since

$$\frac{[K_o]}{[K_i]}=\frac{[Cl_i]}{[Cl_o]}.$$

Yet at this external potassium concentration, potassium efflux is about double the linear component of potassium influx. It follows, therefore, that the whole of potassium efflux and the linear potassium influx cannot occur through a single inactive permeability channel. If about one-half of the efflux were by some other route, however, the remaining part of the efflux and the linear influx might occur by simple diffusion through a channel, although, to account for the high Q_{10}, it would be necessary to suppose that a certain energy was required for a potassium ion to enter.

Sodium efflux in the absence of external potassium

The sodium efflux which continues in the absence of external potassium accounts for between one-half and two-thirds of the total sodium efflux. The sodium efflux into potassium-free solution is unlikely to be active, since it is not affected by glucose deprivation, but the question can be tested by seeing whether cells can show a net loss of sodium into a solution free from potassium and rich in sodium. Cells incubated with a potassium-free glucose-Ringer's solution certainly show a gain in sodium (see Fig. 10), but here the disparity between intra- and extracellular sodium concentrations is very great. A more stringent test can be arranged by putting the cells in a solution with a sodium concentration greater than that within the cells but much smaller than that in standard Ringer's solution. The solution must be isotonic, and, to avoid changing the potential across the cell membrane, should contain the same concentration of chloride ions as does Ringer's solution. A suitable solution was obtained by preparing a Ringer's solution in which all but 21 mM-NaCl was replaced by choline chloride, and from which potassium was omitted. Fig. 12 shows the changes in sodium content of glucose-depleted cells incubated in such a solution. Glucose-depleted cells were used, in spite of the demonstrated lack of action of glucose in the absence of potassium, in an attempt to reduce the effect of potassium leaking from the cells. This leakage was sufficient to raise the external potassium concentration to 0·6 mM during the course of the experiment. The failure of the cells to show any significant sodium loss, even with the external sodium concentration only 2 mM greater than the concentration in the cell water, indicates that no active efflux was occurring. It is just possible that the absence of active efflux was caused by the presence of choline, but the rapid loss of sodium from cells provided with potassium and glucose (Fig. 12, lower curve) makes this extremely unlikely.

If the sodium efflux in the absence of potassium and glucose is not active, the next question to be decided is whether it might be the result of a simple leak. With an internal sodium concentration of 15 m-mole/l. cell water, and an external concentration of 146 m-mole/l., Ussing's equation predicts that an efflux of 1 m-mole/l. cells/hr would be accompanied by an influx of about 14 m-mole/l. cells/hr. This is about five times greater than the observed influx; hence the idea of a simple leak must be rejected.

If efflux is passive and yet Ussing's equation predicts the wrong value for influx, the condition of independence assumed in deriving Ussing's equation must be unsatisfied; in other words, the chance that any individual ion will cross the membrane does depend on the presence of other ions of the same species. The experimental data suggest that there is some interaction between influx and efflux. In the experiment illustrated by Fig. 12, net sodium movement in the absence of potassium and glucose, and with 21 mM-Na outside

was practically zero. It will be shown in the next section that sodium influx under similar conditions is about 0·5 m-mole/l. cells/hr, so that the efflux must have been of roughly this magnitude. With 151 mM-Na outside, sodium efflux in the absence of external potassium is about 2 m-mole/l. cells/hr as determined either directly or from simultaneous measurements of influx and net movements. Taken together, these results show that reduction in external sodium from 151 to 21 mM leads to something like a fourfold reduction in sodium influx. This type of interaction, together with the fact that the ratio

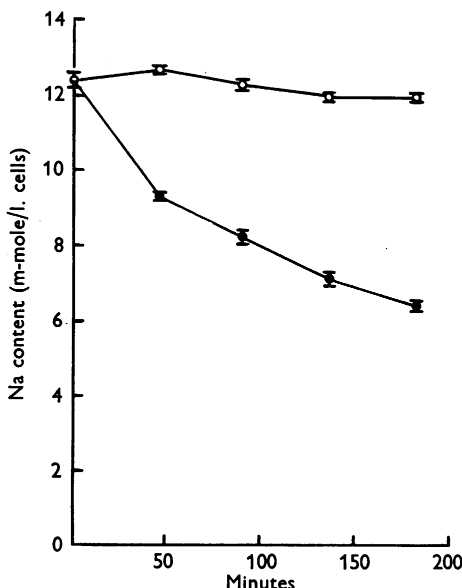

Fig. 12. Expt. no. M.C.L. 1. Net movement of sodium between cells and a solution containing 21 mM-Na. ○, Glucose absent, $[K_o]$ 0 at start and 0·6 mM at end of experiment; ●, glucose 180 mg/100 ml., $[K_o]$ 12 mM. Each point is the mean of three estimations.

