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THE INFLUENCE OF IONS ON THE LABELLING OF ADENOSINE TRIPHOSPHATE IN RED CELL GHOSTS

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SUMMARY

1. The ionic composition of human red cell ghosts and suspending Ringer solutions have been varied independently. Measurements were made of the incorporation of [^{32}P]*o*-phosphate ($^{32}P_i$) into ATP associated with different concentration gradients of Na and K across the membrane.

2. Some incorporation of $^{32}P_i$ was always found irrespective of the ionic composition of ghosts or media. However, additional labelling of energy-rich phosphate occurred when low Na, high K ghosts were incubated in a high Na, K-free medium. This did not occur when there was only a gradient of either Na or K. Downhill movements of both Na into and K out of the ghosts were needed for the extra labelling.

3. Even in the presence of suitable ionic gradients, the extra incorporation was prevented by ouabain or by adding a small amount of external K sufficient to facilitate normal operation of the Na pump.

4. Increase in internal P_i stimulated the incorporation.

5. The results show that the conditions for forward and backward running of the ATPase system associated with the Na pump are such that both reactions cannot proceed optimally at the same time.

INTRODUCTION

Hydrolysis of ATP by an ATPase within the cell membrane provides energy for the active transport of Na and K in human red cells, and the question arises: can energy liberated by downhill movements of ions be utilized to synthesize ATP? Garrahan & Glynn (1967) demonstrated labelling of ATP in red cell ghosts when there were large concentration gradients of Na and K between the ghosts and external media. They suggested that the Na pump could run backwards and synthesize ATP with energy derived from ionic concentration gradients. On the other hand,

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incorporation of $^{32}P_i$ into ATP was slight with preparations of broken cells which were rich in ATPase activity (Skou, 1960; Fahn, Koval & Albers, 1966). Since the cation concentrations must have been the same on both sides of the cell membranes, ATP break-down would be favoured. Experiments on fragmented membranes of other tissues have clarified the ATPase reaction in showing that the turnover of a high-energy phosphorylated intermediate may be involved. The labelling by [γ-^{32}P]ATP of a protein in the membrane requires Na whilst its hydrolysis requires K. If this phosphorylated intermediate is an integral part of the Na pump, it follows that ATP splitting also may involve a two-step reaction (see Glynn, 1968).

In the present work, we have studied the incorporation of P_i into ATP when the pump was operating normally and when downhill movements were occurring of both Na and K or of only one ion. The main conclusions are that simultaneous movements of both Na inwards and K outwards were needed to reverse the ATPase reaction of the Na pump, and that, even when there were gradients of both ions across the membrane, reversal was prevented by forward running of the pump.

A preliminary account of the results has been published (Lant & Whittam, 1968).

METHODS

Procedure

Preparation of ghosts. The procedure employed was based on methods described previously (Hoffman, 1958; Hoffman, Tosteson & Whittam, 1960; Whittam, 1962). Citrated human blood aged 5–21 days (supplied in part by the Sheffield Regional Blood Transfusion Service) was centrifuged at 1500 *g* for 8 min. The white cells and plasma were removed and the erythrocytes (1 vol.) were washed 4 times in 3–4 vol. of 0·15 M-KCl, NaCl or choline chloride depending on the type of ghost being prepared. The cells were then packed at 2700 *g* for 8 min at room temperature and were squirted into 10 vol. of a suitable lysing solution stirred vigorously and maintained at 5° C. The composition of the lysing fluid differed in the different experiments. Its osmolarity was never more than 50 ideal m-osmole/l. It always contained (mM): ATP, 1; Mg, 7; fluoride, 2; iodoacetate (IAA), 0·2; $^{32}P_i$ (pH 6·5–7·5), 1–5.

Unless otherwise stated, isotonicity was restored by the addition of sufficient 3 M-KCl, NaCl or choline chloride. The haemolysate was divided into equal samples (usually of 35 ml.) and incubated in polypropylene tubes at 37° C for 30 min to allow the membranes to regain a low permeability to cations and ATP. The reconstituted ghosts were then sedimented by centrifugation at 18,000 *g* for 1 min (Sorvall superspeed centrifuge; Ivan Sorvall Inc., Norwalk, Conn., U.S.A.). In most ^{32}P incorporation experiments, 10 ml. samples of the post-reversal supernatant were retained for use as controls. The remainder of the supernatant was discarded, and the ghosts were washed twice at 5° C in 30 ml. of the medium in which they were subsequently to be incubated. Where required, ouabain was added in suitable amounts of a freshly prepared 5 mM aqueous solution.

Incubation. The washed ghosts were resuspended in 30 ml. of suitable medium and incubated for 15 min or longer at 37° C in a shaking water-bath. At the end of the incubation, the suspensions were centrifuged at 18,000 *g* for 2 min. The clear supernatants were sucked off, 10 ml. samples being retained for analysis. In ^{32}P incorporation studies, the ghosts were

lysed in 10 ml. of water containing 5 μmoles of unlabelled ATP, 5 μmoles of unlabelled P_i to act as carriers for the labelled material. In other experiments, the ghosts were lysed in 10 ml. water.

Deproteinization of ghost extracts. The haemolysates were transferred completely to small graduated polypropylene tubes containing 1 ml. of 50% (w/v) perchloric acid immersed in an ice-bath. The perchloric acid supernatants, containing one drop BDH universal indicator (British Drug Houses, Ltd.), were neutralized to pH 6·5–7·5 by the addition of sufficient 2 N-KOH. After a further 10 min at 5° C, the sparingly soluble $KClO_4$ was removed by centrifugation. The extracts were then ready for analysis.

Chemical analytical methods

Two methods were used, namely a chromatographic technique employing an anion exchange resin and an extraction technique employing organic solvents.

Chromatographic technique. The neutralized perchloric acid extracts were diluted with water, usually to 40 ml., to reduce the chloride concentration to less than 2 mM. Separations were carried out essentially as described by Bartlett (1959*a*, *b*; 1968) on columns of Dowex 1-X8 chloride resin (200–400 mesh) (BioRad Laboratories, Richmond, California, U.S.A.), 10 cm long and 0·8 cm in diameter. Before use, the columns were washed with 1 N-HCl until the effluents had an absorbance of less than 0·02 at 260 mμ; backwashing was then carried out with water until the pH of the effluents exceeded 4·5. Samples (1–2 ml.) of the diluted extracts were kept for determination of total radioactivity and the remainder was run through the columns at a flow rate of 0·5 ml./min. The composition and volume of the eluting solutions and the results of the chromatographic separation of P_i and nucleotides in a typical experiment are shown in Fig. 4. Effluents were collected in 10 ml. fractions which were then assayed for radioactivity and ultraviolet absorbance at 230–290 mμ (Unicam spectrophotometers, SP 500 and SP 800; Unicam Instruments Ltd., Cambridge). Application of the extracts was always followed by an equal volume of water; analysis of these initial effluent fractions showed them to be free of radioactivity and of absorption due to adenine-containing compounds. In all experiments, column chromatography was carried out at room temperature; column operations were usually interrupted after completion of the 0·02 N-HCl elution, were held at 5° C overnight, and were then completed the next day.

Significant radioactivity was found only in the fractions containing P_i, ADP and ATP. Since the labelling of ADP can be presumed to have arisen from [γ-^{32}P]ATP through adenylate kinase activity, the combined radioactivity in the ADP and ATP fractions, expressed as a fraction of the total counts in the neutralized $HClO_4$ extracts, was used to give a measure of how much of the original phosphate had been esterified and become incorporated into 'high-energy phosphate'.

