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The Influence of External Sodium Ions on the Sodium Pump in Erythrocytes

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1. A study has been made of the interaction between Na^+ and K^+ on the adenosine triphosphatase activity of erythrocyte 'ghosts', and on the K^+ influx and Na^+ efflux of intact erythrocytes. The adenosine triphosphatase activity and the ion movements were greater at a low external K^+ concentration in the absence of Na^+ than they were in the presence of 150mM-Na^+. The inhibition by external Na^+ of K^+ influx had an inhibitory constant of 5–10mM. 2. Activation by K^+ of kidney microsomal adenosine triphosphatase was retarded by Na^+, and activation by Na^+ was retarded by K^+. Fragmented erythrocyte membranes behaved similarly. 3. These observations suggest that there is competition between Na^+ and K^+ at the K^+-sensitive site of the membrane.

The ATPase* activity of cell membranes is stimulated by Na^+ and K^+, and each ion does not activate without the other. In preparations of fragmented membranes the activity shows a sigmoid response to Na^+ when K^+ is present in excess and vice versa (Skou, 1957; Post, Merritt, Kinsolving & Albright, 1960; Green & Taylor, 1964). This observation has been interpreted in two ways. One is that the ion in excess competes with the ion in smaller concentration for sites of activation (Skou, 1957; Post *et al.* 1960). The other view is that enzymic activity involves an allosteric interaction between ions and the enzyme, which produces a change in the protein conformation (Squires, 1965; Robinson, 1967), similar to the effects described by Monod, Wyman & Changeux (1965). A relevant fact is that enzymic stimulation requires that the ions are located on opposite sides of the membrane, whereas competition requires that they are on the same side. Separation of ions cannot be achieved with fragmented membranes, and in order to distinguish clearly between these two possibilities it is necessary to work with intact membranes so that Na^+ can activate from inside and K^+ from outside. Whittam & Ager (1964) and Schatzmann (1965) showed with intact 'ghosts' that activation of the transport ATPase by external K^+ is impeded by external Na^+. Further, Garrahan & Glynn (1967) found that small amounts of Na^+ at the outer surface of the cell membrane interfered with K^+ influx. The present study employed erythrocytes and 'ghosts' for investigating the influence of external Na^+ on ATPase activity, K^+ influx and Na^+ efflux. The main finding is that each activity was decreased by external Na^+, and the results can be explained in terms of competition between Na^+ and K^+ for the K^+-sensitive site on the external surface of the membrane.

* Abbreviation: ATPase, adenosine triphosphatase.

MATERIALS AND METHODS

Materials and procedure

Preparation of kidney microsomal ATPase. This preparation was based on the procedure of Epstein & Whittam (1966). The cortex (20g.) of rabbit kidneys was homogenized in 200ml. of solution containing (final concns.) 0·25M-sucrose, 2mM-EDTA (sodium salt) and 1mM-imidazole–HCl, pH 7·4, first in an Ato-Mix blender (Measuring and Scientific Equipment Ltd., London, S.W. 1) at full speed for 90sec. and then with 20 strokes of an all-glass homogenizer. After filtration through muslin, the homogenate was centrifuged for 15min. at 5000*g* and the sediment was discarded. The supernatant was centrifuged again for 30min. at 110000*g* and the resulting pellet of microsomes was resuspended in a solution containing 0·25M-sucrose and 1mM-imidazole–HCl, pH 7·4, to give about 10mg. dry wt./ml. The preparation was stored at −10° and the activity was retained over a period of 6–7 weeks. The dry weight was always measured and the values for ATPase activity were comparable with those described previously (Wheeler & Whittam, 1964; Epstein & Whittam, 1966). Protein was not usually determined, but an approximate relationship is that 1mg. dry wt. is equivalent to 0·8mg. of protein.

Erythrocyte membrane ATPase. This was prepared as described by Wheeler & Whittam (1964). The suspension of washed fragmented membranes was frozen in a freezing bath of acetone and solid CO_2 and thawed gently. This was done twice before storage at −10°.