TABLE 9. Effect of temperature on the K-linked and K-independent components of sodium efflux

Temperature (° C)	Na efflux (m-mole/l. cells/hr) $[K_o]$ = 0–0·031 mM	$[K_o]$ = 7·7 mM
29·9	1·02 ± 0·02	2·13 ± 0·03
37·7	1·85 ± 0·01	3·92 ± 0·02

Flux	Apparent E_A (cal/mole)	Q_{10}
Total	15,000 ± 400	2·24 ± 0·03
K-linked	14,500 ± 800	2·18 ± 0·07
K-independent	15,300 ± 600	2·28 ± 0·05

Experiment no. S.O.O. 1. Duration 20 min; glucose, 100 mg/100 ml.
Each figure for sodium efflux is the mean of three estimations ± the S.E. of the mean.

of the fluxes is much less than the ratio of concentrations, is suggestive of the type of mechanism described by Ussing under the name of 'exchange diffusion'.

Temperature coefficients. The temperature coefficients of the potassium-linked and potassium-independent sodium efflux were found by measuring the sodium efflux from ^{24}Na-loaded cells at two different temperatures in the presence and absence of potassium (see Table 9). The figure of 15,000 ± 400 cal/mole for the apparent energy of activation of the total sodium efflux agrees well with Solomon's (1952) figure of 14,900 ± 3400 cal/mole. For the potassium-linked and potassium-independent fluxes the apparent activation energies work out as 14,500 ± 800 and 15,300 ± 600 cal/mole respectively. These values are not significantly different but the agreement may well be fortuitous.

Sodium influx

Although a downhill flux, sodium influx cannot be due to a simple leak since it does not vary linearly with the external sodium concentration (Solomon, 1952), it has a high temperature coefficient (Solomon, 1952), and it appears to be connected with sodium efflux—see previous section. Solomon measured sodium influx over a range of concentrations from 40 to 180 mM, keeping

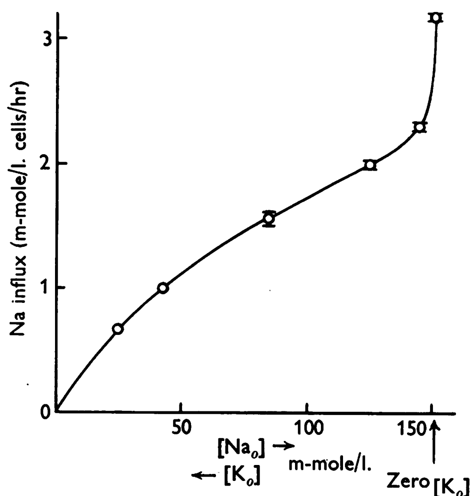

Fig. 13. Expt. no. D.T. 1. The effect of external sodium concentration on sodium influx. Potassium replacing sodium. Each point is the mean of two determinations.

the concentration of the other cations constant at about half their normal values and maintaining the isotonicity of the solution with di- or tri-saccharides. This method has the disadvantage that the chloride concentration differs in the different test solutions, and so the membrane potential and the internal pH will vary from one solution to another. Fig. 13 gives the results of an experiment designed to avoid these difficulties. Washed cells were incubated in solutions containing complementary amounts of sodium and

potassium with fixed, roughly normal, amounts of chloride, calcium, magnesium and phosphate. The sodium was labelled with ^{24}Na and the entry of activity into the cells over a 20 min period was measured. The gradual rise in influx over the first three-quarters of the curve is presumably due to the increasing sodium concentration, but the abrupt rise at the end accompanies a change in sodium concentration of only a few per cent, while the potassium concentration falls from about 20 mM to zero. This suggests that removal of potassium from the solution increases sodium influx, a finding in agreement with the results of Shaw (1954) on horse cells, although in contradiction to a result of Harris & Maizels (1951) on human cells.