Extraction technique. The extraction technique employed was based on the method of P_i determination of Berenblum & Chain (1938*a*, *b*) as modified by Martin & Doty (1949), Weil-Malherbe & Green (1951) and Nielsen & Lehninger (1955). This involves the conversion of P_i into phosphomolybdate, which is then extracted into organic solvents whilst phosphate esters remain in the aqueous residue. To 5 ml. of the neutralized perchloric acid extract in a separating funnel were added 1 ml. 5% (w/v) ammonium molybdate in 4 N-H_2SO_4, 1 ml. of 10 mM-KH_2PO_4 and 7 ml. of a water-saturated mixture of 1:1 ethyl acetate and isobutanol (2-methylpropan-1-ol). The mixture was allowed to stand for 5 min and then shaken vigorously for 30 sec. After the two layers had separated, the aqueous layer was run off into a stoppered centrifuge tube. More carrier P_i (1 ml. of 5 mM-KH_2PO_4) was added and 8 ml. of a water-saturated mixture of 4:1 (v/v) ethyl acetate and isobutanol. The mixture was allowed to stand for 2 min and then shaken vigorously for 30 sec. The tubes were then centrifuged briefly to facilitate complete separation of the two phases, and the upper organic layer was withdrawn by means of a fine capillary tube attached to a water-suction pump and discarded. The capillary tube was rinsed with 95% ethanol from a wash-bottle after each

use. Following the second extraction, exactly 5 ml. of the aqueous layer was withdrawn by pipette for determination of radioactivity. For each set of experiments, a control determination of 'non-extractable' radioactivity was carried out to determine the bound ^{32}P largely arising from radiochemical impurities in the $^{32}P_i$. The mean value $\pm$ S.E. of mean for this labelling was $0{\cdot}116 \pm 0{\cdot}008$ % (35) of the total radioactivity in the original perchloric acid extract. It was not decreased further by acid hydrolysis. As a check on the reliability of the extraction procedure, three experiments were undertaken in which six extractions were carried out on deproteinized samples derived from parallel incubations. The precision of the values for bound ^{32}P, based on S.D., was within the range of $\pm 3{\cdot}5$ % of the mean.

Measurement of radioactivity. Radioactivity was measured either by a liquid sample Geiger counter linked to an IDL 1700 scaler (Isotope Developments, Ltd., Reading), or by liquid scintillation counting employing an automatic coincidence unit (IDL Tritomat model 6020). With the latter technique, advantage was taken of the Čerenkov phenomenon, and counting performed without the addition of organic scintillant (see Braunsberg & Guyver, 1965; Garrahan & Glynn, 1966). With each method of measurement, counting was continued on individual samples for a time sufficient for at least 10,000 counts to have accumulated; the standard error of counting was thus approximately 1 %.

Orthophosphate. This was estimated in deproteinized extracts by the methods of either Fiske & Subbarow (1925) or Weil-Malherbe & Green (1951). Acid-labile phosphate was determined as the decrease in esterified $^{32}P_i$ obtained with the organic extraction method when extracts were heated with an equal volume of 2 N-HCl in a boiling-water-bath for 10 min.

Haemoglobin (Hb). This was estimated as oxyhaemoglobin from the extinction at 540 mμ of suitably diluted samples clarified with weak ammoniated water as described by Wootton (1964).

Na and K. These elements were measured either by an EEL flame photometer (Evans Electroselenium Ltd., Halstead) or by a Unicam atomic absorption spectrophotometer (SP 90) (Unicam Instruments, Ltd., Cambridge). Samples were diluted to contain less than 0·2 mM-Na^+ or K^+ and measurements undertaken in triplicate alternating with standard solutions of NaCl or KCl. The cation concentration in the ghosts was calculated by multiplying the concentration in each lysed sample by the ratio of the haemoglobin concentration in unit volume of packed ghosts to that in the sample.

Materials

ATP was obtained from Sigma, London, Ltd. as the crystalline disodium salt. ^{32}P was obtained from the Radiochemical Centre, Amersham, as a sterile solution of very high specific activity in dilute HCl, pH 2–3 (reference PBS. 1). The solution was neutralized before use to around pH 7 with unbuffered Tris.

Ouabain (strophanthin G) and iodoacetic acid were laboratory reagent products from British Drug Houses, Ltd. The latter was neutralized with either NaOH or KOH before use.

Other salts, *o*-phosphoric acid, were of Analar grade, wherever possible, and all solutions were made up in glass-distilled water.

RESULTS

Cation content of ghosts

Tonicity at reversal. Variations in the preparative procedure have profound effects on the permeability to cations of erythrocyte ghosts (Hoffman, 1962). Since it was important in the present study to have intact membranes across which cation gradients could be maintained, the influence of conditions at the time of haemolysis on the characteristics of

the resultant ghosts was investigated first. The aim was to define the conditions which would lead to reliable yields of low Na, high K ghosts possessing low permeability to these cations. Red cells were completely lysed in a solution containing (mM): ATP, 1; P_i, 1–2; Mg, 2–7; Na, 2. The osmotic pressure was then changed over a wide range by adding different amounts of 3 M-KCl. After 30 min incubation to allow low permeability to cations to be regained (Hoffman *et al.* 1960), the ghosts were washed and then incubated for 15 min in an isotonic Na medium. The degree of sealing to haemoglobin and K was then assessed (Fig. 1).

The amount of K and haemoglobin found in the ghosts depended on the

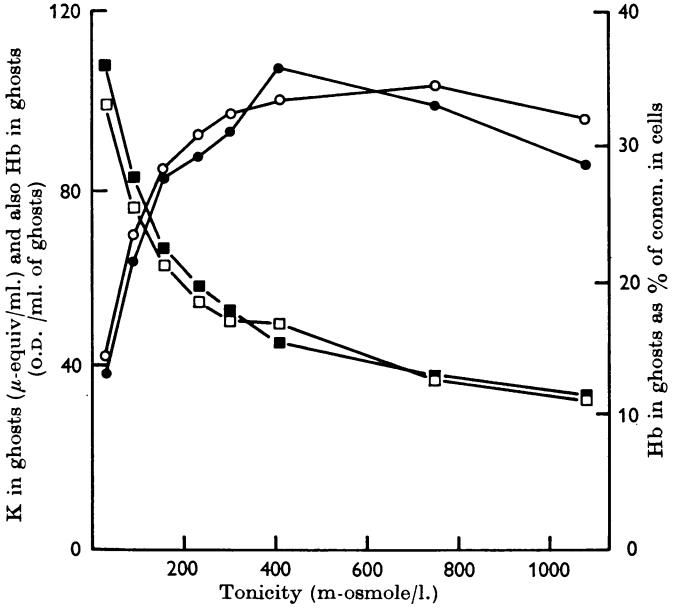

Fig. 1. The relationship between tonicity of the lysing medium at reversal, and cation and haemoglobin content of resealed ghosts. 150 mM-KCl-washed red cells were lysed (1:10 vol.) in a solution of the following composition (mM): ATP, 1; P_i, 1; Na, 2; Mg, 7; K, 4; iodoacetate, 0·2; fluoride, 2. Lysate was kept at 0° C and divided into equal portions; amounts of 3 M-KCl were then squirted in calculated to increase total osmolarity over the range 40–1040 m-osmole/l. Resealing was then completed by incubating for 30 min. at 37° C. The harvested ghosts were washed twice in Na medium of composition (mM): Na, 142; Mg, 7; Cl, 152; Tris (pH 7·3 at 37° C), 5; iodoacetate, 0·2; fluoride, 2. A portion of each final wash suspension was spun at 5° C (18,000 *g*) for 1 min to yield packed ghost sample. Remainder of ghosts resuspended in Na medium and incubated for 15 min. Post-incubation ghosts sampled and analysed for Hb and K content. In calculating the Hb in the ghosts as a percentage of the original cell Hb, a value of 280 has been taken as unit Hb concentration of packed red cells. K concentrations: ○, before incubation; ●, after incubation. Unit optical density (540 mμ): □, before incubation; ■, after incubation.

osmotic pressure of the haemolysate from which the ghosts were obtained. The highest concentration of haemoglobin was in the spontaneously resealed ghosts formed when no KCl was added to the haemolysate (Hoffman's Group I ghosts, see Hoffman, 1962). These ghosts contained about 35% of the original cell haemoglobin. The level of haemoglobin in the ghosts then declined steadily as the tonicity of the haemolysate was raised at reversal so that the haemoglobin concentration in ghosts formed under isotonic conditions was only 18% of the original cell haemoglobin. Since haemoglobin reaches diffusion equilibrium at the time of cell lysis (Hoffman, 1958), and the original cell:lysing fluid ratio was 1:10, the haemoglobin concentration (in optical density units) in the ghosts before resealing must have been one eleventh of that in the cells (280/11 = 25·5). The final haemoglobin concentration in the ghosts formed under isotonic conditions was 50, indicating that there was a shrinkage of approximately 50% between the time of lysis and final separation of the ghosts.

The K concentration (in μ-equiv/ml. ghosts) was about 40 when no electrolyte was added to the haemolysate and 98 when isotonicity had been restored with 3 M-KCl. Although the K concentration in the haemolysate was subsequently raised threefold above isotonicity, there was no further increase in the internal K of the final ghosts over 100 μ-equiv/ml. Assuming that there was a state of equilibrium at the time of ghost reversal, the K concentration inside the ghosts would have been high whilst they were in the haemolysate. The fact that constant values of K concentration of between 95 and 100 μequiv/ml. were obtained after washing in an isotonic Na medium could indicate that substantial amounts of K leaked out during the washing stages. There is some support for this explanation since there was occasional evidence in the supernatant of rehaemolysis after washing ghosts which had been sealed under hypertonic conditions. Another possibility is that ghosts formed under hypertonic conditions of reversal swell to dilute internal K. This is unlikely to be a major factor, however, because the change in volume which would have had to occur to lower the apparent concentration of K would have been accompanied by a much larger fall in unit haemoglobin concentration than was actually observed.