ATPase activity. This was measured in mixtures containing 20mM-imidazole–HCl buffer, pH 7·6, various amounts of

the chlorides of the cations being investigated, and ATP as the sodium or tris salt. $MgCl_2$ was added at the same concentration as ATP. The reaction was started by adding the membrane preparation and, after incubation at 37° in a shaking water bath for 60 min., it was stopped by the addition of trichloroacetic acid to a final concentration of 5% (w/v).

Erythrocyte 'ghosts'. These were isolated as previously described (Whittam, 1962) from a haemolysate containing 3 mM-ATP to which sufficient 3 M-NaCl was added to raise the osmotic pressure to that of plasma. The 'ghosts' were washed three times in either ice-cold 0·15 M-NaCl or ice-cold 0·15 M-choline chloride containing 10 mM-tris, pH 7·6. Samples (1·0 ml.) of a suspension of 'ghosts' were added to 1 ml. of pre-warmed medium and, after 20 min. at 37°, 0·5 ml. of 35% (w/v) trichloroacetic acid was added and the containers were transferred to an ice-bath.

K^+ *influx.* This was measured by the method of Glynn (1956).

Na^+ *efflux.* This was measured by loading the cells with $^{24}Na^+$ and following the time-course of the loss of label on incubation in non-radioactive solutions. Erythrocytes, either fresh or 3–4 weeks old, were incubated at room temperature with $^{24}NaCl$ for 4 hr. The cells were washed five times with ice-cold non-radioactive solution; this contained 10 mM-tris, pH 7·6, and NaCl, choline chloride or LiCl (150 mM) or $MgCl_2$ (100 mM). Portions (5 ml.) of a suspension of the washed radioactive cells were added to 10 ml. of pre-warmed medium to which additions of NaCl, KCl and ouabain were sometimes made. With fresh cells 10 mM-glucose was added and with cold-stored cells 10 mM-inosine was added. The cell suspensions (haematocrit about 3%) were shaken at 37° and samples (about 4·5 ml.) were taken at 15, 45 and 75 min. They were cooled in ice for 3–5 min. and centrifuged, and 2 ml. of supernatant was removed for counting. It was necessary to know the radioactivity initially present in the cells from which the counts in the supernatant were obtained. This was calculated from the haematocrit and the total counts in the cell suspension. The rate constant for the Na^+ efflux was given by the slope of the line between 15 and 75 min. when $\ln(1-N_t/N_0)$ was plotted against time, where N_t is the number of counts in 2 ml. of supernatant at time t, and N_0 is the number of counts in the cells at zero time. The Na^+ efflux was obtained by multiplying the rate constant by the Na^+ concentration in the cells.

Chemical methods

Determination of Na^+ *and* K^+. These were determined by flame photometry with either an EEL or a Unicam SP. 90 flame photometer. When the Na^+ concentration of cells was determined in the efflux experiments, cells without tracer were used in otherwise identical conditions.

Haemoglobin. This was estimated as oxyhaemoglobin from the extinction at 540 mμ of suitable dilutions clarified with 6·7 mN-NH_3 (King, 1951).

Inorganic phosphate. P_i was determined by the method of Berenblum & Chain (1938) when high concentrations of choline were present, but otherwise by the method of Fiske & Subbarow (1925). Choline was found to interfere with the simpler method by giving a yellow precipitate.

Chemicals used. ATP was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. $^{24}NaCl$ and ^{42}KCl were obtained as sterile iso-osmotic solutions from The Radiochemical Centre, Amersham, Bucks. Other materials were A.R. grade wherever possible. During this work replacements other than magnesium and choline were used. Tetramethylammonium chloride, tris hydrochloride and LiCl were similar to $MgCl_2$ and choline chloride in being apparently without effect on the sodium pump. The ouabain-sensitive K^+ influx and Na^+ efflux were approximately the same with the various substituents. However, with tetraethylammonium chloride the active K^+ influx was inhibited and this compound apparently behaved as a poison; thus it caused a fall of about 70% in the K^+ influx.

