Vectorial Aspects of Adenosine-Triphosphatase Activity in Erythrocyte Membranes

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Directional effects of ions on a chemical mechanism are implied by the complex way in which active transport processes depend on the ionic composition of the fluids bathing the cell membranes. There appears to be an interdependence of cation movements, for an ion is transported in one direction only when another ion is moved in the opposite direction. The net extrusion of Na^+ ions from erythrocytes requires the presence in the medium of K^+ ions, which are simultaneously taken up (Harris & Maizels, 1951; Glynn, 1956; Post & Jolly, 1957). It is this coupled movement of Na^+ and K^+ ions that is inhibited by cardiac glycosides, probably from the external surface of the cell (Glynn, 1957). Other ions, notably Rb^+, NH_4^+, Cs^+ and Li^+, may replace K^+ ions both in being accumulated and in facilitating Na^+ ion efflux (Solomon, 1952; Love & Burch, 1953; Post & Jolly, 1957; Kahn, 1962; McConaghey & Maizels, 1962). There is, in contrast, a strict requirement for internal Na^+ ions, because no active transport occurs in their absence (McConaghey & Maizels, 1962). The Na^+ ions are always moved outwards and the other alkali-metal ions inwards. A further example of the dependence of cation transport on the cell's ionic environment is the retardation by external Na^+ ions of the uptake of K^+ ions when the K^+ ion concentration is low relative to that of Na^+ ions (Post, Merritt, Kinsolving & Albright, 1960). It follows that Na^+ ions affect K^+ ion uptake differently, depending on where they are, since from inside the cell they promote K^+ ion uptake and from outside they retard it. These effects illustrate the marked directionality of the cell membrane in the way it catalyses ion movements.

The question arises: does the enzyme system responsible for the supply of energy for active transport show the same spatial orientation as regards activation and inhibition by ions? The enzyme system utilizes ATP in intact cells, since K^+ ion influx falls in a way parallel to the ATP concentration and, further, ATP hydrolysis is diminished in the presence of ouabain as active transport is inhibited (Whittam, 1958; Dunham, 1957; Lindemann & Passow, 1960). Again, active Na^+ ion and K^+ ion transport is supported by ATP in erythrocyte 'ghosts' (Gardos, 1954; Hoffman, 1960). These two ions together stimulate the adenosine-triphosphatase activity of fragmented erythrocyte membranes, and the stimulation is prevented by ouabain (Post *et al.* 1960; Dunham & Glynn, 1961). Moreover, this stimulated activity, like the transport system, is spatially asymmetrical in requiring internal Na^+ ions and external K^+ ions, an effect of one ion being seen only when the other ion is on the opposite side of the membrane (Glynn, 1962; Whittam, 1962*a*; Laris & Letchworth, 1962). The similar directionality shown by the membrane-bound adenosine triphosphatase and the active transport system suggests a close relationship.

The aim of the present work was to elucidate the response of the membrane adenosine triphosphatase of human erythrocytes to Li^+, Na^+, K^+, Rb^+ and Cs^+ ions. 'Ghosts' with a low permeability to cations allowed the internal and external ionic concentrations to be varied independently (Hoffman, 1958; Hoffman, Tosteson & Whittam, 1960; Whittam, 1962*a*). The main result is that enzymic activity is stimulated by internal Li^+ or Na^+ ions acting synergically with external Li^+, K^+, Rb^+ or Cs^+ ions. The concentrations of external Li^+, K^+, Rb^+ and Cs^+ ions required for half-maximal stimulation are similar to those required for their uptake.

Preliminary accounts of some of this work have been published (Whittam, 1962*b*; Whittam & Ager, 1962).

METHODS

Preparation of 'ghosts'. The procedure is based on methods described previously (Gourley, 1957; Hoffman, 1958; Hoffman *et al.* 1960; Whittam, 1962*a*). Human blood aged 4–45 days (kindly supplied by the Blood Transfusion Centre at the Churchill Hospital, Oxford) was centrifuged at 1700*g* for 10 min. ($g_{max.}$ is given throughout). The white cells and plasma were removed, and the erythrocytes (1 vol.) washed twice in 3–4 vol. of 0·15 M-NaCl or choline chloride. Then 1 vol. of washed cells was added with vigorous stirring to 5–60 vol. of either distilled water or a solution containing equimolar concentrations of $MgCl_2$ and

ATP (disodium salt) in the range 0·25–4 mM. Sufficient 3 M-NaCl, -LiCl, -RbCl or -CsCl (approx. 5 ml./100 ml. of haemolysis fluid) was added to raise the osmotic pressure of the haemolysate to about 0·3 osmolar. The haemolysate, now iso-osmotic with plasma, was incubated for 30 min. at 37° to allow the membranes to regain a low permeability to Na^+ and K^+ ions and to ATP (Hoffman *et al.* 1960; Whittam, 1962*a*). The reconstituted 'ghosts' were sedimented by centrifugation for 5 min. at 17000*g* (Servall centrifuge; Ivan Servall Inc., Conn., U.S.A.) and were washed once or twice followed by centrifugation in the same way as above. These 'ghosts' contained in high concentration the cation of the alkali-metal chloride that was added to the haemolysate. The 'ghosts' were finally suspended in the medium in which they were to be incubated. The volume of 'ghosts' (about 0·2 ml./ml. of suspension) was calculated accurately from the ratio of the haemoglobin concentration in the suspension and in the packed 'ghosts' after their final wash.

Determination of volume of reconstituted 'ghosts'. To relate the volume of 'ghosts' to the initial cell volume, two 40 ml. samples of the final haemolysate were centrifuged at 17000*g* for 5 min. After removal of the supernatant fluid, the haemoglobin concentration in the 'ghosts' ($[Hb]_{G2}$) was determined on one sample and the total haemoglobin (Hb_T) in the 'ghosts' on the other sample. The volume of 'ghosts' in 1 ml. of haemolysate (V_{G2}) was calculated from the relationship:

$$V_{G2} = \frac{Hb_T}{40 \times [Hb]_{G2}}$$

Another estimate of V_{G2} was obtained from determination of the haemoglobin concentration in the whole haemolysate ($[Hb]_H$, per ml.) and in the supernatant fluid after the final centrifugation ($[Hb]_{S2}$):

$$[Hb]_H = V_{G2} \times [Hb]_{G2} + (1 - V_{G2}) \times [Hb]_{S2}$$

$$V_{G2} = \frac{[Hb]_H - [Hb]_{S2}}{[Hb]_{G2} - [Hb]_{S2}}$$

The estimates of V_{G2} obtained by these two methods were in good agreement. The volume of cells added to the haemolysing fluid was determined as follows. A sample of washed erythrocytes was centrifuged for 30 min. at 2000*g*, and the haemoglobin concentration of the packed cells determined ($[Hb]_C$). Since the fluid trapped between the cells under these conditions probably occupied only about 2% of the total volume (Jackson & Nutt, 1951; Maizels & Remington, 1959), $[Hb]_C$ was taken to represent the actual cell haemoglobin concentration. The volume of cells from which the 'ghosts' in 1 ml. of haemolysate were derived was therefore $[Hb]_H/[Hb]_C$.