TABLE 10. The effect of $[Na_o]$ and $[K_o]$ on sodium influx

$[Na_o]$ (mM)	Uptake of Na (m-mole/l. cells/hr)	
	K absent	$[K_o] = 10$ mM
5	0·143 ± 0·003	0·153 ± 0·002
10	0·257 ± 0·003	0·297 ± 0·007
20	0·485 ± 0·005	0·459 ± 0·005
60	1·34 ± 0·02	1·14 ± 0·02
120	2·41 ± 0·03	1·85 ± 0·01

Expt. no. M.W. 1. Choline replacing sodium; duration 22 min; glucose, 100 mg/100 ml.
Each figure for sodium uptake is the mean of two estimations ± S.E. of the mean.

In order to test this point further, and also to determine the effect of sodium concentration on sodium influx without the complicating effects of potassium, cells were incubated in two series of flasks, each covering a range of sodium concentrations, one series being K-free and the other containing 10 mM-K. Choline chloride was used to maintain isotonicity, and the concentrations of calcium, magnesium, chloride and phosphate were fixed and normal. The results are given in Table 10. Removal of potassium from the outside solution clearly increases sodium influx, and the extra flux increases with increasing sodium concentration, although the results are not accurate enough to determine the form of this relationship.

One way of explaining these results is to suppose that the extra sodium which enters in the absence of potassium is carried in on the potassium carriers. On this theory one might expect the extra sodium influx to be glucose sensitive, and a demonstration of this would be evidence for the theory. On the other hand, insensitivity to glucose would not disprove the theory since one could postulate that the potassium carriers were only 'pumped' when they were carrying potassium. Table 11 gives the results of an experiment to measure the flux of sodium into cells depleted of glucose and incubated in Ringer's solutions with and without glucose and with and without potassium. Glucose appeared to have no effect whether potassium was present or not.

TABLE 11. The effect of glucose on sodium influx in the presence and absence of external potassium

$[K_o]$ (mM)	Na influx (m-mole/l. cells/hr) Glucose absent	Glucose 200 mg/100 ml.
0	2·84 ± 0·03	2·76 ± 0·03
10	1·86 ± 0·02	1·82 ± 0·02

Expt. no. R.H. 1. Duration 20 min.
Each figure for sodium influx is the mean of three estimations ± S.E. of the mean.

DISCUSSION

The main conclusions from the experiments that have been described may be summarized with the help of a diagram—see Fig. 14.

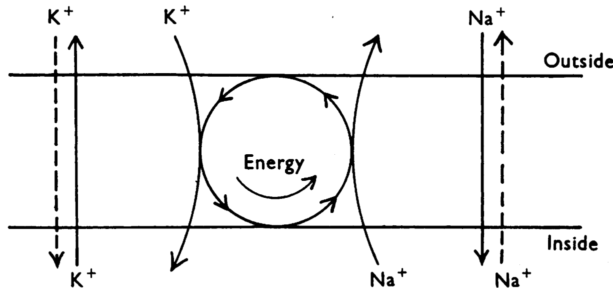

Fig. 14. Sodium and potassium fluxes in the human red cell.

The curved arrows connected by a circle represent the active fluxes. They appear to be tightly linked, since progressive lowering of the external potassium concentration leads to parallel decline in active potassium uptake and active sodium extrusion until, with no potassium outside, both active fluxes are abolished. The fact that removal of glucose leads to a decrease of roughly the same magnitude in potassium influx and in sodium efflux suggests that the linkage involves a one-to-one exchange of sodium for potassium.

The passive fluxes, represented in the diagram by straight arrows, occur in both directions but are predominantly outwards for potassium and inwards for sodium. They cannot be explained entirely by free diffusion through holes in the membrane, but depend partly on mechanisms in which the chance of penetration by any individual ion is affected by the presence of other ions of the same species. For sodium there is evidence suggestive of interaction, of the 'exchange diffusion' type, between efflux and influx.

Maizels (1954) has pointed out that an apparent linkage between sodium and potassium transport would exist if the cell were merely able to hold its

crystalloid osmotic pressure constant, since 'the osmotic and electrical requirements of cells and plasma are such that sodium and potassium in the cell phase and also in the plasma phase are necessarily complementary'. The effect of external potassium concentration on sodium efflux in the present experiments cannot be explained on this basis, since the experiments were of such short duration that the internal potassium concentration did not change significantly. The maintenance of a roughly constant crystalloid osmotic pressure under conditions in which the sodium content and potassium content vary widely would appear to be the consequence, rather than the cause, of the cell's ability to exchange sodium ions for potassium ions in a one-to-one ratio.