RESULTS

Activation by Na^+ *and* K^+ *of the membrane ATPase*

Kidney. To ascertain the response of the ATPase to K^+, measurements were made of the enzymic activity with different small concentrations of K^+ and sufficient Na^+ to give maximum stimulation. The experiment was designed to test whether the activity would increase roughly linearly with small concentrations of K^+, or whether there would be an inflexion in the response, as described by Squires (1965).

The results show that K^+ stimulated the activity about fourfold (from 6·5 to 23·6 μmoles of P_i liberated/mg. dry wt./hr.) and that maximum activity was elicited with about 5 mM-K^+ (Fig. 1). There was a small but consistent lag in the activity at very low concentrations (less than 0·5 mM), and the curve could not be described by a rectangular hyperbola. The line drawn is that for the equation:

$$y = \alpha x^n/(\beta + x^n)$$

where y is the reaction velocity (μmoles of P_i liberated/mg. dry wt./hr.), x is the K^+ concentration and α is the maximum velocity of the K^+-activated reaction. Values of n and β were obtained by plotting $\log(\alpha/y - 1)$ against $\log x$, as first described by Hill (1910). The value of n was 1·3 and β, which nearly corresponds to the concentration for half-maximum activation when n is near unity, was 1·4 mM.

A similar experiment in which the Na^+ concentration was varied (0–100 mM) with a constant K^+ concentration (10 mM) also showed an inflexion in the activity at low Na^+ concentrations (less than 10 mM) (Fig. 2). Again, the points could be described by the above equation, and the values were $n = 1·7$, $\beta = 83$ mM. As n becomes increasingly greater than 1 the value of β is clearly not the concentration required for half-maximum activation and, from the graph, the latter value was about 12 mM. These results agree with earlier work in showing that the response of the transport ATPase of fragmented membranes to Na^+ and K^+ is not as simple as would be expected from straightforward

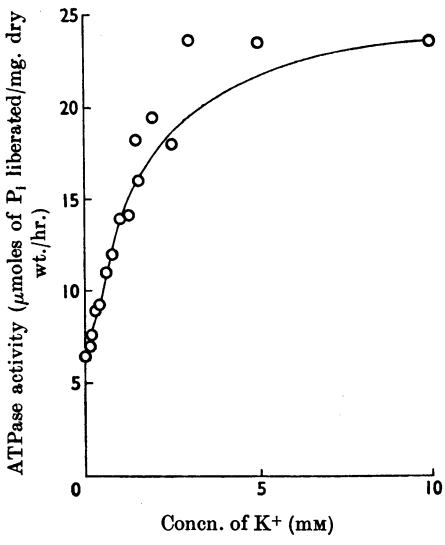

Fig. 1. Stimulation of the ATPase of kidney microsomes by K^+ in the presence of Na^+. Rabbit kidney microsomes (about 0·1 mg. dry wt.) were incubated for 1 hr. at 37° with 3 mM-ATP, and the P_i liberated was determined. The medium contained (mM): NaCl, 100; imidazole–HCl buffer, pH 7·5, 20; $MgCl_2$, 3; and KCl as appropriate. The line drawn is from the equation:

$$y = \alpha x^n/(\beta + x^n)$$

where y is the reaction velocity and α the maximum velocity for the K^+-stimulated reaction, and x is the concentration. Values of $n = 1{\cdot}3$ and $\beta = 1{\cdot}4$ mM, obtained from a Hill (1910) plot of the data, were used.

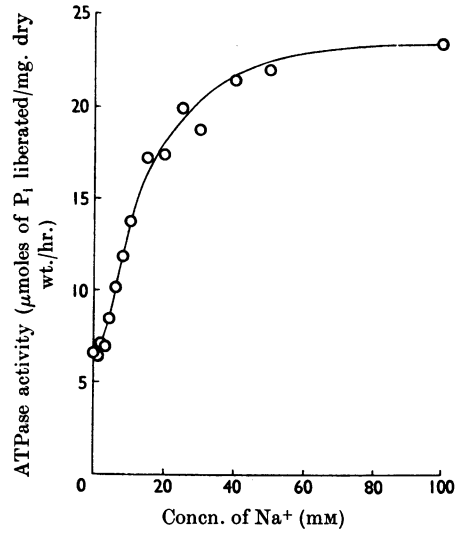

Fig. 2. Stimulation of ATPase of kidney microsomes by Na^+ in the presence of K^+. The conditions were those of Fig. 1 except that the medium contained 10 mM-KCl and appropriate amounts of NaCl. The line is drawn from the equation given in Fig. 1 by using values of $n = 1{\cdot}7$ and $\beta = 83$ mM obtained in a similar way; y and α are the velocity and maximum velocity of the Na^+-sensitive reaction respectively, and x is the Na^+ concentration.