Determination of shrinkage during reconstitution. Cells swell during haemolysis to form spherical 'ghosts', the haemoglobin concentration of which ($[Hb]_{G1}$) was determined in the same way as that of the final 'ghosts' ($[Hb]_{G2}$). The spherical 'ghosts' shrink when the osmolarity of the haemolysate is restored to that of plasma, and, provided that the amount of haemoglobin in the 'ghosts' remains constant, the values of $[Hb]_{G1}$ and $[Hb]_{G2}$ are related to the initial (V_{G1}) and final (V_{G2}) 'ghost' volumes as follows:

$$\frac{V_{G1}}{V_{G2}} = \frac{[Hb]_{G2}}{[Hb]_{G1}}$$

The shrinkage of 'ghosts' during reconstitution was therefore calculated from the relationship:

$$\text{Percentage shrinkage} = \frac{(V_{G1} - V_{G2})}{V_{G1}} \times 100$$

$$= \left(1 - \frac{[Hb]_{G1}}{[Hb]_{G2}}\right) \times 100$$

Assay of adenosine-triphosphatase activity. This was carried out essentially as described by Whittam (1962*a*). Reconstituted 'ghosts' containing ATP were suspended in medium of composition NaCl (140–160 mM), KCl (0–12 mM), $MgCl_2$ (2 mM) and tris–HCl buffer, pH 7·6 (10 mM). Samples of suspension (2 ml.) were incubated at 37° for 15, 30 or 60 min., and 0·2 ml. of 50% or 0·5 ml. of 25% (w/v) trichloroacetic acid was added. The amount of orthophosphate produced was linear with time over the range employed (Whittam, 1962*a*) and has been taken as a measure of adenosine-triphosphatase activity. In some experiments the NaCl in the washing or incubation medium was replaced partially or completely by LiCl, RbCl, CsCl, choline chloride, tris hydrochloride, tetramethylammonium chloride or $MgCl_2$. In other experiments, 'ghost' suspensions were centrifuged for 5 min. at 3400*g* before and after incubation, for separate phosphate analyses of 'ghosts' and supernatant fluids.

Chemical estimations. Orthophosphate was estimated on the trichloroacetic acid extracts by the method of Fiske & Subbarow (1925), in which there is no appreciable hydrolysis of ATP (Bartlett, 1959). The S.E. for a mean of 3·39 μmoles/ml. of cells for five estimations was 0·01 with a range from 3·37 to 3·43. Choline, tetramethylammonium and tris, when present in low concentrations, did not interfere with this estimation. Acid-labile phosphate was determined as the increase in orthophosphate when extracts were heated for 7 min. at 100° with an equal volume of 2 N-HCl. Haemoglobin was estimated according to the method of King (1951), and potassium with an EEL flame photometer.

Chemicals. ATP (disodium salt) and tris (Sigma 7–9) were obtained from the Sigma Chemical Co. Other chemicals were of AnalaR quality (British Drug Houses Ltd.) wherever possible.

RESULTS

General characteristics of the 'ghost' preparation

Attachment of haemoglobin to the membrane. Table 1 shows that 'ghosts' produced by haemolysis in water had a haemoglobin concentration after centrifugation ($[Hb]_{G1}$) greater than that in the surrounding fluid ($[Hb]_{S1}$). The absorption spectrum of the haemoglobin in the 'ghosts' had the characteristics described by Lemberg & Legge (1949). The value of $[Hb]_{G1} - [Hb]_{S1}$ remained constant and was equal to 3·8–4·7 % of the cell haemoglobin concentration. A small fraction of the cellular haemoglobin seems to remain attached to the membrane during haemolysis in water.

Volume of reconstituted 'ghosts'. When erythrocytes aged 28 days were haemolysed with various proportions of water, the 'ghosts' appeared

Table 1. *Inequality of haemoglobin concentration in 'ghosts' and supernatant fluid*

Erythrocytes aged 28 days were washed three times with 0·15 M-NaCl and 1 vol. of cell suspension (haematocrit 0·75) was added to 5–59 vol. of distilled water. The haemolysates were centrifuged and the haemoglobin concentrations of sedimented 'ghosts' ($[Hb]_{G1}$) and supernatant fluid ($[Hb]_{S1}$) were determined. The second column shows the $[Hb]_{G1}/[Hb]_{S1}$ ratio, and the third column the

$$100 \times ([Hb]_{G1} - [Hb]_{S1})/[Hb]_C \text{ ratio,}$$

where $[Hb]_C$ is the haemoglobin concentration in cells packed for 30 min. at 2000*g*. Similar results were obtained with cells aged 4, 9, 26, and 45 days. For a total of 18 values for the difference in the third column the range was 2·5–5·5, the mean 4·0 and S.E.M. 0·2.

Volume of cells / Volume of water	Concn. of haemoglobin in 'ghosts' / Concn. of haemoglobin in supernatant fluid	Difference in haemoglobin concn. between 'ghosts' and supernatant fluid (% of concn. of cell haemoglobin)
1:8	1·4	4·4
1:17	1·8	3·8
1:35	2·5	3·8
1:54	3·8	4·7
1:80	4·5	4·3

Table 2. *Shrinkage of 'ghosts' during reconstitution*

Erythrocytes aged 4–45 days were washed two or three times with 0·15 M-NaCl and 1 vol. of cell suspension (haematocrit 0·7–0·8) was added to 5–20 vol. of vigorously stirred distilled water. After 10–15 min. 3 M-NaCl was added (5 ml./100 ml. of water) and the haemolysate was incubated for 30 min. at 37°. Samples of haemolysate were centrifuged for 5 min. at 17000*g* before and after this reconstitution, and from the initial ($[Hb]_{G1}$) and final ($[Hb]_{G2}$) haemoglobin concentrations in the sedimented 'ghosts' shrinkage was calculated as

$$(1 - [Hb]_{G1}/[Hb]_{G2}) \times 100\%.$$

The volume of reconstituted 'ghosts'/ml. of haemolysate was calculated from their total haemoglobin content and from $[Hb]_{G2}$; the volume of erythrocytes from which they were derived was determined as the ratio of the haemoglobin concentrations in haemolysate and packed cells (see the Methods section). Similar results were obtained with cells aged 9 and 26 days.

Cell age (days)	Volume of cells / Volume of water	Percentage shrinkage of 'ghosts'	Volume of reconstituted 'ghosts' (% of initial cell volume)
28	1:8	76	33
	1:17	79	27
4	1:6	74	36
	1:12	78	27
45	1:7	77	27
	1:14	77	24
	1:26	75	24

spherical under the phase-contrast microscope. After the osmolarity of the haemolysates had been raised to that of plasma by the addition of 3 M-sodium chloride, the 'ghosts' were crenated and shrunken, as found by Ponder & Barreto (1957). Determination of 'ghost' haemoglobin concentration before and after the addition of 3 M-sodium chloride showed that the spherical 'ghosts' shrank by 76 % when the ratio of cells to water was 1:8 and by 79 % when it was 1:17 (Table 2). The degree of shrinkage was the same irrespective of a fourfold change in the volume of water in which the cells were haemolysed. In consequence, reconstituted 'ghosts' occupied only a quarter to a third of the volume of the cells from which they were derived (Table 2). Similar results were obtained with cells aged 4 and 45 days. 'Ghosts' prepared by haemolysis in ATP solution shrink in the same way. Shrinkage was unaffected when 3 M-lithium chloride, -potassium chloride, -rubidium chloride or -caesium chloride was used in place of sodium chloride to raise the osmolarity of haemolysates.