Harris (1954) has argued that the link between the active fluxes must involve a two-to-one exchange of sodium for potassium. He assumes that the entire sodium influx and potassium efflux occur through leaks, and, by applying the Ussing relationship, calculates the sodium efflux and potassium influx that would occur through these leaks. He subtracts these calculated fluxes from the figures for the total uphill fluxes, and what is left he calls active. The 'active' flux of sodium derived in this way is roughly twice that of potassium. The results described in the present paper show that it is not permissible to apply Ussing's relationship in this way.

It is interesting to compare the pattern of fluxes found in red cells with those found in other tissues. A link between active sodium extrusion and potassium influx has been demonstrated in frog muscle and in cephalopod nerves. Thus Steinbach (1940, 1952) showed that when frog muscles are soaked in potassium-free Ringer's solution they gain sodium and lose potassium, and that if they are subsequently transferred to a potassium-rich medium they are able to expel some of their internal sodium. Keynes (1954), working with tracers, found that sodium efflux from frog muscles was reduced in potassium-free Ringer's solution, but was increased if the potassium level was raised above normal. Similar effects have been described in *Sepia* nerves by Hodgkin & Keynes (1955*a*). It is possible that the widespread occurrence of a higher K:Na ratio in intracellular than in extracellular fluid reflects the widespread existence of an active sodium–potassium exchange mechanism, but this is not necessarily so. Certainly active movements of sodium and potassium are not always linked. In frog skin, Koefoed Johnsen (unpublished work, quoted by Ussing, 1955) has shown that absence of potassium ions brings about a very marked reduction of sodium transport, but the mechanism does not transport potassium measurably. Scott & Hayward (1954) have evidence that in *Ulva lactuca* the active influx of potassium and extrusion of sodium occur by separate mechanisms, since it is possible to inhibit the fluxes independently. Dobson (1955) has shown that in the cells lining the rumen sodium uptake is active and accompanied by passive uptake of chloride. Kidney tubules seem to be able to transport sodium and potassium

independently, and the isolated gill of *Ereiocheir* pumps sodium and potassium indiscriminately, according to Koch (1954).

Interaction between the passive movements of individual ions of a single species is also found in cells other than red cells. Such interaction is indicated by a discrepancy between the observed flux ratio and that predicted from the Ussing relationship. The discrepancy may be in the direction described for the passive sodium and potassium fluxes of the human red cell, where the flux ratio is closer to unity than the ratio of activities, or it may be in the opposite direction. More formally:

$$\left|\frac{m_i}{m_o}-1\right| < \left|\frac{C_o}{C_i}\,e^{zEF/RT}-1\right|,$$

$$\left|\frac{m_i}{m_o}-1\right| > \left|\frac{C_o}{C_i}\,e^{zEF/RT}-1\right|,$$

where m_i and m_o are the inward and outward fluxes, C_o and C_i are the external and internal concentrations, z is the valency, and the other symbols have their usual meanings. It is assumed that the activity coefficient of the ion is the same on the two sides of the membrane.

Behaviour of the former type was first described by Levi & Ussing (1948) as a result of their investigations of sodium fluxes in frog muscle, although later work (Keynes, 1954) has suggested that the fluxes they were considering were not wholly passive. Clearer examples of this type of interaction are provided by the work of Mitchell (1954) on phosphate movements in *Micrococcus pyogenes*, and of Croghan (1955) on sodium movements in the brine shrimp *Artemia*. A flux ratio greater than that predicted from the Ussing relationship has so far been described only in *Sepia* axons poisoned with dinitrophenol (Hodgkin & Keynes, 1955*b*).

It is usually possible to decide whether a given flux is 'uphill' or 'downhill', active or passive, 'independent' or 'non-independent' on fairly good evidence. A discussion of the mechanism responsible must remain almost entirely speculative. The specificity of ion transport makes it seem extremely likely that formation of a complex between the transported ion and some cell constituent is commonly involved. It has been stated (Ussing, 1949) that for net transport to be brought about it is necessary that the formation and splitting of the complex be spatially separated. This is not quite true since it is possible to conceive of an active transport mechanism in which ions were split from a complex in such a way that they had a high kinetic energy or an orientation favourable to penetration. If, however, spatial separation of the formation and splitting of a complex were part of the mechanism, then energy could be introduced either in the movement of the complex across the membrane, or by being used to alter the specificity of the complex in a cyclical manner. The former supposition is made in Goldacre's (1952) contractile protein hypothesis, and

in hypotheses involving rotating molecules or molecular segments—see, for example, Lundegardh (1954)—while the latter forms the basis of numerous schemes put forward by a variety of workers since Osterhout—see, for example, Hodgkin & Keynes (1955*a*), Solomon (1952), Franck & Mayer (1947).