When the ghosts were incubated for 15 min there was little change in haemoglobin concentration. The K concentrations in the ghosts also stayed close to the pre-incubation values, though in the ghosts formed under hypertonic conditions, the K losses tended to be greater and rehaemolysis was often evident to the eye. Thus, spontaneously resealed ghosts and those formed under conditions of extreme hypertonicity lost about 10% of their initial K concentration after 15 min incubation, whereas for the ghosts sealed at isotonicity the figure was 5–6%.

In a parallel experiment, ghosts were incubated in an isotonic choline chloride medium. Cells were lysed as before except that labelled $^{32}P_i$ was included. The pattern of haemoglobin and K concentrations in the ghosts produced at different levels of tonicity was the same as in ghosts washed in a Na medium. The $^{32}P_i$ concentration of the ghosts closely followed that of haemoglobin, being highest in the spontaneously resealed ghosts. On incubation for 15 min, the absolute losses of K (as % of initial K) amounted to 18% in the spontaneously resealed ghosts; 6% in the ghosts formed under isotonic conditions; and 47% in the ghosts formed under conditions of extreme hypertonicity. Since, in this experiment, the washing medium was choline chloride, it was possible to measure the Na concentration in the ghosts (in μ-equiv/ml.). This amounted to 11 in the spontaneously resealed ghosts, about 6 in ghosts formed under isotonic conditions, and 3·5 in ghosts formed under extreme hypertonic conditions. These results show that the ghosts prepared from an isotonic haemolysate had regained the lowest permeability to Na and K.

Rate of K loss from high K, low Na ghosts. To check further on the low permeability of isotonically resealed ghosts, the loss of K to an all Na medium was followed as a function of time in two experiments. The K loss from the ghosts was calculated from the amount of K that appeared in the medium (Fig. 2). After 15 min the mean concentration of K in the medium amounted to 1·00 mM, of which 0·08 mM was present initially in the nominally K-free medium. This is equivalent to a rate of K loss (μ-equiv/ml. ghosts/min) of 0·88; the rate fell to a value of 0·15 over the subsequent 45 min of incubation. It is not clear why the rate of K loss should have been greater in the first 15 min and then level off with time. One possibility is that the high initial loss of K was derived from a proportion of ghosts which were either imperfectly sealed to K or became leaky on increasing the temperature from 5 to 37° C at the start of incubation. A possible interpretation of the levelling off after 15 min is that, as external K increased due to K loss from the ghosts, a significant K influx then began to offset the unidirectional K efflux and thereby reduce net K loss. In this connexion K efflux from red cells does not seem to be inhibited by external K (Glynn, 1956).

In five experiments where low Na, high K ghosts were washed and incubated in isotonic Na or choline medium, the concentrations of K in the media after 15 min incubation were compared. The mean values and S.E. obtained (mM-K) were $0·81 \pm 0·13$ in the Na medium and $0·39 \pm 0·05$ in the choline medium, indicating that loss of K was twice as great into the Na as into the choline medium. Although choline is known to traverse the cell membrane (Martin, 1968), this result implies that it does not penetrate as readily as Na in exchange for internal K.

Ghosts with different Na and K content. Ghosts rich in Na or choline instead of K were also prepared, and their Na and K content was compared with that of the low Na, high K ghosts (Table 1). The results show that whereas in the low Na, high K ghosts, Na concentration (μ-equiv/ml. ghosts) was as low as 5 ± 2 associated with a K concentration of 111 ± 19, these proportions were essentially reversed in the high Na, low K ghosts, which contained 113 Na and 9 K. In the low Na, low K ghosts, rich in

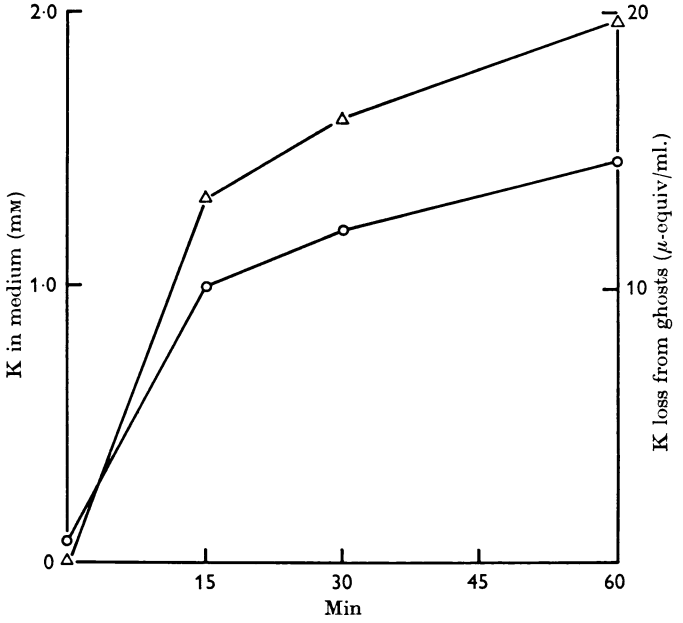

Fig. 2. The net loss of K from low Na, high K, isotonically resealed ghosts incubated in Na medium. The solution in which the ghosts were sealed contained (mM): ATP, 1; $^{32}P_i$, 1; Mg, 7; K, 147; Cl, 149; Na, 2; iodoacetate, 0·2; fluoride, 2. The sodium incubation medium contained (mM): Na, 142; Mg, 7; Cl, 152; Tris (pH 7·3 at 37° C), 5; iodoacetate, 0·2; fluoride, 2. The plotted values are means from two experiments. ○, K in medium; △, K loss from ghosts.

choline, the ghosts must have had a low permeability to choline, since the internal concentrations were 9 for Na and 15 for K, and there was no evidence of rehaemolysis. Measurements of Na and K were also made when ghosts were washed in either isotonic K or Na medium, and the values obtained agreed closely with those found after washing with isotonic choline chloride. With the high Na ghosts, the Na concentration was lowered by 5–10 % on incubation for 15 min in K or choline medium, and this change is comparable to the K loss from high K ghosts.

TABLE 1. Na and K content of isotonically resealed ghosts

Concentration in ghosts (μ-equiv/ml.)

Kind of ghosts ...	Low Na								High Na			
	High K				Low K, high choline							
Incubation ...	Before		After		Before		After		Before		After	
Medium	Na	K	Na	K	Na	K	Na	K	Na	K	Na	K
Choline	5±2* (7)	111±19 (7)	—	—	9	15	4	12	113	9	102	7
		103†		97								
Na	—	100	—	89	—	13	—	8	—	8	—	7
K	6	—	5	—	13	—	4	—	103	—	98	—

* These values represent the mean ± S.E. of 18 incubations in 7 separate experiments.

† This value and the remaining results in the Table represent the means of duplicate incubations carried out with two lots of ghosts of each kind.

Low Na ghosts were prepared by lysing cells in a solution of the following composition (mM): ATP, 1; Na, 2; K, 2; *o*-phosphoric acid adjusted to pH 7·6 with Tris base, 1; Mg, 7; Cl, 14; iodoacetate, 0·2; fluoride, 2. Sufficient 3 M salt solution added to restore tonicity to 310 m-osmolar.

For low Na, high K ghosts, 3 M-KCl was used; for low Na, low K ghosts, 3 M choline chloride. High Na ghosts were prepared by using a lysing solution containing (mM): ATP, 1; Na, 6; P_i, 1; Mg, 7; Cl, 14; iodoacetate, 0·2; fluoride, 2. Isotonicity was restored with 3 M-NaCl. The washing and incubating media were of the following composition (mM): (*a*) choline, 140; Cl, 140; Tris (pH 8·3 at 5° C), 10; (*b*) Na, 144; Cl, 156; Mg, 7; Tris (pH 8·3 at 5° C), 5; iodoacetate, 0·2; fluoride, 2; (*c*) same as Na medium with K replacing Na. Incubation time: 15 min.