Preparation of 'ghosts' of varied adenosine triphosphate content. 'Ghosts' with a range of ATP concentrations were prepared by haemolysis in solutions containing 0·25–4 mM-ATP. The acid-labile phosphate concentration of the 'ghosts' was determined as a convenient indication of their ATP content. It was roughly proportional to the ATP content of the haemolysate in the lower range (0·25–2 mM-ATP) but was not further increased when the haemolysing fluid contained 4 mM-ATP (Table 3). Thus 'ghosts' contained a maximum of about 12 μmoles of acid-labile phosphate/ml. of 'ghosts'. Further increase in ATP concentration in the haemolysing fluid did not result in further increase in the acid-labile phosphate concentrations in the 'ghosts', and some rehaemolysis occasionally occurred during incubation which was evident by eye. The assay of adenosine-triphosphatase activity in most experiments was therefore made with 'ghosts' prepared from haemolysing fluid containing 2 mM- or 4 mM-ATP.

Adenosine triphosphate hydrolysis in 'ghosts' of different adenosine triphosphate content. Table 3 shows that, with an increase in acid-labile phosphate concentration from 1·7 to 12·1 μmoles/ml. of 'ghosts', the adenosine-triphosphatase activity (in μmoles of orthophosphate/ml. of erythrocytes/hr.) rose from 0·2 to a maximum of 1·5. Ouabain-sensitive activity rose from 0·1 to 0·8. The activity may also be expressed in μmoles of orthophosphate/ml. of 'ghosts'/hr. and again there was the same increase with acid-labile phosphate concentration, the total increasing from 1·0 to 6·8 and the ouabain-sensitive activity from 0·4 to 3·0. Although an accurate determination of the dependence of adenosine-triphosphatase activity on ATP concen-

tration was not possible under the present conditions, in three experiments the total enzymic activity appeared to flatten off, with half-maximal activity at an acid-labile phosphate concentration of 2·2–5·0 μmoles/ml. of 'ghosts'.

pH-dependence of adenosine-triphosphatase activity. Adenosine-triphosphatase activity was determined at pH values from 5·2 to 9·5. When 'ghosts' with a high concentration of Na^+ ions were incubated in medium containing 10 mM-K^+ ion, the activity (in μmoles of orthophosphate/ml. of 'ghosts'/hr.) increased from 3·1 at pH 5·2 to 4·6 at pH 7·5 (Fig. 1). It then rose sharply to a maximum rate of 6·6–6·7 at pH 8·5–9·0. The activity fell off as the pH was raised to 9·5. Adding ouabain to the complete system or omitting K^+ ions from the medium caused a marked depression of activity at all pH values. A difference curve relating K^+ ion-dependent or ouabain-sensitive adenosine-triphosphatase activity to pH was different from the total activity in having a broad maximum at pH 7–8·5 (Fig. 1). Thus, in contrast with the total activity, the K^+ ion-dependent ouabain-sensitive activity was significantly greater at pH 7·9 [2·48 ± 0·25 (8)] than at pH 9·0 [1·48 ± 0·24 (8)]. At pH 7·5 the K^+ ion-dependent activity was about 50 % of the total. There appear to be two enzymic mechanisms for ATP hydrolysis: one dependent on external K^+ ions and inhibited by ouabain, with a pH optimum of 7–8·5, and the other not sensitive to K^+ ions and ouabain, with a pH optimum at 8·5–9.

Asymmetrical activation of adenosine triphosphatase by alkali-metal ions

The dependence of ATP hydrolysis on the location of cations was studied for all the alkali-metal ions by independently varying the nature of the internal and external ionic environment.

Activation by internal Na^+ *and* Li^+ *ions.* When 'ghosts' were incubated in medium containing 10 mM-K^+ ion, the rate of ATP hydrolysis (in μmoles of orthophosphate/ml. of 'ghosts'/hr.) depended on the kind of cation within the 'ghosts'. There is some spread in the results, but there is a gradation of activity dependent on internal cation in the sequence:

$$Na^+ > Li^+ > K^+ > Rb^+ > Cs^+$$

The effect of internal cation is more clearly brought out by comparing the fall in activity due to lack of K^+ ions in the medium or to ouabain. Omission of K^+ ions from the medium depressed enzymic activity in Na^+ ion- and Li^+ ion-rich 'ghosts' to 1·24 and 1·11 respectively. In contrast, there was no effect on the activity in 'ghosts' containing predominantly K^+ (1·46), Rb^+ (1·25) or Cs^+ (0·99) ions. The addition of ouabain to medium containing 10 mM-K^+ ion had the same effect as the omission of K^+ ion in causing a greater fall in adenosine-triphosphatase activity in 'ghosts' containing chiefly Na^+ or Li^+ ions than in 'ghosts' containing mainly K^+, Rb^+ or Cs^+ ions. The activity sensitive to ouabain was about 1·5 in Na^+ ion- or Li^+ ion-rich ghosts compared with about 0·8 in 'ghosts' rich in

Table 3. *Adenosine triphosphate hydrolysis in 'ghosts' of different adenosine triphosphate content*

Washed erythrocytes aged 32 days were added (1 vol.) to 5 vol. of solution containing equimolar ATP (disodium salt) and $MgCl_2$ (0·25–4 mM). Then 3 M-NaCl was added (5·3 ml./100 ml. of ATP solution), and the haemolysates were incubated for 30 min. at 37° and centrifuged for 5 min. at 17000*g*. The supernatant fluid was removed, the 'ghosts' were resuspended in medium containing NaCl (160 mM), KCl (10 mM), $MgCl_2$ (2 mM) and tris–HCl buffer, pH 7·6 (10 mM), and centrifuged as before. The 'ghosts' were suspended in this medium (about 0·2 ml. of 'ghosts'/ml. of suspension), samples of suspension (2 ml.) were incubated for 10–30 min. at 37° in the presence and absence of ouabain (0·1 mM), and 50% (w/v) trichloroacetic acid (0·2 ml.) was added. Adenosine-triphosphatase activity was taken as the increase in orthophosphate between initial and incubated samples. Acid-labile phosphate was determined on the initial samples. This was one of a series of five experiments, in two of which the effect of ouabain was examined.