Fig. 15 summarizes a cyclical carrier hypothesis put forward by Shaw (1954) to explain his findings in horse red cells. This hypothesis is of particular interest since it provides a ready explanation of many of the findings in human red cells described in the present paper.

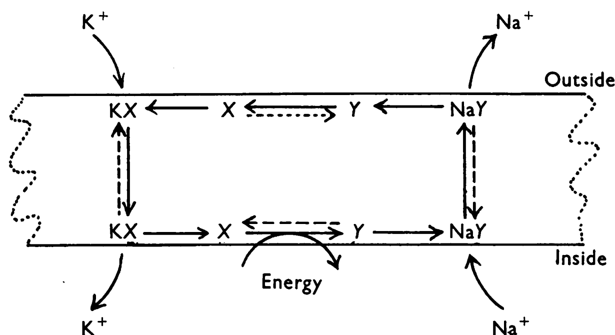

Fig. 15. A carrier hypothesis to explain the link between sodium and potassium movements. Redrawn from Shaw (1954).

It is supposed that K^+ and Na^+ can only cross the membrane in combination with the carriers X and Y, X being K^+ specific and Y being Na^+ specific. The complexes KX and NaY are supposed to be freely diffusible and in equilibrium respectively with K^+ and X, and Na^+ and Y, at the two surfaces of the membrane. X and Y are, by hypothesis, interconvertible and are in equilibrium at the outer surface of the membrane; at the inside surface, X is converted into Y with the expenditure of metabolic energy.

In the presence of glucose and external potassium, the system will carry out an active one-to-one exchange of Na^+ for K^+; because of the 'backwash' (represented by dotted arrows) there will also be a small influx of sodium and efflux of potassium. Saturation of the potassium carriers will cause levelling off of the active potassium influx at high external potassium concentrations. In the absence of glucose and potassium, the Na^+ specific carriers will still be able to shuttle backwards and forwards, and can thus be responsible for the passive part of the sodium efflux. Furthermore, the ratio of the inward and outward passive fluxes of both sodium and potassium will be less than that predicted from the Ussing relationship, and will approach unity more or less closely depending on how saturated the carriers are. The effect of external potassium on sodium influx can be explained by supposing either that, in the absence of potassium, sodium is carried in on the potassium carriers; or that

removal of potassium leads to accumulation, at the outer surface of the membrane, of X and hence, by displacement of the equilibrium, of Y.

There are, however, certain unsatisfactory features. Removal of glucose from human red cells causes a fall in the magnitude of the Michaelis component of potassium influx, but leaves the half-saturation constant β unaffected. This is most readily explained by supposing that β represents the affinity constant of the potassium-specific carrier, and that in the absence of glucose there are fewer carriers crossing the membrane. However, to explain the absence of any active sodium efflux at zero external potassium concentration, it is necessary to postulate that the carriers cannot cross the membrane without attached cations. If this assumption is made, then the half-saturation value of the Michaelis component of potassium influx cannot be interpreted simply as the dissociation constant of the carrier-ion complex, since the amount of carrier present on the outside of the membrane will itself depend on the external potassium concentration. Another difficulty is the difference between horse and human red cells, since Shaw found that removal of glucose from horse cells led to a considerable *increase* in both the magnitude and half-saturation value of the Michaelis component of potassium influx. It is possible that this difference is related to the fact that horse cells, unlike human cells, are relatively impermeable to glucose (Wilbrandt, 1938), and glycolyse very slowly (Meyerhof, 1932).