Homogeneity of ghosts

The homogeneity with respect to K and haemoglobin content of low Na, high K ghosts was studied in three experiments by taking samples at descending levels from columns of packed ghosts in haematocrit tubes. In the first place, the ratio K/Hb was less in the ghosts than in the haemolysate, suggesting that some ghosts were completely leaky to cations. When ghosts were fractionated, those in the lowest third of each column were

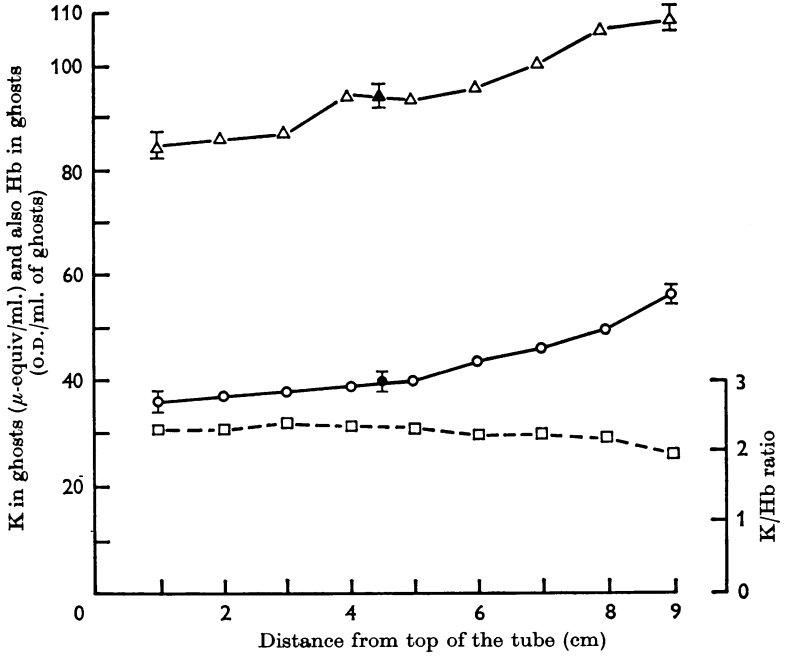

Fig. 3. K and Hb concentrations at different heights of columns of packed ghosts. Low Na, high K, isotonically resealed ghosts, sealed in a medium containing (mM): ATP, 1; P_i, 1; K, 140; Mg, 7; Cl, 149; Na, 2; iodoacetate, 0·2; fluoride, 2. The resealed ghosts were incubated at 37° C for 30 min and then washed twice in Na medium before packing residue in five haematocrit tubes for 30 min at 1500 *g*. Consecutive 25 μl. samples were taken from each packed column for analysis. Plotted figures are means of five samples. The mean K and Hb concentration of four mixed, but unseparated, samples of packed ghosts from the same batch are shown. The S.E. of mean for the mean values of top, bottom and mixed samples are also shown. Packed ghost samples △, K; ○, Hb; unseparated ghosts ▲, K; ●, Hb □, K/Hb ratio.

richest in K and haemoglobin (Fig. 3). The mean K and haemoglobin concentrations between top and bottom of the column differed by 26 and 54 %, respectively. The ratio of K to Hb at each level, however, remained

approximately constant, decreasing slightly in the lowest third of the column. This inhomogeneity could be explained by some ghosts being more leaky than others, the leaky ones being less dense and collecting in the upper part of the column. If this were the case, however, haemoglobin and K would have had to be lost in the same proportions in order for the ratio of K to Hb to remain about the same. An alternative reason is that differences in ghost volumes are responsible. The ghosts at the top of the column may be larger, and hence the apparent concentrations of Hb and K smaller. This latter explanation agrees with some of the observations obtained from differential centrifugation of intact red cells (Prentice & Bishop, 1965; Piomelli, Lurinsky & Wasserman, 1967). A similar pattern of inhomogeneity in reconstituted ghosts to that described here was noted by Hoffman (1958), who attributed its origin to occurrence of a variable degree of rehaemolysis in the ghost population.

A mixed sample of packed low Na, high K ghosts gave values for K and Hb concentrations which were close to the arithmetical mean of the concentrations at the top and bottom of a packed column. No attempt has been made to partition ghosts by differential centrifugation in subsequent experiments, and isotonically resealed ghosts were used throughout the present study. For purposes of sampling, mixed samples of packed ghosts were used.

Effect of the ionic composition of the medium on labelling of ATP

Comparison of high Na and high K media. The first aspect of the relationship between cation gradients and incorporation of $^{32}P_i$ into ATP that we investigated was whether such labelling was related to the amount of Na and K in the external medium. Low Na, high K ghosts containing (mM): $^{32}P_i$, 1–5, and ATP, 1, were prepared and were incubated in media of differing ionic composition. After 15 min all enzymic activity was stopped by the addition of perchloric acid. The amount of ^{32}P-labelling of ADP and ATP was analysed by column chromatography or extraction technique, as outlined in Methods. The level of incorporation was expressed as a percentage of the total radioactivity in the ghosts, the latter being kept as uniform as possible throughout individual experiments. The results show that with chromatography there was complete uptake of ^{32}P by the column, satisfactory separation of the labelled products and reproducibility of results (Fig. 4). In a typical experiment with low Na, high K ghosts incubated in a high Na, K-free and in an all K medium, counts were found in ATP after incubation in both media, but the incorporation in the Na medium (7238 counts/100 sec) was about double that in the K medium (3039 counts/100 sec). Under both conditions there were approximately 5 times as many counts in ATP as in ADP. Although the incorporation,

as a percentage of the counts in P_i, was only 1·74 in the Na medium as compared with 0·76 in the K medium, the difference in labelling is highly significant. As adenylate kinase is known to be present in red cell membranes, the counts in ADP can be assumed to have arisen from labelled

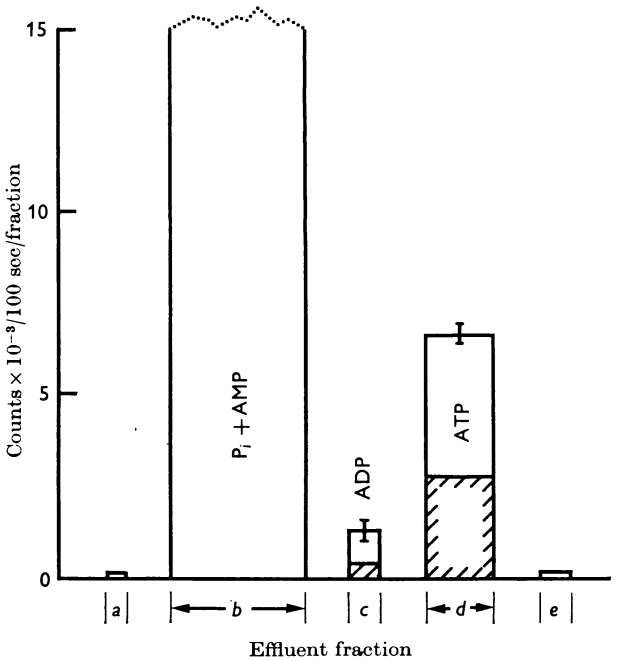

Fig. 4. Elution chart of P_i, ADP and ATP on Dowex columns. Extracts were from an experiment (II[8]) where low Na, high K ghosts were incubated for 15 min in a high Na, K-free medium and in an all K medium.

The incubations in Na medium (clear areas) were done in triplicate and the results in the figure are means ± S.E. of mean. The incubations in the K medium (hatched areas) were in duplicate and the result is the mean.

Effluent fractions	Volume (ml.)
(*a*) application and water	60
(*b*) 0·01 N-HCl	450
(*c*) 0·02 N-HCl	100
(*d*) 0·5 N-NH_4Cl	220
(*e*) 1·0 N-HCl	100

The ghosts were sealed in a medium containing (mM): ATP, 1; $^{32}P_i$, 5; Mg, 7; K, 144; Cl, 158; iodoacetate, 0·2; fluoride, 2.

The incubation media contained (mM): Na or K, 142, Mg, 7; Tris (pH 8·0 at 5° C), 5; Cl, 156; iodoacetate, 0·2; fluoride, 2. After incubation in the Na medium, the mean radioactivity in the combined ADP and ATP fractions equalled 8586 counts/100 sec. This represented 1·741 % of the mean total counts in the extracts (492,900 ± 28,300 counts/100 sec). The equivalent mean percentage incorporation from incubation in potassium medium was 0·764 %.

ATP (Overgaard-Hansen, 1957). For this reason, in these and subsequent incorporation studies, it was more satisfactory to consider the combined labelling in ADP and ATP, referring to these as counts in energy-rich phosphate.