Concn. of ATP in haemolysing fluid (mM)	Concn. of acid-labile phosphate in 'ghosts' (μmoles/ml.)	Adenosine-triphosphatase activity (μmoles of orthophosphate/ml. of original cells/hr.) Total	Ouabain-sensitive activity	(μmoles of orthophosphate/ml. of 'ghosts'/hr.) Total	Ouabain-sensitive activity
0·25	1·7	0·2	0·1	1·0	0·4
0·5	2·8	0·4	0·3	1·8	1·1
1	6·3	1·0	0·5	5·3	2·7
2	12·1	1·5	0·6	6·8	3·0
4	12·2	1·3	0·8	3·7	2·3

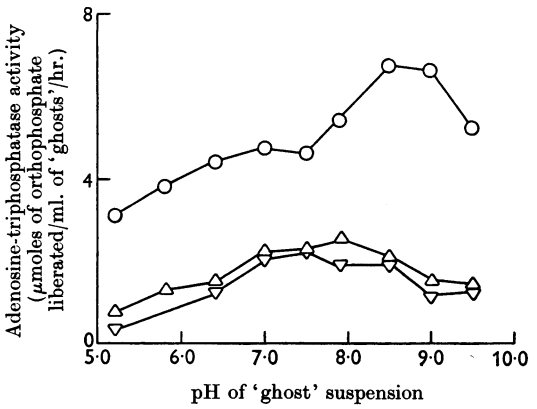

Fig. 1. pH-dependence of adenosine-triphosphatase activity. Washed erythrocytes aged 3–4 weeks were haemolysed in 5 vol. of a solution containing ATP (2 mM) and $MgCl_2$ (2 mM). Then 3 M-NaCl was added (5·3 ml./100 ml. of ATP solution), and the haemolysate was incubated for 30 min. at 37°. The 'ghosts' were washed and suspended in medium containing NaCl (140 mM), $MgCl_2$ (2 mM), KCl (10 mM, if present) and tris–HCl buffer, pH 7·6 (30 mM). Then N-HCl or N-NaOH was added dropwise to give suspensions of pH 5·2 to 9·5 (measured with a glass electrode). Samples of suspension (2 ml.) were incubated for 15–30 min. at 37° in the presence and absence of ouabain (0·1 mM) and KCl (10 mM), and 25% (w/v) trichloroacetic acid (0·5 ml.) was added. The pH was constant during incubation, except for a fall of 0·2 pH unit at pH 9·0–9·5. Rehaemolysis was under 3%, except at pH 5·2 where it reached 3–10%. The graph shows mean values from five to twelve experiments for the increase in orthophosphate during incubation in the presence of 10 mM-KCl (○), and for the fall in orthophosphate liberation caused by ouabain (△). The decrease in orthophosphate liberation caused by the omission of K^+ ions from the medium (▽) is the mean of two or three experiments.

K^+, Rb^+ or Cs^+ ions. When K^+ ions were omitted from the medium the decrease in activity caused by ouabain (0·44–0·61) was unaffected by the nature of the internal cation (Table 4). Internal Rb^+ and Cs^+ ions thus resemble internal K^+ ions in not stimulating ATP hydrolysis, whereas internal Li^+ ions resemble internal Na^+ ions in activating enzymic activity in a way dependent on external K^+ ions. The synergic stimulation by internal Li^+ or Na^+ ions acting in concert with external K^+ ions was abolished by ouabain.

Synergic activation of adenosine triphosphatase by internal Li^+ *or* Na^+ *ions, and by external* Li^+, K^+, Rb^+ *or* Cs^+ *ions.* To find out whether external Li^+, Rb^+ and Cs^+ ions activate the membrane adenosine triphosphatase as well as K^+ ions, the concentrations in the medium were varied. 'Ghosts' rich in Na^+ or Li^+ ions were incubated in media containing 150 mM-sodium chloride, -lithium chloride, -rubidium chloride or -caesium chloride, or 75 mM-sodium chloride together with 75 mM-lithium chloride, -rubidium chloride or -caesium chloride. The amount of the adenosine-triphosphatase activity inhibited by ouabain was measured as an indication of the enzymic activity dependent on directional effects of ions. In 'ghosts' containing a high concentration of Li^+ ions, this activity was 0·4 in medium containing only sodium chloride but was raised to 0·8 or 1·0 when the media contained 75 mM-Li^+, -Rb^+ or -Cs^+ ion (Table 5). In the same experiment, 'ghosts' containing predominantly Na^+ ions behaved in the same way as 'ghosts' rich in Li^+ ions. The activity sensitive to ouabain of 0·5 in medium containing Na^+ ions was increased to 1·0 or 1·1 in media containing 75 mM-Li^+, -Rb^+ or -Cs^+ ion. The same increase in activity for both kinds of 'ghost' was also found when 10 mM-K^+

Table 4. *Synergic stimulation of the membrane adenosine triphosphatase by internal* Li^+ *or* Na^+ *ions and external* K^+ *ions*

A haemolysate was made by adding 1 vol. of erythrocytes to 5 vol. of a solution of 4 mM-ATP (disodium salt) and 4 mM-$MgCl_2$. A solution (3 M) of LiCl, NaCl, KCl, RbCl or CsCl was then added to restore iso-osmolarity with plasma. After 30 min. incubation at 37° the 'ghosts' were centrifuged, washed in medium containing NaCl (150 mM), $MgCl_2$ (2 mM) and tris–HCl buffer, pH 7·6 (10 mM), and suspended in the same medium. In one lot of suspension, 1 M-KCl was added to give a final concentration of 10 mM. The suspensions were incubated for 1 hr. in the presence and absence of 0·1 mM-ouabain, and orthophosphate was then estimated. Means ± S.E.M. are given and the numbers of observations are in parentheses.

	Orthophosphate produced from ATP (μmoles/ml. of 'ghosts'/hr.)			
	K^+ ion-free medium		Medium containing 10 mM-K^+ ion	
Alkali-metal ion in 'ghosts'	Control	Decrease in presence of ouabain	Control	Decrease in presence of ouabain
Li^+	1·11 ± 0·07 (12)	0·61 ± 0·11 (6)	1·99 ± 0·13 (11)	1·44 ± 0·11 (10)
Na^+	1·24 ± 0·08 (24)	0·59 ± 0·07 (15)	2·32 ± 0·13 (18)	1·61 ± 0·09 (14)
K^+	1·46 (3)	0·61 (3)	1·67 ± 0·12 (8)	0·87 ± 0·10 (8)
Rb^+	1·25 (4)	0·60 (2)	1·23 (4)	0·75 (4)
Cs^+	0·99 (3)	0·44 (1)	1·10 (4)	0·71 (4)

Table 5. *Synergic stimulation of adenosine-triphosphatase activity by internal* Li^+ *and* Na^+ *ions and external* Li^+, K^+, Rb^+ *and* Cs^+ *ions*

'Ghosts' containing Li^+ or Na^+ ions were prepared as described in Table 4, but the NaCl of the incubation medium was partially replaced by LiCl, RbCl or CsCl. Orthophosphate was estimated after incubation for 1 hr. in the presence and absence of K^+ ions (10 mM) and ouabain (0·1 mM).

Main cation in 'ghosts'	Concn. of cations in medium (mM)				Ouabain-sensitive adenosine-triphosphatase activity (μmoles of orthophosphate liberated/ml. of 'ghosts'/hr.)		
	Na^+	Li^+	Rb^+	Cs^+	K^+ ion-free medium	Medium containing 10 mM-K^+ ion	Increase due to K^+ ions
Li^+	150	—	—	—	0·4	1·0	0·6
	75	75	—	—	0·8	1·1	0·3
	75	—	75	—	0·8	0·8	0
	75	—	—	75	1·0	1·0	0
Na^+	150	—	—	—	0·5	1·1	0·6
	75	75	—	—	1·1	—	—
	75	—	75	—	1·0	—	—
	75	—	—	75	1·0	—	—

ion was added to medium containing 150 mM-sodium chloride. The addition of 10 mM-K^+ ion to media already containing 75 mM- or 150 mM-Li^+, -Rb^+ or -Cs^+ ion caused little or no further stimulation of enzymic activity (Table 5). Thus the ouabain-sensitive activity was the same with 75 mM-Li^+ ion, -Rb^+ ion or -Cs^+ ion in the medium as with 10 mM-K^+ ion. These results suggest that external Li^+, Rb^+ and Cs^+ ions activate the same enzyme system as K^+ ions.