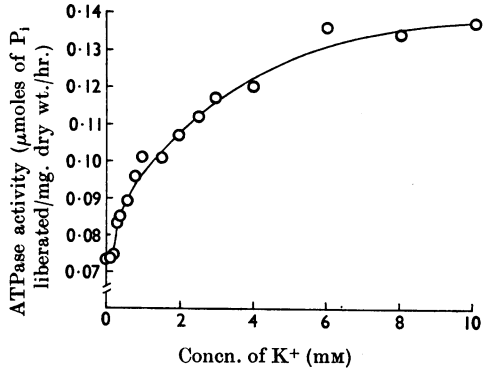

Fig. 3. Stimulation of fragmented erythrocyte membrane ATPase by K^+ in the presence of Na^+. Erythrocyte membranes (about 9 mg. dry wt.) were incubated for 1 hr. at 37° with 3 mM-ATP, and the P_i liberated was determined. The medium contained (mM): NaCl, 100; imidazole–HCl buffer, pH 7·6, 20; $MgCl_2$, 3; and KCl in appropriate amounts. The ATPase activity can be fitted to the curve of the equation given in Fig. 1, where y and α are the velocity and maximum velocity of the K^+-sensitive reaction respectively and x is the K^+ concentration, and $n = 1{\cdot}6$ and $\beta = 1{\cdot}6$ mM.

activation by one ion when there is an abundant amount of the other ion.

Erythrocyte membranes. The activation of the ATPase activity of fragmented erythrocyte membranes by small concentrations of K^+ also showed an inflexion over the range 0–0·5 mM (Fig. 3). The points could again be represented by the above equation, the constants being $n = 1{\cdot}6$, $\beta = 1{\cdot}6$ mM. There would appear to be two possible explanations for these results with fragmented membranes at small concentrations of an activating cation. First, the results might mean that there is a requirement for more than one ion of K^+ before synergistic stimulation is obtained with Na^+, and similarly that more than one Na^+ ion is required before stimulation is obtained with K^+ (Squires, 1965). An alternative view is that the lack of response to low concentrations of an activating ion arises from competition between the two ions, such that activation by one ion at low concentrations is impeded by the other ion at high concentrations. Interpretation of results with fragmented membranes is difficult since it is not possible to make independent changes in the ionic composition of the fluids bathing the inner and the outer surface of the membranes.