The chromatographic technique of separation was compared with the simpler extraction technique which measures the sum of ADP plus ATP. The counts for both methods are shown as those in ADP plus ATP. In the column chromatography the two compounds were eluted separately (see Fig. 4). In three experiments, with the same type of low Na, high K ghosts, both techniques of analysis were employed simultaneously (Table 2). There were some differences (of not more than 25 %) from experiment to experiment in the absolute magnitude of incorporation, but there was always more labelling (from 30 to 90 %) in Na than in K media. With both methods of analysis, the results agreed closely (within 8 %), in showing the same ratio between the incorporation with the 2 media (e.g. 1·95 and 1·88; 1·44 and 1·33; 1·41 and 1·52).

In Expt. III[3] of Table 2, high Na, low K ghosts were also prepared. Their behaviour was in contrast with that of the low Na, high K ghosts made from the same batch of original cells, in that they no longer showed the extra incorporation in the Na medium. The ratio of incorporation in Na medium/incorporation in K medium was 1·11 with the column chromatography technique, and 1·06 with the extraction technique. Taken as a whole, these results justified use of the chemical extraction method as an alternative to column chromatography.

Effect of 10 *mM external K*. The smaller extent of labelling in the K medium as compared with the Na medium could have arisen from so having increased the external K concentration that internal K would no longer leak outwards. On the other hand, external K would enable the Na pump to work in its usual way. In order to distinguish between these possibilities, ghosts were incubated in a high Na medium to which 10 mM-K was added to allow maximal stimulation of the forward running of the Na pump under conditions where large ionic gradients were still present. Addition of 10 mM-K caused the same decrease in labelling as that found with 150 mM-K, namely to 65 and 67 %, respectively, of the amount of labelling achieved in the Na medium (Expt. III[5], Table 3). Since the greatest incorporation was found in the high Na, K-free medium, the amount of labelling under other conditions of incubation was referred in each experiment to that achieved in the Na medium and the latter arbitrarily called 100 %. In the two other experiments in Table 3, 10 mM-K caused a decrease in labelling from 100 to 68 % and from 100 to 58 % of incorporation in Na medium.

Effect of ouabain. In order to test whether inhibition of the normal

TABLE 2. The incorporation of $^{32}P_i$ into energy-rich phosphate by isotonically resealed ghosts: comparison of column chromatography and organic extraction methods of analysis

Expt.	Type of ghost	Method of analysis	Main cation in medium	^{32}P counts/100 sec (a) Total	^{32}P counts/100 sec (b) ADP+ATP	% incorporation into energy-rich phosphate (b)/(a) × 100	Ratio of fractional incorporation Na medium / K medium
III[2]	Low Na	Column	Na	85,000	1532	1·802	1·95
	High K		K	86,100	795	0·923	
		Extraction	Na	89,000	1952	2·193	1·88
			K	90,000	1050	1·66	
III[3]	Low Na	Column	Na	63,000	1075	1·706	1·44
	High K		K	63,600	756	1·188	
		Extraction	Na	66,600	1430	2·147	1·33
			K	67,200	1084	1·613	
	High Na	Column	Na	78,000	765	0·980	1·11
	Low K		K	77,200	682	0·883	
		Extraction	Na	83,400	1044 (182)*	1·231	1·06
			K	86,000	1012 (170)	1·176	
III[6]	Low Na	Column	Na	89,000	925	1·039	1·41
	High K		K	90,000	663	0·736	
		Extraction	Na	95,000	1196	1·258	1·52
			K	94,200	778	0·825	

Cells were lysed in a solution containing (mM): ATP, 1; *o*-phosphoric acid adjusted to pH 7·4 with Tris base, 5; Mg, 7; Na, 2; Cl, 14; iodoacetate, 0·2; fluoride, 2. The inhibitors were added as Na or K salts depending on whether low or high Na ghosts were being prepared. Isotonicity was restored with either 3 M-KCl or 3 M-NaCl. Washing and incubation media contained (mM): Na or K, 144; Mg, 7; Cl, 161; Tris (pH 8·0 at 5° C), 5; iodoacetate, 0·2; fluoride, 2. Incubation time: 15 min.

* These figures show the counts remaining in the extracts after acid hydrolysis. They indicate that approx. 75% of ^{32}P in this fraction was acid-labile.

TABLE 3. The incorporation of $^{32}P_i$ into energy-rich phosphate by low Na, high K, isotonically resealed ghosts

Expt.	Method of analysis	Medium	^{32}P counts/100 sec (a) Total in extract	^{32}P counts/100 sec (b) ADP + ATP	% incorporation into energy-rich phosphate (b)/(a) × 100	Incorporation as % of value in Na medium	P_i into energy-rich phosphate n-moles/ml. ghosts/15 min
II[10]	Column chromatography	High Na, K-free	77,100	1783	2·312	100	—
		High Na, 10 K	79,400	1242	1·564	68	—
		High Na + ouabain	77,200	1124	1·455	63	—
III	Extraction	High Na, K-free	96,000	946	0·985	100	40·0
		High Na + ouabain	92,900	594	0·639	65	25·1
		High Na, 10 K	83,900	565	0·673	68	23·9
		High Na, 10 K + ouabain	99,100	566	0·571	58	24·0
		High K	93,000	613	0·655	67	25·9
		High K + ouabain	101,500	626	0·616	63	26·5
III[20]	Extraction	High Na, K-free	112,200	3178	2·832	100	38·0
		High Na + ouabain	124,600	1957	1·570	55	23·4
		High Na, 10 K	121,600	1986	1·633	58	23·8
		High choline, K-free	112,000	2107	1·881	66	25·2

The ghosts were sealed in a medium containing (mM): ATP, 1; $^{32}P_i$, 5; Mg, 7; Na, 2; K, 147; Cl, 149; iodoacetate, 0·2; fluoride, 2. They were washed and incubated for 15 min in media, as described in Table 1.

operation of the Na pump affected the labelling, ouabain (10–50 μM) was added. The incorporation was not significantly changed with either 10 or 150 mM-K in the medium (Table 3). Ouabain was also added to the high Na, K-free medium to see if it decreased the labelling as described by Garrahan & Glynn (1967). Addition of the glycoside caused a fall of 35–40 % in the labelling, comparable with the decrease found in the presence of external K (Expt. III[5], Table 3).

Replacement of external Na with choline. In order to test whether a downhill gradient of K was enough to produce labelling of energy-rich phosphate, ghosts were incubated in a medium in which external Na had been replaced by choline. Deprivation of external Na reduced the labelling by 34 % (Table 3), comparable with the decrease caused by raising external K or internal Na, or by adding ouabain. Although the effect seems most likely to be due to Na deprivation, some decrease in labelling might be caused by external K. Thus there was some leakage of K from ghosts that gave rise to a concentration in the medium of about 0·4 mM-K after 15 min. K is more effective in activating ATPase in a Na-free medium than in a Na medium (Whittam & Ager, 1964) and the K that leaked into the choline medium could have contributed to the decrease in labelling. It seems unlikely, however, that this K on its own could have completely prevented the extra incorporation of $^{32}P_i$ into ATP. A relevant consideration is that extra labelling was found in the same high K, low Na ghosts incubated in a high Na, K-free medium despite leakage of K to give an external concentration after 15 min of 0·8–1·0 mM.

It is useful to have a measure of incorporation in absolute units relating P_i to the volume of ghosts. By determining the specific activity of $^{32}P_i$ in the haemolysate, it was possible to ascribe the counts in energy-rich phosphate to the incorporation of an absolute quantity of P_i. The values again show the differences described above between the various conditions. It was not possible to measure the net synthesis of ATP that is implied by these results because of the very small quantities involved. The rate of incorporation was only 1–2 % of the rate of ATPase activity, both rates being calculated under optimum conditions (see Discussion).

Absolute magnitude of labelling. In some experiments 1 mM P_i was added instead of the usual 5 mM (Table 4). The same pattern of incorporation was found at both concentrations. The absolute quantity of P_i converted into energy-rich phosphate, however, was decreased with decrease in P_i. Thus, whereas 47·3 n-moles P_i/ml. ghosts were converted into energy-rich phosphate at a level of 5 mM P_i in the lysing fluid, the comparable value with 1 mM P_i in the lysing fluid was 25·8. Ghosts were also analysed before incubation under the various conditions, and appreciable labelling occurred whilst the ghosts were being prepared (Table 4).