Concentrations of external cations required for half-maximal stimulation. To discover the concentrations at which external Li^+, K^+, Rb^+ and Cs^+ ions produce half-maximal stimulation of the adenosine triphosphatase, 'ghosts' rich in Na^+ ions were incubated in media containing 0–98 mM-lithium chloride, -potassium chloride, -rubidium chloride or -caesium chloride, and sodium chloride to make a total concentration of 150 mM. The activity, expressed as the stimulation due to the ion other than Na^+ ion, increased steeply as the concentration of external K^+ or Rb^+ ions was raised from 0 to 5 mM, and little further increase occurred at higher concentrations (Fig. 2). Greater concentrations of Cs^+ and Li^+ ions were required for stimulation, but at concentrations above 40 mM-Cs^+ ion a plateau was again observed at about the same magnitude as was found with K^+ and Rb^+ ions. The same level was reached with 98 mM-Li^+ ion. Half-maximal activation would be elicited with 1–2 mM-K^+ or -Rb^+ ion, 8 mM-Cs^+ ion or 20 mM-Li^+ ion.

Stimulation by K^+ *ions in* Na^+ *ion-free medium.* To test whether external Na^+ ions interfere with the activation of adenosine-triphosphatase activity by K^+ ions, the concentration of K^+ ions required for half-maximal stimulation was examined in

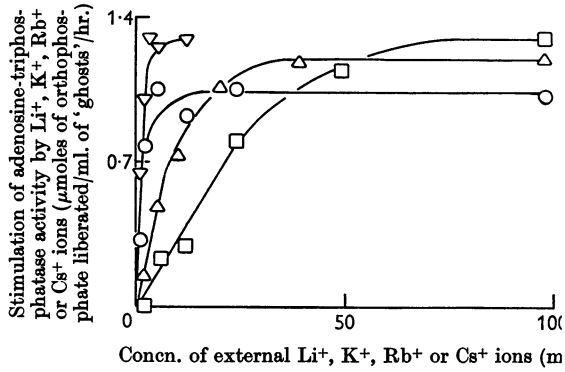

Fig. 2. Stimulation of adenosine-triphosphatase activity by external Li^+, K^+, Rb^+ and Cs^+ ions. The Na^+ ion-rich 'ghosts' were prepared as described in Table 4, suspended in media containing 0–98 mM-LiCl, -KCl, -RbCl or -CsCl, and incubated for 1 hr. at 37°. The graph shows the difference in orthophosphate liberation in media containing Rb^+ (○), K^+ (▽), Cs^+ (△) or Li^+ (□) ions and in medium containing only Na^+ ions. The curve for K^+ ions is typical of a series of six, and the other curves are from an experiment typical of a series of three.

both choline-containing and Na^+ ion-containing media. Fig. 3 shows that in choline-containing medium the activity increased more steeply than in Na^+ ion-containing medium as the K^+ ion concentration was raised to about 2 mM, but it became the same in both media as the concentration was raised to 11 mM. After the addition of ouabain the activity was independent of the K^+ ion concentration in the medium. The rectangular-hyperbolic shape suggested by the points in Fig. 3 made it worth while to see if the adenosine-triphosphatase

activity could be described by simple equations of the type:

$$A = A_1 + \frac{\alpha[K]_0}{\beta + [K]_0}$$

The maximum rate of activity is given by α and the K^+ ion concentration at which half-maximal stimulation occurs by β. A_1 is equal to the rate in the presence of 0·1 mM-ouabain. The curves actually drawn in Fig. 3 represent the equations:

$$A = 3{\cdot}07 + \frac{2{\cdot}4[K]_0}{0{\cdot}5 + [K]_0} \text{ and } A = 2{\cdot}57 + \frac{3{\cdot}5[K]_0}{2{\cdot}2 + [K]_0}$$

for choline-containing and Na^+ ion-containing media respectively. In the total of four experiments made on these lines the values of β in choline-containing medium were 0·5, 0·3, 0·5 and 0·8 (mean 0·5), whereas those in Na^+ ion-containing medium were at the higher values of 2·2, 1·4, 2·7 and 3·0 respectively (mean 2·3). These differences in the K^+ ion concentration required for half-maximal stimulation show that K^+ ions activate more effectively in the absence of Na^+ ions in the medium. It appears that Na^+ ions interfere with the activation by external K^+ ions.

Variation of external Na^+ *ion concentration.* To test the effect of various concentrations of external Na^+ ions on K^+ ion-activation of the membrane adenosine triphosphatase, 'ghosts' were incubated in mixtures of Na^+ ion-containing and choline-containing media. The Na^+ ion concentration was from 0 to 160 mM and the K^+ ion concentrations were chosen to elicit maximum activity with 12 mM or sub-maximal activity with 1·2 mM. In media containing 12 mM-K^+ ion, the activity was unaffected by the external Na^+ ion concentration (Fig. 4). In contrast, the activity in the presence of

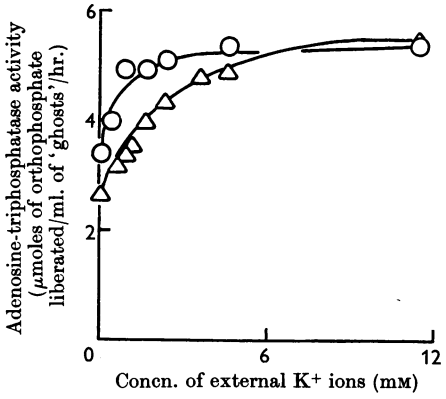

Fig. 3. Stimulation of adenosine-triphosphatase activity by K^+ ions in the presence and absence of external Na^+ ions. The Na^+ ion-rich 'ghosts' were prepared as described in Table 4 and washed twice in Na^+ ion-containing or choline-containing medium. The 'ghosts' were suspended in medium containing K^+ ions (0–11·5 mM) and ouabain (0·1 mM, if present), and incubated for 1 hr. at 37°. The graph shows orthophosphate liberation in choline-containing (○) and Na^+ ion-containing (△) medium in one of four similar experiments. The total adenosine-triphosphatase activity in choline-containing medium fits the curve:

$$A = 3{\cdot}07 + \frac{2{\cdot}4\,[K]_0}{0{\cdot}5 + [K]_0}$$

and the activity in Na^+ ion-containing medium the curve:

$$A = 2{\cdot}57 + \frac{3{\cdot}5\,[K]_0}{2{\cdot}2 + [K]_0}$$

where $[K]_0$ is the concentration of K^+ ions added to the medium. No correction has been made for loss or gain of K^+ ions by the 'ghosts'. This was probably small, since media initially free from K^+ ions contained about 0·1 mM-K^+ ion after incubation, derived from leakage and from rehaemolysis of 3–4% of the 'ghosts'.