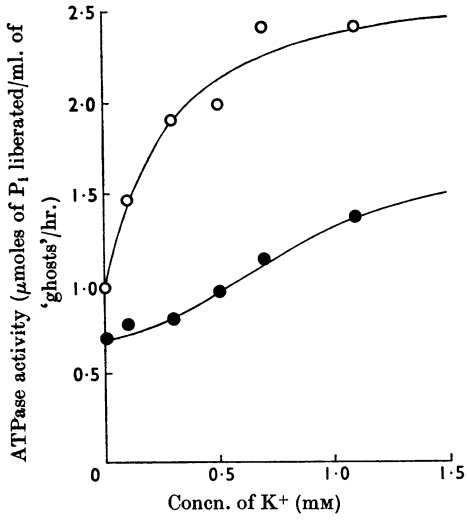

Fig. 4. Stimulation of ATPase of erythrocyte 'ghosts' by K^+ in the presence (●) and absence (○) of external Na^+. Na^+-rich 'ghosts', loaded with ATP, were washed three times in either Na^+ medium or choline medium containing (mM): tris–HCl buffer, pH 7·6, 10; with either NaCl, 150, or choline chloride, 150. The 'ghosts' were suspended (10% haematocrit) in the same media containing KCl (0–10 mM) and incubated for 20 or 30 min. at 37°. The graph shows the P_i liberated in choline medium (○) and in NaCl medium (●). Results shown for 0 mM-K^+ were obtained in the presence of 0·25 mM-ouabain. The points show the mean values from four experiments. The final K^+ concentrations of the original K^+-free media were about 0·2 mM, and allowance was made for this increase by adding the mean increase (0·1 mM) to the initial K^+ concentration. The ATPase activity in choline medium can be fitted to the curve of the equation given in Fig. 1 if $n=1$ and $\beta=0{\cdot}25$ mM, i.e. it is a hyperbola. The activity in NaCl medium fits the equation if $n=1{\cdot}6$ and $\beta=1{\cdot}5$ mM. In both cases y is the K^+-stimulated reaction velocity and α the K^+-stimulated maximum velocity.

Erythrocyte 'ghosts'. To test whether activation by K^+ is impeded by Na^+ it is necessary to work with intact membranes so that any competition at the external surface of the membrane can be distinguished from the synergistic activation of the transport ATPase that depends on Na^+ being located inside and K^+ outside. If therefore there is competition between the two ions it follows that this will occur when the two ions are on the same side. Previous work with erythrocyte 'ghosts' (Whittam & Ager, 1964) was extended by incubating 'ghosts', loaded with ATP, in solutions containing very low concentrations of K^+ (0–1 mM).

There was a marked difference between the ATPase activities at the same K^+ concentration in the presence and in the absence of Na^+. Thus with 0·5 mM-K^+ the activity in the absence of Na^+ was double that in the presence of Na^+ (Fig. 4). There was some leakage of K^+ from the 'ghosts' during the incubation, and the final K^+ concentration was about 0·2 mM. in the originally K^+-free medium Allowance was made for the mean increase in the K^+ concentration during the incubation by adding 0·1 mM to the initial K^+ concentration in the calculations. ATPase activity in the complete absence of Na^+- and K^+-affected ATPase activity was taken as the value in the presence of 0·25 mM-ouabain.

Fig. 4 shows the effect of external Na^+ on the activation by external K^+. The points for ATPase activity in the absence of external Na^+ could be fitted to a rectangular hyperbola and the value for half-maximum activation is 0·25 mM-K^+ (Fig. 4). There was no evidence of the sigmoid response (Fig. 3) found with fragmented membranes. A higher K^+ concentration (about 1·5 mM) was required for half-maximum activation in the presence of 150 mM-Na^+. Activation by K^+ was evidently impeded by Na^+. These values are somewhat lower than those of 0·5 mM- and 2·3 mM-K^+ earlier obtained in the absence and presence of Na^+ respectively (Whittam & Ager, 1964); they are the result of making measurements with a lower K^+ concentration than that previously used, and also of making allowance for the amount of K^+ leakage from the 'ghosts'. It appears from these results that the sigmoid curves obtained for the ATPase activity of fragmented membranes arise from a combination of mutual inhibition and activation by Na^+ and K^+. The work with fragmented membranes was not extended because, as shown in Fig. 4, ATPase was stimulated in a straightforward way by external K^+ when Na^+ ions were absent from the Ringer solution.

Interference by Na^+ *with* K^+ *influx and* Na^+ *efflux*

Since the ATPase activity is linked to ion movements, measurements of K^+ influx and Na^+ efflux in normal erythrocytes were made to find out whether external Na^+ also retards the activation of these ion movements by external K^+ in a manner parallel to the results described above.