TABLE 4. The incorporation of ^{32}P-labelled P_i into energy-rich phosphate by low Na, high K, isotonically resealed ghosts

P_i concentration in lysing fluid ...	5 mM		1 mM			
					Activity of Na pump	
Medium	Incorporation as % of value in Na medium	P_i into energy-rich phosphate n-moles/ml. ghosts/15 min	Incorporation as % of value in Na medium	P_i into energy-rich phosphate n-moles/ml. ghosts/15 min	Forwards	Backwards
Na	100 (11)	47·3 ± 6·5 (4)	100 (7)	25·8 ± 4·0 (7)	None	High
Na (not incubated)	42·4 ± 7·8 (3)	20·7 (2)	26·0 ± 4·1 (3)	6·5 ± 1·9 (3)	—	—
Na + ouabain (10 μM)	68·0 ± 4·0 (5)	25·1 (2)	—	—	Inhibited	Inhibited
Na + ouabain (50 μM)	64·2 ± 8·5 (4)	32·0 ± 4·9 (3)	60·6 (2)	23·4 (2)	Inhibited	Inhibited
Na + K (10 mM)	71·0 ± 4·6 (5)	32·4 ± 5·2 (4)	54·6 ± 4·4 (6)	16·6 ± 3·4 (6)	High	None
K	61·2 ± 3·9 (7)	23·8 (2)	—	—	High	None
K + ouabain (50 μM)	70·0 (2)	24·9 (2)	—	—	Inhibited	Inhibited
'Cold' lysing fluid	66·9 ± 3·1 (3)	—	—	—	High	None
Choline	71·0 (1)	35·0 (1)	67·9 (2)	23·4 (2)	None	None

The ghosts were sealed in a solution containing (mM): ATP, 1; $^{32}P_i$, 1–5; Mg, 7; Na, 2; K, 145; Cl, 149; iodoacetate, 0·2; fluoride, 2. The washing and incubation media were of the following composition (mM): Na, K or choline, 144; Mg, 7; Cl, 158; Tris (pH 7·3 at 37° C, 5; iodoacetate, 0·2; fluoride, 2. The 'cold lysing fluid used in three experiments was prepared by sealing a parallel lot of cells from the same donor in a fluid of identical composition to the above, containing 5 mM unlabelled P_i. After incubating at 37° C for 30 min, the suspension was centrifuged at 18,000 *g* for 1 min at 5° C and the supernatant pooled. This served as a 'cold' medium for subsequent washing and incubation of $^{32}P_i$ labelled ghosts. Free $^{32}P_i$ and nucleotide-bound $^{32}P_i$ were separated by ion exchange chromatography in five experiments and by organic extraction in the remainder. In four experiments, both techniques were employed simultaneously (see Table 2). The individual results obtained in each experiment were means of incubations in the different media carried out in duplicate or triplicate. The total number of experiments is indicated in parenthesis, and the results in the Table are means ± S.E. of mean.

Even after two washes in Na medium and no incubation at all, some 42% of the incorporation level attained after incubating in the Na medium was found with 5 mM-P_i in the lysing fluid; with 1 mM P_i, the comparable value was 26%. To test whether this labelling increased with time, ghosts were incubated in a fluid identical in composition with the radioactive haemolysate, except that it did not contain ^{32}P. This fluid was obtained as the supernatant from a 'cold' haemolysate made by lysing some of the same cells in the usual hyptonic lysing fluid to which ^{32}P had not been added. Incubation for 15 min in this medium caused an increase in labelling to the level achieved when ghosts were incubated in high K medium (Table 4). The results suggest that ionic gradients had no effect on the uniform level of labelling when the Na pump was operating normally, or was inhibited in its reverse or forward reaction.

Effect of changes in internal Na and K content of ghosts

Having demonstrated that the K concentration gradient was not itself enough to cause extra incorporation of $^{32}P_i$ into energy-rich phosphate, the next step was to ascertain whether a downhill movement of Na into ghosts, without accompanying K moving out, was effective. The need for a Na gradient was first tested by measuring incorporation in ghosts where the internal Na was raised to over 100 μ-equiv/ml. (see Table 1). In each experiment where alterations in internal Na and K content of ghosts were made, a parallel set of low Na, high K ghosts was prepared from the same batch of cells and these were incubated as control. Direct comparison of absolute levels of incorporation is difficult in these paired experiments because, although all the ghosts in a particular study were made from one batch of cells and lysed in one lysing fluid, the quantity of radioactivity trapped within each type of ghost at reversal was not always the same. For each lot of ghosts, the amount of incorporation in the Na medium has, therefore, been called 100%, in order to allow valid comparison of the effect of changes in medium upon the behaviour of different types of ghost.

When the internal Na concentration was raised, the labelling was the same in the presence and absence of 10 mM-K, and when external Na was replaced with choline. The absolute labelling of energy-rich phosphate was less (12·3 n-moles P_i/ml. ghosts/15 min) in the high Na, low K ghosts than in low Na, high K ghosts (15·2 n-moles P_i/ml. ghosts/15 min). The level in high Na ghosts was in the region of that found when the low Na ghosts were incubated in medium containing 10 mM-K (11·5 n-moles P_i/ml. ghosts/15 min). The results show that raising internal Na decreased labelling of ATP. The effect could arise from abolishing the Na gradient or the K gradient or both. In order to test the effect of a Na gradient alone, high

TABLE 5. The incorporation of $^{32}P_i$ into energy-rich phosphate by isotonically resealed ghosts with differing internal sodium and potassium content

Expt.	Type of ghost	Medium	^{32}P counts/100 sec. (a) Total in extracts	^{32}P counts/100 sec. (b) ADP+ATP	Counts in energy-rich phosphate / Total counts (b)/(a) × 100	^{32}P incorporation as % of value in Na medium	P_i into energy-rich phosphate n-moles/ml. ghosts/15 min
III[26]	Low Na	Na	45,900	755	1·644	100	15·2
	High K	Na+K	43,800	573	1·308	80	11·5
		Choline	42,200	549	1·300	79	11·0
	High Na	Na	45,600	608	1·333	100	12·3
	Low K	Na+K	47,400	598	1·261	95	12·0
		Choline	43,400	576	1·327	100	11·6
III[33]	Low Na	Na	418,000	12221	2·921	100	23·7
	High K	Na+K	420,000	7531	1·793	61	14·6
	Low Na	Na	353,000	8787	2·489	100	17·0
	Low K High choline	Na+K	337,000	7952	2·359	95	15·5
III[35]	Low Na	Na	284,300	4910 (1065)*	1·727	100	13·1
	High K	Na+K	287,800	3387 (896)	1·176	68	9·0
	Low Na	Na	256,700	4232 (710)	1·648	100	11·3
	½K ½ choline	Na+K	241,900	2915 (621)	1·205	73	7·8
	Low Na	Na	203,700	2994	1·469	100	8·0
	Low K High choline	Na+K	207,600	2899	1·396	95	7·7

Cells were lysed in a solution containing (mM): ATP, 1; Mg, 7; *o*-phosphoric acid adjusted to pH 7·6 with Tris base, 1; Na, 2; Cl, 14; iodoacetate, 0·2; fluoride, 2. (The inhibitors were added as Na or K salts depending on whether low or high Na ghosts were being prepared.) From each batch of cells, one lot of low Na, high K ghosts was made and served as control. Isotonicity was restored by addition of 3 M-KCl, 3 M-NaCl, 3 M-choline chloride or, in the case of half K–half choline ghosts, equal proportions of 3 M-KCl and 3 M-choline chloride. The washing and incubating media were of the following composition: choline, K or Na, 142; Cl, 161; Mg, 7; Tris (pH 8·3 at 5° C), 5; iodoacetate, 0·2; fluoride, 2. The inhibitors were added as Na salts in the choline and Na media; as K salts in the K medium. Free $^{32}P_i$ and nucleotide-bound $^{32}P_i$ were separated by organic extraction. The results in the Table are means of duplicate or triplicate incubations carried out with each lot of ghosts.

* These figures represent the counts remaining in the extracts after acid hydrolysis. They indicate that 76–80% of the ^{32}P in this fraction was acid-labile.

choline ghosts, low in both Na and K (see Table 1), were incubated so that external Na could leak inwards without outward movement of K. In a high Na medium, these ghosts incorporated approximately the same amount of $^{32}P_i$ into energy-rich phosphate whether or not external K (10 mM) was present (15·5 and 17·0 n-moles P_i/ml. ghosts/15 min). Again, control ghosts rich in K showed a marked difference with and without external K (14·6 and 23·3 n-moles P_i/ml. ghosts/15 min, respectively). To check whether choline might be acting as a poison, ghosts were made with an internal composition of half choline and half K. Incubation of these ghosts in Na media showed a decrease in labelling of 27 % with 10 mM external K (Expt. III[35] Table 5). Ghosts containing half K–half choline showed less incorporation (n-moles P_i/ml. ghosts/15 min), 11·3, than that achieved in the high K ghosts, 13·1, in keeping with the difference in internal K content. As before, the high choline ghosts in this experiment showed the same degree of labelling, irrespective of presence of external K (8·0 and 7·7 n-moles P_i/ml. ghosts/15 min). It seems unlikely that choline itself was inhibiting labelling.