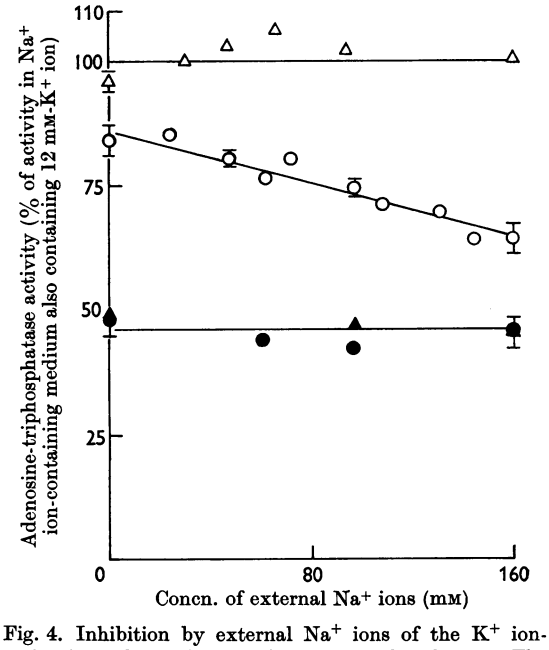

Fig. 4. Inhibition by external Na^+ ions of the K^+ ion-activation of membrane adenosine triphosphatase. The Na^+ ion-rich 'ghosts' were prepared as described in Table 4 and washed twice in Na^+ ion-containing or choline-containing medium. They were suspended in mixtures of these media containing 0–160 mM-Na^+ ion, and KCl was added to a final concentration of 1·2 or 12 mM. The suspensions were incubated for 1 hr. at 37° in the presence and absence of ouabain (0·1 mM) and orthophosphate was then determined. Since the total adenosine-triphosphatase activity varied between experiments, the results have been expressed as percentages of ATP hydrolysis in Na^+ ion-containing medium containing 12 mM-K^+ ion. Each point represents the mean of at least two experiments, and the S.E.M. is given for points from four or more experiments. The media contained 1·2 mM-K^+ ion (○), 1·2 mM-K^+ ion and 0·1 mM-ouabain (●), 12 mM-K^+ ion (△), or 12 mM-K^+ ion and 0·1 mM-ouabain (▲).

1·2 mM-K^+ ion depended on the Na^+ ion concentration, as would be expected from Fig. 3. As a percentage of the rate with 12 mM-K^+ ion the activity with 1·2 mM-K^+ ion fell from 84 to 64 % as the Na^+ ion concentration was raised from 0 to 160 mM. The corresponding ouabain-sensitive activity fell by 45 % from 1·41 ± 0·15 (5) in choline-containing medium to 0·77 ± 0·07 (5) μmole of orthophosphate/ml. of 'ghosts'/hr. in Na^+ ion-containing medium. The activity in the presence of ouabain was unaffected by either the Na^+ or K^+ ion concentrations. This result shows that gradual replacement of Na^+ ions with choline led to a graded increase in enzymic activity. Table 6 shows that other substituents, tris, tetramethylammonium and Mg^{2+} ions, gave the same effect. In medium containing 160 mM-Na^+ ion and 1·2 mM-K^+ ion, the activity (in μmoles of orthophosphate/ml. of 'ghosts'/hr.) was 2·4, but it increased on replacement of Na^+ ions by other substituents. Thus on lowering the Na^+ ion concentration to 65 mM it was 2·8–3·1, and further lowering to 30 mM caused a rise in activity to 3·0–3·5. As was found with choline, the replacement of Na^+ ions by other substituents had little effect on adenosine-triphosphatase activity when the medium contained 12 mM-K^+ ion (Table 6). Thus the stimulating effect of low K^+ ion concentrations is enhanced when the Na^+ ions in the medium are replaced by choline, tris, tetramethylammonium or Mg^{2+} ions. The effect is not specific to choline, and as Na^+ ions are the common variable the results suggest that external Na^+ ions interfere with the activation of adenosine-triphosphatase activity by K^+ ions.

Location in which orthophosphate is released from adenosine triphosphate. In view of the possibility that the membrane adenosine triphosphatase may be due to the combined action of a phosphokinase and a phosphatase, acting respectively at the inner and outer faces of the membrane, it was decided to determine on which side of the membrane orthophosphate is released during adenosine-triphosphatase activity. A difficulty in testing this point is that orthophosphate will move across the membrane to lower a concentration gradient that may be established owing to preferential liberation on one side or the other. The orthophosphate concentration in 'ghosts' and medium was therefore measured when the medium initially contained three different concentrations of orthophosphate. With 3·6 mM-orthophosphate in the medium, in the face of a gradient favouring leakage, the concentration in the 'ghosts' (in μmoles/ml. of 'ghost' water) rose from 6·6 to 6·8 (Expt. 7) and from 5·1 to 6·2 (Expt. 8). On the other hand, in the presence of ouabain the internal concentration fell from 6·6 to 5·5 (Expt. 7) or from 5·1 to 4·9 (Expt. 8) (Table 7). The effect of ouabain in lowering the orthophosphate concentration in the 'ghosts' indicates that at least part of the orthophosphate liberated during ATP hydrolysis must have been preferentially directed to the inside of the 'ghosts'. When the medium contained only 0·2 mM-orthophosphate, the concentration in the 'ghosts' fell from 5·3 to 4·0, and a greater fall to 3·0 was found in the presence of ouabain. These changes were accompanied by a rise in the external orthophosphate concentration to 0·9 or 1·1 mM. Again, with 2 mM-orthophosphate, the concentration in the 'ghosts' fell during incubation, from 6·6 to 5·5 in the control and to 4·7 in the presence of ouabain. These results show that the ouabain-sensitive component of the adenosine-triphosphatase activity releases orthophosphate to the inside of the 'ghosts'.

Table 6. *Inhibition by external* Na^+ *ions of* K^+ *ion-activated adenosine-triphosphatase activity*

'Ghosts' rich in Na^+ ions were prepared as described in Table 4, and were washed twice and suspended in Na^+ ion-containing medium. Media in which NaCl was replaced by 160 mM-choline chloride, -tris hydrochloride or -tetramethylammonium chloride, or by 130 mM-$MgCl_2$, were added to give final Na^+ ion concentrations of 30–160 mM. 'Ghosts' were also washed and suspended in choline-containing medium. Orthophosphate was determined before and after incubation for 1 hr. at 37° in the presence of 1·2 mM- or 12 mM-KCl. A further experiment gave an identical result.

External concn. (mM)		Adenosine-triphosphatase activity (μmoles of orthophosphate liberated/ml. of 'ghosts'/hr.)				
			Media in which NaCl was replaced by:			
K^+	Na^+	Na^+ ion-containing medium	Choline chloride	Tris hydrochloride	Tetramethyl-ammonium chloride	$MgCl_2$
1·2	160	2·4	—	—	—	—
1·2	65	—	2·9	2·8	2·9	3·1
1·2	30	—	3·0	3·0	3·1	3·5
1·2	0	—	3·5	—	—	—
12	160	3·8	—	—	—	—
12	30	—	3·4	3·6	4·0	3·8

Table 7. *Location in which orthophosphate is released from adenosine triphosphate*

The Na^+ ion-rich 'ghosts' were prepared as described in Fig. 1, and were washed and suspended in medium containing 0–3·6 mM-orthophosphate. Samples of the suspension were centrifuged before and after incubation for 20 min. at 37°, and orthophosphate was determined on 'ghosts', supernatant fluid and whole suspension. The haemoglobin concentration of each sample of sedimented 'ghosts' was determined to discover the volume of trapped fluid. When the orthophosphate content of whole suspension was computed from the orthophosphate concentrations in 'ghosts' and supernatant fluid, the values agreed with direct estimations. Orthophosphate concentration in the 'ghost' water was calculated from the percentage of water in the 'ghosts' (71–73 %), determined by weighing samples before and after drying overnight at 105°.