The cyclical carrier hypothesis just considered depends on the existence of substances which form more or less specific undissociated complexes with sodium and potassium: such substances are unfamiliar. A hypothesis of rather a different type is that the discrimination between sodium and potassium results from the activation or inhibition of an enzyme by Na^+ or K^+. It is not necessary that the enzyme should have a different affinity for the two ions, but merely that it should be more active when binding one than the other. The attractiveness of this hypothesis lies in the fact that several such enzymes have already been described. A list of these is given in Table 12, together with the ions that activate or inhibit them. The antagonism between the effects of K^+ and Rb^+ and the effects of Na^+ and Li^+ is striking, and it may also be significant that several of the enzymes catalyse reactions involving the transfer of large amounts of energy. If energy were fed into the active transport mechanism through the reactions of a potassium-activated enzyme situated on the cell surface, the abolition of active sodium extrusion when the external potassium concentration was reduced would be easily explained. It would be interesting to determine the intracellular localization of potassium-activated enzymes, and to find whether the external potassium concentration does affect the energy metabolism of the cell. However, it may be worth pointing out that, although energy-rich phosphate bonds (or similar high energy bonds) are quite probably involved in active transport, it is rather

unlikely on energetic grounds that one such bond is expended each time a Na^+ is exchanged for a K^+. If the glucose consumption of red cells is taken to be 1·5 m-mole/l. cells/hr (Raker *et al.* 1950; Maizels, 1951) and each molecule of sugar glycolysed is assumed to give rise to two new energy-rich phosphate bonds, then the expenditure of one of these bonds for each Na^+–K^+ exchange would require between one-third and two-thirds of the total production.

TABLE 12. Enzymes activated by univalent cations

Enzyme	Source	Activated by	Inhibited by	Reference
Pyruvic-phosphoferase	Muscle and viscera of a variety of species	K, Rb, NH_4	Na*, Li, Ca	Kachmar & Boyer (1953)
Fructokinase	Beef liver	Mg + K or NH_4 weakly or Na very weakly	—	Hers (1952)
Phospho-fructokinase	Yeast	K, NH_4, Mg, Mn	Na in high concn.	Muntz (1947)
Acetate activating system	Pig heart	K, Rb, NH_4	Na, Li	von Korff (1953)
'Choline acetylase'	Rat brain	K	—	Nachmansohn & John (1945)
Phospho-transacetylase	*Clostridium Kluyveri*	K, NH_4, ? Divalent ions	Na, Li	Stadtman (1952)
Aldehyde dehydrogenase	Yeast	K, Rb, NH_4	Na*, Li, Cs	Black (1951)
Galactosidase	*Bacillus coli*	K, Rb, NH_4	Na*, Li	Cohn & Monod (1951)
Apyrase	Rat brain	Mg + Na	—	Utter (1950)

* In the absence of other ions sodium is weakly activating.

SUMMARY

1. Radioactive tracers have been used to study the movements of sodium and potassium in washed human red cells suspended in buffered physiological salt solutions.

2. It has been found that the relationship between potassium influx (m_i^K) and the concentration of potassium outside the cell ($[K_o]$) may be described by Shaw's (1955) equation

$$m_i^K = \frac{\alpha[K_o]}{\beta + [K_o]} + \gamma[K_o],$$

where α, β and γ are constants.

3. Removal of glucose decreases α by about 50% but leaves β and γ unaffected.

4. These results suggest that potassium influx consists of a small passive component, proportional to the external potassium concentration, and a large active component depending on a mechanism which becomes saturated at high external potassium concentrations.

5. Sodium efflux remains constant at external potassium concentrations greater than about 10 mM, but below 10 mM the efflux decreases. With no potassium outside, sodium efflux is reduced by about one-third of its value at high potassium concentrations.

6. Removal of glucose has no effect on sodium efflux in the absence of potassium, but abolishes about three-quarters of the extra efflux which occurs in the presence of potassium.

7. This extra sodium efflux and the active potassium influx vary in the same way with the external potassium concentration.

8. In a medium free from glucose and potassium, red cells do not show a *net* loss of sodium when the external sodium concentration is greater than the internal.

9. The proposed interpretation of these results is that sodium efflux consists of two components; an active component tightly linked to potassium influx and driven by energy from glucose; and a passive component, not linked to potassium influx and unaffected by glucose deprivation. The fact that removal of glucose leads to a decrease of roughly the same size in potassium influx and in sodium efflux suggests that the linkage between the active fluxes involves a one-to-one exchange of Na^+ for K^+.

10. The temperature coefficients of both components of potassium influx and sodium efflux have been measured.

11. Sodium influx varies with the external sodium concentration in a non-linear manner. It is also affected by the concentration of potassium outside the cell.

12. The ratio of sodium influx to the passive component of sodium efflux, and the ratio of potassium efflux to the passive component of potassium influx, have been compared with the ratios to be expected if the passive fluxes were brought about by free diffusion in aqueous solution. The results indicate that the passive fluxes must depend, partly, on mechanisms in which the chance of penetration of any given ion is affected by the presence of other ions of the same species. For sodium there is some evidence of interaction, of the 'exchange diffusion' type, between efflux and influx.