K^+ *influx*. K^+ influx was stimulated by raising the external K^+ concentration. In the absence of external Na^+ half-maximum stimulation was given with 0·4 mM-K^+ (Fig. 5) and there was an immediate increase in K^+ influx at small concentrations. In contrast, in the presence of 150 mM-Na^+ the response to external K^+ was not so great and the concentration for half-maximum stimulation was 1·5 mM, in agreement with the results of Garrahan & Glynn (1967). The inhibition of K^+ influx by external Na^+ is also shown as the difference between

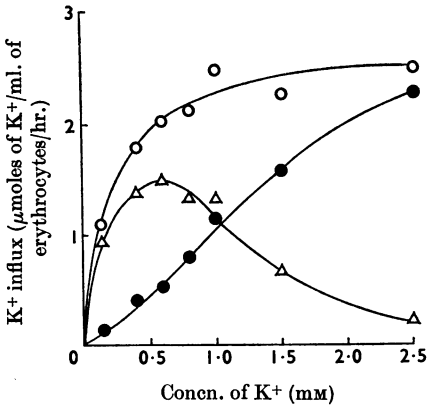

Fig. 5. Stimulation of K^+ influx by external K^+ with and without external Na^+. Erythrocytes (4% haematocrit) were incubated for 1 hr. at 37° in Na^+ medium or Mg^{2+} medium containing (mM): tris–HCl buffer, pH 7·6, 10; with either NaCl, 150, or $MgCl_2$, 100; and suitable amounts of KCl, including some $^{42}K^+$. The Figure shows the K^+ influx in Na^+-free medium (○) and in 150 mM-NaCl (●). The difference in the influxes at each K^+ concentration used is also shown (△).

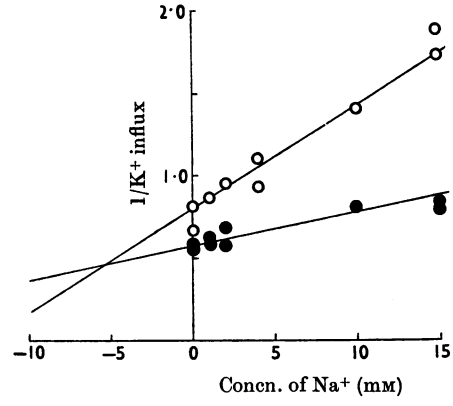

Fig. 6. Inhibition of K^+ influx by Na^+. Erythrocytes (5% haematocrit) were incubated for 1 hr. at 37° in solutions containing (mM): tris–HCl buffer, pH 7·6, 10; NaCl, 0–150; $MgCl_2$ to maintain the same osmotic pressure of the solution, and KCl, including some $^{42}K^+$, 0·15 or 0·5. The Figure shows the reciprocal of the Na^+-sensitive K^+ influx (total influx − the influx in the presence of 150 mM-Na^+) plotted against the Na^+ concentration. The K^+ concentrations were 0·15 mM (○) and 0·5 mM (●). The intercept of the two lines gives the value for the inhibition constant.

the values in the absence and presence of Na^+ at each K^+ concentration, and it appears that the difference was greatest at 0·6 mM-K^+. It seems that K^+ influx is like the transport ATPase as regards competition between external Na^+ and K^+.

A quantitative indication of the inhibitory effect of Na^+ on K^+ activation was obtained. Two concentrations of external K^+ were chosen (0·15 and 0·5 mM) and the K^+ influx was measured at different Na^+ concentrations. Fig. 6 shows the decrease in influx caused by external Na^+. The magnitude of the fall (μmoles/ml. of cells/hr.) caused by 150 mM-Na^+ was 1·35 with 0·15 mM-K^+, and 1·64 with 0·5 mM-K^+. In another experiment falls of 0·83 with 0·15 mM-K^+ and 1·29 with 0·5 mM-K^+ were observed. Values for the inhibitor constant (the concentration of Na^+ that gives half-maximum inhibition) were obtained by plotting the reciprocal of the Na^+-sensitive influx against the Na^+ concentration. Two straight lines were obtained in both cases and their points of intersection gave values of 9–10 mM- and 4–5 mM-Na^+ for the inhibitor constant in the two experiments. These results show that at low K^+ concentrations the K^+ influx was markedly affected by the concentration of external Na^+.