	Concn. of orthophosphate in 'ghost' water (μmoles/ml.)				Concn. of orthophosphate in medium (mM)		
		After incubation				After incubation	
Expt. no.	Before incubation	Control	With 0·1 mM-ouabain	Difference due to ouabain	Before incubation	Control	With 0·1 mM-ouabain
7	5·3	4·0	3·0	1·0	0·2	1·1	0·9
	6·6	5·5	4·7	0·8	2·0	2·7	2·5
8	6·6	6·8	5·5	1·3	3·6	4·1	3·8
	5·1	6·2	4·9	1·3	3·6	4·2	4·0

DISCUSSION

Attachment of haemoglobin to erythrocyte membranes. When erythrocytes were haemolysed with 8–80 times their volume of water, the haemoglobin concentration in the resulting 'ghosts', after separating them by centrifuging, was higher than that in the surrounding fluid. In spite of the tenfold range in the cells: water ratio, the concentration difference was constant and represented about 4 % of the cell haemoglobin. The apparent failure of haemoglobin to reach diffusion equilibrium conflicts with the findings of Hoffman (1958) and Weed, Reed & Berg (1963). Hoffman (1958) found that ^{59}Fe-labelled haemoglobin became evenly distributed during haemolysis and Weed *et al.* (1963) that haemolysis under the gentle conditions of Danon (1961) produced 'ghosts' virtually free of haemoglobin. On the other hand, our results are compatible with the fact that repeated washing with distilled water, and even homogenization, failed to remove the last 3 % of the cell haemoglobin (Moscowitz & Calvin, 1952; Anderson & Turner, 1960). Removal of haemoglobin below this concentration caused a substantial loss of lipid. These observations suggest that some 3–4 % of the cell haemoglobin fails to equilibrate during haemolysis. If this amount remains attached to the membranes, it is of quantitative significance with regard to membrane structure, for it has been calculated that 2 % of the cell haemoglobin would be needed to form a monomolecular layer on the inner surface of the cell membrane (Drabkin, 1945). Moreover, a chemical binding of haemoglobin has been demonstrated with phospholipids such as kephalin (Chargaff, Ziff & Hogg, 1939), suggesting that haemoglobin molecules in the region of the membrane are probably relatively highly oriented. An inner layer of haemoglobin that may be an integral part of the ultrastructure of the membrane surface layer was postulated by Ponder (1955), who also (Ponder, 1961) critically reviewed the microscopic evidence supporting this concept with which the present results are consistent.

Nature of 'ghost' preparation. When the osmolarity of a haemolysate is raised to that of plasma, under suitable conditions, the erythrocyte 'ghosts' regain a low permeability to cations (Teorell, 1952; Hoffman, 1958, 1962; Hoffman *et al.* 1960; Whittam, 1962*a*). Such reconstitution or repair of the membranes is accompanied by considerable shrinkage of a variable kind and gives rise to a heterogeneous 'ghost' population (Ponder & Barreto, 1957). With cells aged 4–45 days such shrinkage was about 75 % and the final 'ghost' volume was about 30 % of the cell volume. 'Ghosts' containing ATP shrink in the same way and also regain a low cation permeability (Whittam, 1962*a*). These results illustrate the remarkable changes that the membrane can undergo without suffering irreversible damage. Haemoglobin is lost during haemolysis, yet the membrane can be so repaired that a low permeability to small cations is achieved. Large tears in the membrane arising during haemolysis would appear to be unlikely, and mild stretching of the membrane at the critical haemolytic volume appears to suffice to allow haemoglobin to be liberated and for restoration of the initial permeability (see Rand & Burton, 1963).

Synergic stimulation of adenosine-triphosphatase activity by internal Na^+ *or* Li^+ *ions, and external* Li^+, K^+, Rb^+ *or* Cs^+ *ions.* Erythrocyte 'ghosts'

facilitate the study of the directional effects of ions on membrane enzyme systems that retain their natural spatial orientation. The main conclusion of the present paper is that the rate of ATP hydrolysis within the 'ghosts' is dependent on the nature of the internal and external cations. As shown previously, Na^+ ions activate from inside and K^+ ions from outside (Glynn, 1962; Whittam, 1962*a*; Laris & Letchworth, 1962). Optimum activity has now been achieved with other alkali-metal ions, Li^+ ions acting like Na^+ ions inside the 'ghosts', and Li^+, Rb^+ or Cs^+ ions like K^+ ions in the medium. The synergic activation by these ions was seen only when they were on opposite sides of the membrane, and it was abolished by ouabain. The locations from which the ions activated the adenosine triphosphatase were the same as those from which they are actively transported in the intact erythrocyte. Internal K^+, Rb^+ and Cs^+ ions and external Na^+ ions, which move passively across the erythrocyte membrane, fail to activate ATP hydrolysis. Just as the efflux of Na^+ ions requires the presence of one of the other alkali-metal ions in the medium, so stimulation of the adenosine-triphosphatase activity requires one of these ions in the medium. The addition of K^+ ions to media already containing high concentrations of Li^+, Rb^+ or Cs^+ ions caused no significant increase in activity, indicating that all four ions activate the same enzyme system. A common pathway for the entry of these ions into erythrocytes has been shown, in as much as Li^+, Rb^+ and Cs^+ ions compete with K^+ ions for uptake (Solomon, 1952; Love & Burch, 1953; Kahn, 1962).

The Li^+ ions are unusual in activating from both sides of the membrane, when only active Li^+ ion influx is thought to occur (Post, 1957; McConaghey & Maizels, 1962). Other tissues, however, show some capacity to treat Li^+ ions like Na^+ ions, for Li^+ ions are extruded from frog muscle, although more slowly than Na^+ ions (Keynes & Swan, 1959), and in frog skin Li^+ ions may replace Na^+ ions (Zerahn, 1955).

It has already been shown that the adenosine-triphosphatase activity of fragmented erythrocyte membranes is stimulated half-maximally by 3 mM-K^+ ion in medium containing about 140 mM-Na^+ ion (Post *et al.* 1960). Active Na^+ ion and K^+ ion transport is also half-maximal at about this concentration (Glynn, 1956; Post & Jolly, 1957), as is enzymic activation by external K^+ ions (Fig. 3). The comparable concentrations of Rb^+, Cs^+ and Li^+ ions are also those needed to stimulate Na^+ ion efflux in erythrocytes (McConaghey & Maizels, 1962).