13. The type of mechanism which could be responsible for cation transport in red cells is discussed.

This work was done during the tenure of a Medical Research Council Scholarship. Expenses were met out of a grant from the Nuffield Foundation.

REFERENCES

BLACK, S. (1951). Yeast aldehyde dehydrogenase. *Arch. Biochem.* **34**, 86–97.

COHN, M. & MONOD, J. (1951). Purification et propriétés de la β galactosidase (lactase) d'*Escherichia coli*. *Biochim. biophys. acta*, **7**, 153–174.

CROGHAN, P. C. (1955). *The osmotic acid and ionic regulation of* Artemia salina. Cambridge University Ph.D. Thesis.

DAVIDSEN, H. G. & KJERULF-JENSEN, K. (1950). K uptake of normal and low K human red corpuscles. *Proc. Soc. exp. Biol., N.Y.*, **74**, 477–480.

DOBSON, A. (1955). The forces moving Na ions through rumen epithelium. *J. Physiol.* **128**, 39*P*.

FLYNN, F. & MAIZELS, M. (1949). Cation control in human erythrocytes. *J. Physiol.* **110**, 301–318.

FRANCK, J. & MAYER, J. E. (1947). An osmotic diffusion pump. *Arch. Biochem. Biophys.* **14**, 297–313.

Glynn, I. M. (1954). Linked Na and K movements in human red cells. *J. Physiol.* **126**, 35*P*.

Gold, G. L. & Solomon, A. K. (1955). The transport of Na into human erythrocytes *in vivo*. *J. gen. Physiol.* **38**, 389–404.

Goldacre, R. J. (1952). The folding and unfolding of protein molecules as the basis of osmotic work. *Int. Rev. Cytol.* **1**, 135–164.

Harris, E. J. (1954). Linkage of K and Na active transport in human erythrocytes. In *Active Transport and Secretion*, ed. Brown, R. & Danielli, J. F. Cambridge University Press.

Harris, E. J. & Maizels, M. (1951). The permeability of human erythrocytes to Na. *J. Physiol.* **113**, 506–524.

Harris, E. J. & Maizels, M. (1952). Distribution of ions in suspensions of human erythrocytes. *J. Physiol.* **118**, 40–53.

Harris, E. J. & Prankerd, T. A. J. (1953). The rate of Na extrusion from human erythrocytes. *J. Physiol.* **121**, 470–486.

Hers, H. G. (1952). Role de magnesium et du potassium dans la reaction fructokinasique. *Biochim. biophys. acta*, **8**, 424–430. See also *Symposium on Phosphate Metabolism*, ed. McElroy, W. D. & Glass, B., **1**, p. 490. Baltimore: Johns Hopkins University Press.

Hodgkin, A. L. & Keynes, R. D. (1953). The mobility and diffusion coefficient of K in giant axons from *Sepia*. *J. Physiol.* **119**, 513–528.

Hodgkin, A. L. & Keynes, R. D. (1955*a*). Active transport of cations in giant axons from *Sepia* and *Loligo*. *J. Physiol.* **128**, 28–60.

Hodgkin, A. L. & Keynes, R. D. (1955*b*). The K permeability of a giant nerve fibre. *J. Physiol.* **128**, 61–88.

Kachmar, J. F. & Boyer, P. D. (1953). The K activation and Ca inhibition of pyruvic phosphoferase. *J. biol. Chem.* **200**, 669–682.

Keynes, R. D. (1954). The ionic fluxes in frog muscle. *Proc. Roy. Soc.* B, **142**, 359–382.

Klinghoffer, K. A. (1935). Permeability of the red cell membrane to glucose. *Amer. J. Physiol.* **111**, 231–242.

Koch, H. J. (1954). Cholinesterase and active transport of sodium chloride through the isolated gills of the crab *Eriocheir sinensis* (M. Edw.). In *Recent Developments in Cell Physiology. Proc. 7th Symp. Colston res. Soc., Bristol*, ed. Kitching, J. A. London: Butterworth.

Le Fevre, P. G. (1948). Evidence of active transfer of certain non-electrolytes across the human red cell membrane. *J. gen. Physiol.* **31**, 505–527.

Levi, H. & Ussing, H. H. (1948). The exchange of Na and Cl ions across the fibre membrane of the isolated frog sartorius. *Acta physiol. scand.* **16**, 232–248.