Na^+ *efflux*. Na^+ efflux was stimulated by external K^+, and the efflux was greater in the absence of Na^+ in the medium than when 150 mM-Na^+ was present (Fig. 7). In Na^+-free medium the K^+ concentration for half-maximum stimulation was about 0·2 mM, whereas in the presence of 150 mM-Na^+ the comparable value was 0·9 mM. This result shows that competition between external Na^+ and

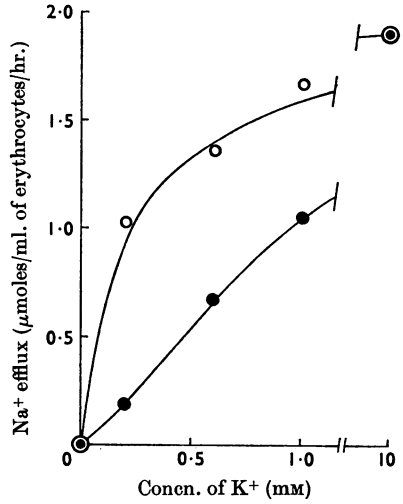

Fig. 7. Stimulation of Na^+ efflux by external K^+ with and without external Na^+. Cells loaded with $^{24}Na^+$ were incubated (5% haematocrit) at 37° in solutions containing (mM): tris–HCl buffer, pH 7·6, 10; with either NaCl, 150, or $MgCl_2$, 100; and suitable amounts of KCl. The Na^+ efflux was calculated from the rate of appearance of $^{24}Na^+$ in the supernatant between 15 and 75 min. after the start of the incubation. The K^+-sensitive Na^+ efflux is shown with 150 mM-Na^+ (●) and in Na^+-free medium (○).

K^+ affects Na^+ efflux in the same way that it does the ATPase activity and the K^+ influx.

DISCUSSION

These results with the Na^+- and K^+-activated ATPase of fragmented membranes confirm that the stimulation by one ion at low concentrations is impeded by the other ion in excess. The experiments were designed to test the suggestion that protein-conformational changes arise from a synergistic action of Na^+ and K^+, as indicated by the sigmoid responses of the enzymic activity to ions (Squires, 1965; Robinson, 1967). It is attractive to suppose that ions are transferred from one side of the membrane to the other by some such mechanism (Whittam, 1962; Tosteson, 1963; Skou & Hilberg, 1965). However, the evidence with fragmented membranes on the inhibition and stimulation by Na^+ and K^+ is equivocal, and it seems unwarranted to draw conclusions about intact cells under physiological conditions.

The present findings for the ATPase activity of 'ghosts' and the K^+ influx and Na^+ efflux of whole erythrocytes show that external Na^+ greatly decreases the stimulation caused by low K^+ concentrations. In each of these three properties of the erythrocytes there was a marked increase in the K^+ concentration required for half-maximum stimulation when external Na^+ was present. The inhibition of the active-transport system by external Na^+ was great and the inhibitor constant was about 5–10mM. Moreover, in the absence of external Na^+ the stimulation of activity appeared to follow a rectangular hyperbola in each case, whereas with external Na^+ there was a more complex relationship similar to that found with fragmented membranes. This is in keeping with the findings of Sachs & Welt (1967) and Garrahan & Glynn (1967) that K^+ influx is not related to the external K^+ concentration by a rectangular hyperbola in the presence of external Na^+. In these studies, as in the present work, care was taken to control the external Na^+ concentration, and each result shows the great sensitivity to Na^+ of the external K^+ site of the transport system. This sensitivity is greater than would have been expected from earlier work on ion movements in cells (Post *et al.* 1960) and on ATPase activity in 'ghosts' (Whittam & Ager, 1964).

Ahmed, Judah & Scholefield (1966) described with a preparation of brain microsome ATPase a sigmoid response to Na^+ similar to that found with kidney microsomes. They concluded that the brain ATPase is activated as a result of the combination of the enzyme with one ion of K^+ and two of Na^+. A comparable proposal by Sachs & Welt (1967) is that there are two co-operative K^+-sensitive site on the outer surface of the membrane. A mechanism involving a co-operative effect of an internal Na^+ site with more than one external K^+-sensitive sites is not excluded by the present results, but neither is it supported. Support would have consisted in an inflexion in the response of the ATPase, K^+ influx and Na^+ efflux at low K^+ concentrations, but this was not found in the absence of Na^+. A result providing unequivocal support for an allosteric mechanism has yet to be obtained.

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