TABLE 6. The incorporation of $^{32}P_i$ into energy-rich phosphate by isotonically resealed ghosts of differing internal sodium and potassium content

Type of ghost	^{32}P incorporation as % of value in Na medium (100 %)		
	Medium		
	Na + ouabain	K	Ionic gradients present
Low Na High K	−30	—	$[Na]_o > [Na]_i$; $[K]_i > [K]_o$, pump inhibited
	—	−39	Na and K gradients abolished
Low Na Low K High choline	+6	—	$[Na]_o > [Na]_i$; no K gradient, pump inhibited
	—	+6	No Na gradient; $[K]_o > [K]_i$
High Na Low K	−3	—	Na and K gradients abolished, pump inhibited
	—	−6	$[Na]_i > [Na]_o$; $[K]_o > [K]_i$

Ghosts were prepared and incubated as in Table 5. The values are from four paired experiments. In each experiment, one lot of low Na, high K ghosts was prepared and served as control to another lot of different Na and K content derived from the same batch of cells. The washing and incubation media contained (mM): Na or K, 142–144; Cl, 161–163; Mg, 7; Tris (pH 8·3 at 5° C), 5; iodoacetate, 0·2; fluoride (as Na or K salt), 2. Ouabain was added to the Na medium in a concentration of 50 μM.

Table 6 summarizes the findings in a further series of four paired experiments in which the behaviour of low Na, high K ghosts was compared with that of low Na, low K (high choline) and also high Na, low K ghosts. The characteristic reduction in labelling of energy-rich phosphate in low Na, high K ghosts when ouabain was added to the all Na medium or when

external Na was replaced by K, no longer occurred when internal K was markedly lowered or internal Na elevated.

These results demonstrate the need for gradients of *both* Na and K in order to produce the $^{32}P_i$ incorporation into energy-rich phosphate which is apparently associated with reversal of the transport ATPase system.

Basal $^{32}P_i$ incorporation

Apart from the extra labelling found when low Na, high K ghosts were incubated in a high Na, K-free medium, the results thus far show that there occurred a low level of labelling in the different types of ghost irrespective of whether ionic gradients were present across the membrane, whether the pump was operating normally, or whether pump action was inhibited by ouabain. If this low uniform labelling is independent of the Na pump, then adding the glycoside from the start should have no effect.

TABLE 7. The effect of ouabain on labelling of energy-rich phosphate

Lysing solution	Incubation medium	% ^{32}P incorporation into energy-rich phosphate in 15 min
(*a*) With ATP	Na	1·859
	K	0·983
(*b*) With ATP and ouabain	Na + ouabain	1·032
	K + ouabain	0·924
(*c*) Without ATP	Na	0·459
	K	0·448

Three different lots of low Na, high K ghosts were prepared from the same batch of cells. The solution (*a*) in which the ghosts were lysed contained (mM): ATP, 1; $^{32}P_i$, 5; Mg, 7; K, 2; Na, 2; Cl, 14; iodoacetate, 0·2; fluoride, 2. In (*b*) 50 μM-ouabain was present, in (*c*) ATP was omitted. Isotonicity was restored by addition of 3 M-KCl. Na and K media used for washing and incubation were the same as in the experiments of Table 2.

Table 7 shows this to be so and, further, the $^{32}P_i$ incorporation was the same whether the ghosts were incubated in a Na or K medium (1·032 and 0·924 % incorporation, respectively). In contrast, ghosts made from the same lot of cells but without ouabain showed a doubling of labelling in the Na, K-free medium (1·859 % incorporation), as compared with the K medium (0·983 % incorporation). When ghosts were made without ATP, there was still a small amount of labelling, apparently in the form of high-energy phosphate, but the characteristic difference in labelling between Na and K media was not found. The nature of this labelling was not investigated.

Rate of the reversed reaction

In two experiments with low Na, high K ghosts, the labelling of energy-rich phosphate was followed for 1 hr in high sodium media with and without 10 mM added potassium. The amount of incorporation increased

with time, and in the high Na, K-free medium, the mean $^{32}P_i$ incorporation was most rapid in the first 15 min, 1·08 n-moles P_i/ml. ghosts/min, decreasing thereafter to a value of 0·28 n-moles P_i/ml. ghosts/min over the subsequent 45 min of incubation (Fig. 5). Potassium chloride (10 mM) was added to the all Na medium after 15 min and prevented any further labelling. Those ghosts incubated from the start in the high Na medium containing 10 mM-K showed a slight increase in incorporation with time

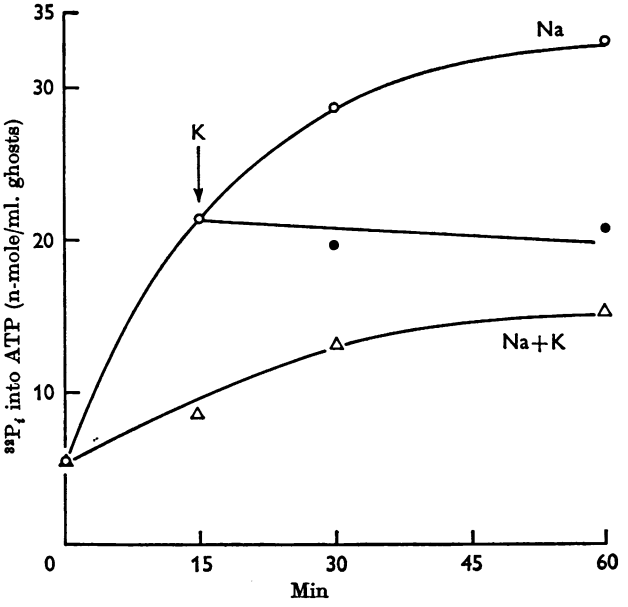

Fig. 5. The incorporation of $^{32}P_i$ into energy-rich phosphate by low Na, high K ghosts as a function of time. The effect of adding 10 mM-K to the all Na, K-free medium, during the course of incubation, was also investigated. The ghosts were sealed in a medium containing (mM): ATP 1; $^{32}P_i$, 1; K, 142; Na, 2; Mg, 7; Cl, 154; iodoacetate, 0·2; fluoride, 2. The medium contained (mM): Na, 142; Mg, M; Tris (pH 8·0 at 5° C), 5; Cl, 156; iodoacetate, 0·2; fluoride, 2. Either at the start or after 15 min incubation, sufficient KCl was added to some of the incubation flasks so as to raise the external K to 10 mM.

and, again, the rate was faster in the first 15 min, 0·31 n-moles P_i/ml. ghosts/min, diminishing to 0·18 n-moles P_i/ml. ghosts/min over the subsequent 45 min incubation.

The latter kind of incorporation was clearly occurring when the sodium pump was operating in a forward direction, and allows a calculation to be made of the rate of the reverse reaction. This is given by the difference at each time interval between incorporation in the high Na, K-free medium and that in the high Na medium containing 10 mM-K. For the first 15 min

of incubation, the mean rate of the reverse reaction (n-moles P_i/ml. ghosts/min ± S.E. of mean) was 0·72 ± 0·11 (6) with 1 mM $^{32}P_i$ in the lysing fluid, and 1·00 ± 0·15 (4) with 5 mM $^{32}P_i$ in the lysing fluid. These values suggest that a fivefold increase in P_i concentration from 1 to 5 mM caused only a small rise in the rate of labelling of ATP.

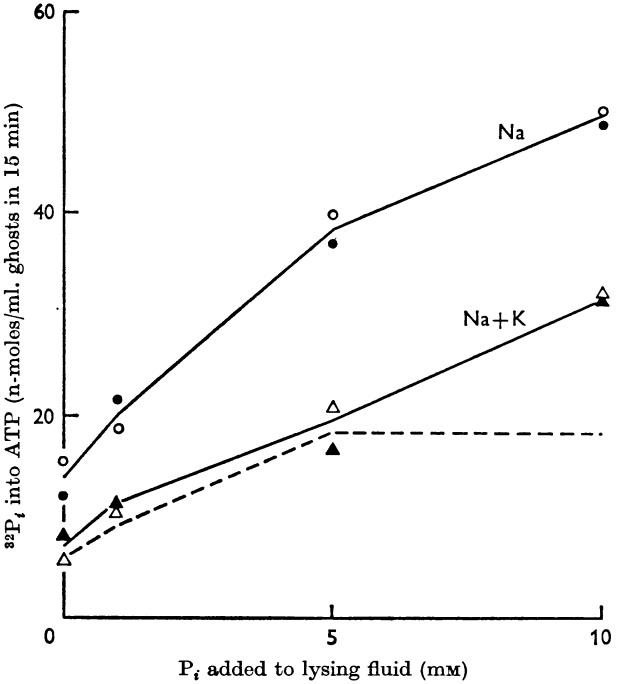

Fig. 6. The effect of increasing internal P_i concentration on $^{32}P_i$ incorporation into energy-rich phosphate by low Na, high K ghosts.