Inhibitory effect of external Na^+ *ions on activation by* K^+ *ions.* The demonstration that the adenosine-triphosphatase activity is stimulated synergically by Na^+ and K^+ ions depended on one ion not being greatly in excess of the other (Post *et al.* 1960; Dunham & Glynn, 1961), because excess of Na^+ ions hinders activation by K^+ ions and vice versa. Figs. 3 and 4 show that, whereas the synergic activation by these ions depends on their being spatially separated, interference by Na^+ ions with K^+ ion-activation occurs from the same external surface of the membrane. Low concentrations of external K^+ ions caused greater stimulation of enzymic activity in various Na^+ ion-free media than in Na^+ ion-containing medium. For instance, in choline-containing medium, half-maximal activation was observed with only 0·3–0·8 mM-K^+ ion, compared with 1·4–3·0 mM-K^+ ion in Na^+-ion-containing medium. It appears that Na^+ ions can displace K^+ ions from their sites of activation when the K^+ ion concentration is low. In medium containing the relatively high K^+ ion concentration of 12 mM, however, there was no inhibition by Na^+ ions. The site where the competition occurs must have a much greater affinity for K^+ than for Na^+ ions. A rough value for the inhibitory constant is 30–50 mM-Na^+ ion. The membrane adenosine triphosphatase thus seems to show a dual response to Na^+ ions depending on their location. Internal Na^+ ions stimulate in concert with external K^+ ions whereas external Na^+ ions interfere with the stimulation by K^+ ions. The active transport system shows the same dual response to Na^+ ions, since external Na^+ ions retard and internal Na^+ ions facilitate active K^+ ion influx (Post & Jolly, 1957; Post *et al.* 1960; McConaghey & Maizels, 1962).

The converse situation of a fall in adenosine-triphosphatase activity when the K^+ ion:Na^+ ion ratio becomes high has been shown with fragmented membranes (Post *et al.* 1960; Dunham & Glynn, 1961). It has not proved possible to prepare 'ghosts' with a sufficiently low Na^+ ion and high K^+ ion concentration to find out whether this effect arises on the inner face of the membranes (Whittam, 1962*a*).

Vectorial features of adenosine-triphosphatase activity in relation to active transport. What is the significance of the differential directional effects of cations on the membrane adenosine triphosphatase? Do the effects imply the operation of two enzymes—a phosphokinase and a phosphatase—located at opposite surfaces of the membrane? Hokin & Hokin (1960, 1963) have suggested that ATP is required for the formation of phosphatidic acid at the inner side of the membrane by the action of diglyceride kinase. The acid is posited to take up Na^+ ions and to diffuse to the outer surface of the membrane where it is hydrolysed by a phosphatase with the liberation of Na^+ ions. These two enzymes are present in erythrocytes and, although the evidence is somewhat inconclusive, it has been

taken to indicate that the enzymes might account for adenosine-triphosphatase activity (Hokin & Hokin, 1961). Most experimental support, however, has come from studies on the uptake of labelled orthophosphate into phospholipids with the avian salt gland. Without going into these aspects, which have themselves been criticized (see Kennedy, 1962), there is the question of the location of the orthophosphate. Our results and those of Sen & Post (1964), described while this paper was in preparation, suggest that orthophosphate liberated by adenosine-triphosphatase activity sensitive to ouabain appears inside the 'ghosts' (Table 7). This is unlikely to result from phosphatase activity at the outer surface of the membrane.

Whatever intermediate is involved, the functional question remains: how do external K^+ ions facilitate internal ATP hydrolysis? Put another way in relation to active transport, how does a chemical reaction bring about the movement of an ion in a direction contrary to its natural diffusion tendency? It has to be appreciated that the spatial asymmetry of the membrane adenosine triphosphatase is observed only when the enzyme system retains its natural spatial orientation. The structural organization of the membrane must be maintained and it is impossible to practise classical isolation and purification, for this would cause a loss of the vectorial features being investigated. The present results are an instance of the control of the rate of enzyme catalysis by spatially separated ions, in which the enzyme system, in turn, determines which ions are selected and rejected by the cell. The metabolic consequences of these events are considerable, since the membrane adenosine triphosphatase acts as a pacemaker of respiration in nucleated cells of kidney and brain through its control of ADP production (Whittam, 1961; Whittam & Willis, 1963; Blond & Whittam, 1964; Whittam & Blond, 1964).

The interpretation of the present results, of necessity, is rather hypothetical, but it may be supposed that Na^+ and K^+ ions are enzymic cofactors that must be in the right location to exert their effects. At first sight it might seem that two sites of a single enzyme must be involved, one for Na^+ ions and one for K^+ ions. On the other hand, the Na^+ ion- and K^+ ion-sensitive sites may not be different entities but may be the same site in a different position. According to this view, the site is exposed alternately to the inside and outside such that its affinity is high for Na^+ and K^+ ions respectively (Whittam, 1962*a*). A configurational change in protein structure is known to arise as an enzyme–substrate complex is formed (Koshland, Yankeelov & Thoma, 1962), and internal Na^+ ions would seem to be involved in inducing the fit of ATP with the enzyme active centre. Overall ATP hydrolysis requires external K^+ ions as well, however, and the associated ion movements may be connected with rearrangement of the enzyme to its initial state. The Na^+ and K^+ ions differ from cofactors in reactions in a homogeneous milieu in being raised to a higher energy level as ATP is hydrolysed. A previous application of the concept of a flexible enzymic active centre has been made by Christensen (1960) in connexion with the transport of amino acids.

The adenosine triphosphatase of erythrocyte membranes is in a situation where vectorial effects of ions determine the rate of the reaction and result in a conversion of chemical energy into osmotic work. As pointed out by Rosenberg (1948) and Katchalsky & Kedem (1962), diffusional flow has a vectorial character and cannot be coupled to a chemical reaction that is not also spatially oriented. The coupling of a chemical reaction to a unidirectional flow of matter thus requires a particular spatial arrangement of the enzymic system where some of the reactants of the energy-yielding process are separated by the membrane. This condition is satisfied by the membrane adenosine triphosphatase when Na^+ and K^+ ions are regarded as enzymic cofactors.

SUMMARY

1. A study has been made of the directional effects of internal and external alkali-metal ions on the hydrolysis of ATP in erythrocyte 'ghosts'.

2. Haemoglobin did not equilibrate completely during haemolysis, as if about 4 % of the cell haemoglobin was attached to the membranes. The volume of the 'ghosts' was about 30 % of that of the initial cells.

3. ATP hydrolysis was maximal at pH 8·5–9·0 in 'ghosts' incubated in medium containing K^+ ions. The part of ATP hydrolysis sensitive to ouabain or to external K^+ ions had maximum activity at pH 7–8·5.

4. Stimulation by external K^+ ions occurred only when Li^+ or Na^+ was the predominant internal cation, and this stimulation was prevented by ouabain. Internal Rb^+ and Cs^+ ions, like internal K^+ ions, failed to activate ATP hydrolysis.

5. Adenosine-triphosphatase activity was stimulated at the external surface of the membrane by Li^+, Rb^+ and Cs^+ ions; the addition of K^+ ions caused no further stimulation. Half-maximal stimulation would be elicited by 1–3 mM-Rb^+ or -K^+ ion, about 8 mM-Cs^+ ion or 20 mM-Li^+ ion.

6. Half-maximal stimulation of ATP hydrolysis in choline- and Na^+ ion-containing medium was found with 0·5 mM- and 2·3 mM-K^+ ion respectively. External Na^+ ions impeded activation by K^+ ions. Thus Na^+ ions exert a dual effect on ATP

hydrolysis, depending on their location, since they activate from inside and hinder from outside.

7. Orthophosphate from the ouabain-sensitive hydrolysis of ATP was liberated inside the 'ghosts'.

8. The directional effects of ions on ATP hydrolysis are closely related to active ion movements.

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