Lundegardh, H. (1954). Anion respiration. In *Active Transport and Secretion*, ed. Brown, R. & Danielli, J. F. Cambridge University Press.

Maizels, M. (1951). Factors in the active transport of cations. *J. Physiol.* **112**, 59–83.

Maizels, M. (1954). Active cation transport in erythrocytes. In *Active Transport and Secretion*, ed. Brown, R. & Danielli, J. F. Cambridge University Press.

Meyerhof, O. (1932). Über die Antrennung des milchsäurebilden Ferments aus Erythrocyten. *Biochem. Z.* **246**, 249–284.

Mitchell, P. (1954). Transport of phosphate through an osmotic barrier. In *Active Transport and Secretion*, ed. Brown, R. & Danielli, J. F. Cambridge University Press.

Muntz, J. A. (1947). The role of K and NH_4 ions in alcoholic fermentation. *J. biol. Chem.* **171**, 653–665.

Nachmansohn, D. & John, H. M. (1945). Studies on choline acetylase. (1) Effect of amino-acids on the dialysed enzyme. Inhibition by α keto-acids. *J. biol. Chem.* **158**, 157–171.

Raker, J. W., Taylor, I. M., Weller, J. M. & Hastings, A. B. (1950). Rate of K exchange of the human erythrocyte. *J. gen. Physiol.* **33**, 691–702.

Scott, G. T. & Hayward, H. R. (1954). Evidence for the presence of separate mechanisms regulating K and Na distribution in *Ulva lactuca*. *J. gen. Physiol.* **37**, 601–620.

Shaw, T. I. (1954). *Sodium and potassium movements in red cells.* Cambridge University Ph.D. Thesis.

Shaw, T. I. (1955). K movements in washed erythrocytes. *J. Physiol.* **129**, 464–475.

Sheppard, C. W. & Beyl, G. E. (1951). Cation exchange in mammalian erythrocytes. *J. gen. Physiol.* **34**, 691–704.

Sheppard, C. W. & Martin, W. R. (1950). Cation exchange between cells and plasma of mammalian blood. *J. gen. Physiol.* **33**, 703–722.

SHEPPARD, C. W., MARTIN, W. R. & BEYL, G. E. (1951). Cation exchange between cells and plasma of mammalian blood. *J. gen. Physiol.* **34**, 411–429.

SHOHL, A. T. & HUNTER, T. H. (1941). The measurement of the cell volume of blood by the Evans Blue dye method. *J. Lab. clin. Med.* **26**, 1829–1837.

SOLOMON, A. K. (1952). The permeability of the human erythrocyte to Na and K. *J. gen. Physiol.* **36**, 57–110.

SOLOMON, A. K. & GOLD, G. L. (1955). K transport in human erythrocytes: evidence for a three-compartment system. *J. gen. Physiol.* **38**, 371–388.

STADTMAN, E. R. (1952). The purification and properties of phosphotransacetylase. *J. biol. Chem.* **196**, 527–534.

STEINBACH, H. B. (1940). Na and K in frog muscle. *J. biol. Chem.* **133**, 695–701.

STEINBACH, H. B. (1952). On the Na and K balance of isolated frog muscles. *Proc. nat. Acad. Sci. Wash.* **38**, 451–455.

STREETEN, D. H. P. & SOLOMON, A. K. (1954). The effect of ACTH and adrenal steroids on K transport in human erythrocytes. *J. gen. Physiol.* **37**, 643–661.

USSING, H. H. (1949). Transport of ions across cellular membranes. *Physiol. Rev.* **29**, 127–155.

USSING, H. H. (1950). The distinction by means of tracers between active transport and diffusion. *Acta physiol. scand.* **19**, 43–56.

USSING, H. H. (1955). Ion transport across biological membranes. In *Ion Transport Across Membranes*, ed. Clarke, H.T. & Nachmansohn, D. New York: Academic Press.

UTTER, M. F. (1950). Mechanism of inhibition of anaerobic glycolysis of brain by Na ions. *J. biol. Chem.* **185**, 499–517.

VON KORFF, R. W. (1953). The effects of alkali metal ions on the acetate activating enzyme system. *J. biol. Chem.* **203**, 265–271.

WILBRANDT, W. (1938). Die Permeabilität der roten Blutkörperchen für einfache Zucker. *Pflüg. Arch. ges. Physiol.* **241**, 302–309.