Four different lots of ghosts were prepared from the same batch of cells by lysing in solutions containing (mM): ATP, 1; Mg, 7; Na, 2; K, 2; iodoacetate, 0·2; fluoride, 2, to which was added $^{32}P_i$, 0, 1, 5 or 10. Isotonicity was restored with 3 M-KCl. The specific activity of ^{32}P was determined in a deproteinized sample of the lysing fluid immediately after reversal. The medium contained (mM): Na, 142; Mg, 7; Tris (pH 8·0 at 5° C) 5; Cl, 156; iodoacetate, 0·2: fluoride, 2. Another lot of medium also had 10 mM-KCl added. The values plotted are the means derived from two identical experiments with different batches of original cells. Open symbols, Expt. III[30]; filled symbols, Expt. III[32]. Each condition of incubation was carried out in duplicate. In one of the experiments, the effect of adding equivalent amounts of P_i to the media was also tested. The results were unaltered. The difference between the two curves is shown by the dotted line, which represents the rate of the reversed action of the pump at each level of P_i.

Effect of internal P_i concentration

In order to test more fully whether incorporation of $^{32}P_i$ into energy-rich phosphate could be augmented by increasing internal P_i, labelling was measured with different P_i concentrations. Two experiments were undertaken in which low Na, high K ghosts were prepared so as to contain different amounts of $^{32}P_i$. In each experiment, four lots of ghosts were made from the same batch of intact cells. The lysing solutions were either free of P_i or contained 1, 5 or 10 mM P_i. The relationship between $^{32}P_i$ incorporation in a high Na medium in the absence and presence of external potassium is shown in Fig. 6. The difference between the curves, which represents the rate of the reversed reaction at each level of P_i, is shown as a dotted line. The mean values for incorporation rates over 15 min derived from the results of both experiments were (n-moles P_i into energy-rich phosphate/ml. ghosts/min): 0·44 (no P_i); 0·69 (1 mM P_i); 1·27 (5 mM P_i); and 1·24 (10 mM P_i). These figures show that an increase in the internal P_i between 1 and 5 mM enhanced the incorporation of $^{33}P_i$ into ADP and ATP, whilst 10 mM P_i did not lead to a further increase.

DISCUSSION

Garrahan & Glynn (1967) showed that an ouabain-sensitive incorporation of $^{32}P_i$ into energy-rich phosphate occurred when suitable gradients across the membranes of red cell ghosts permitted downhill movements of Na and K. We have confirmed and extended this work by independently varying the ionic composition of the internal and external fluids. The results show, first, that the labelling of ATP required the simultaneous downhill movement of both Na and K. Secondly, activating the Na pump by adding 10 mM-K to an all Na medium abolished labelling. The different requirements for maximum forward and backward running of the pump indicate that it cannot work optimally in both directions at once. The over-all reaction involving transport ATPase may be shown as in Fig. 7. It follows that as soon as K leaks out of the ghosts in sufficient quantity, it will start to activate the normal transport ATPase, thereby preventing further occurrence of the reverse reaction. Clearly, between the extreme values of zero and 5 mM external K, optimal for both reverse and forward reactions, respectively, there must be some intermediate value at which P_i incorporation into energy-rich phosphate and ATP hydrolysis occur together, as discussed by Garrahan & Glynn (1967).

If the rate of the reverse reaction is taken as the rate of incorporation of $^{32}P_i$ into ATP that is dependent on simultaneous downhill movements of Na and K, there is a remarkable difference between the maximum rates of

the two reactions. Consider the ATPase activity. The glycoside-sensitive ATPase activity of ghosts containing 1 mM ATP is about 50 n-moles/ml. ghosts/min (Whittam & Ager, 1964). Our present value of 1 n-mole/ml. ghosts/min for the reverse reaction is seen to amount at most to 2 % of the rate of ATP splitting of the pump. Using this value, it should be possible to calculate the stoicheiometry of the reverse reaction (i.e. the number of ions moving associated with the creation of a high-energy phosphate bond). However, it is unlikely that all the ion movements are linked to reversal of the Na pump, and an accurate value for the stoicheiometry and hence efficiency awaits measurement of the extent of coupling of downhill movements.

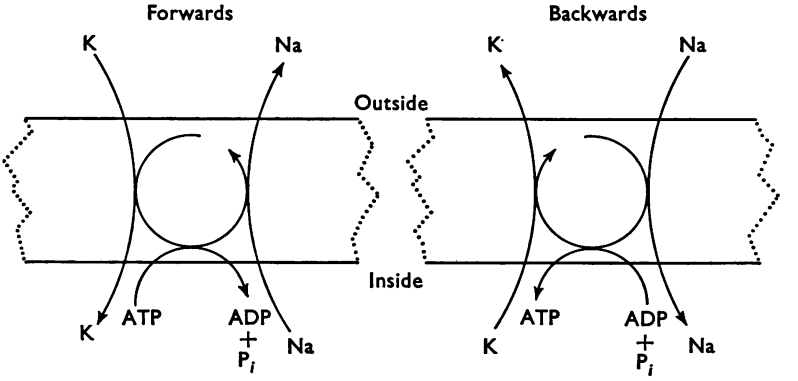

Fig. 7. Model to illustrate forward and backward reactions of the Na pump.

A drawback in the present work is that net synthesis of ATP was not measured. It is not likely to be great in view of the activity of adenylate kinase (Overgaard-Hansen, 1957), and also because of the low rate of labelling of ATP in relation to ATP splitting. The dependence of a part of labelling upon suitable ionic gradients, however, makes it likely that some net synthesis had occurred. It was in order to prevent net synthesis of ATP by glycolytic pathways independent of the Na pump that we included a small amount of iodoacetate and fluoride in the lysing fluid, washing and incubating media, throughout our experiments. Initially we used a concentration of 2 mM iodoacetate but found this led to leakiness of ghosts (Whittam, 1968). A concentration of 0·2 mM was finally used because this blocked lactate production from inosine (H. Lubowitz & R. Whittam, unpublished observations), whilst leaving transport ATPase activity uninhibited (Garrahan & Glynn, 1967).

A complicating feature of the incorporation of $^{32}P_i$ into ATP is that some labelling was always found even when there were no gradients of Na and K across the membrane. This incorporation was more or less uniform

and could not be decreased further by ouabain. Its cause is not clear, but it may be related to the fact that red cell membranes contain tightly bound glycolytic enzymes of which triose phosphate dehydrogenase and phosphoglyceryl kinase are present in highest concentration (Schrier & Doak, 1963; Schrier, 1966). These enzymes catalyse the formation of ATP, and may be involved in the exchange between $^{32}P_i$ and ATP found in the presence of substrate in red cell ghosts (Ronquist & Ågren, 1965) where net synthesis of ATP can occur (Schrier, 1967). In cells also, ATP is synthesized and there is also labelling of ATP with [^{14}C]adenine (Lowy & Williams, 1966; Whittam & Wiley, 1968). Exchange reactions are known in which a complex molecule becomes labelled by a component, and the enzyme needed is the one which catalyses net synthesis (Webster & Varner, 1954; Snoke & Bloch, 1955). The labelling of ATP which occurred independently of the backward running of the Na pump might result from such an exchange reaction that did not involve net synthesis of ATP.

The results have a bearing on the mechanism of the Na pump in that its reversal requires both Na and K. The normal operation of the Na pump may involve a phosphorylated protein intermediate, although this is by no means certain (Skou, 1965). If the turnover of a high-energy intermediate is indeed involved in a way that depends on Na and K, then it seems that its formation and break-down must both be reversible. A useful way of considering the mechanism of active transport is as a coupling between ion movements and a spatially oriented chemical reaction. Energy made available by the hydrolysis of ATP supports ion movements, and the opposite form of energy transformation now appears likely, namely, an increase in chemical energy derived from the downhill movements of ions. From the evidence presented in this work, the general conclusion is that a chemical reaction, or reactions, which result in the incorporation of P_i into ATP can be driven by a coupled flow of Na and K down concentrations gradients.

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