REVERSAL OF THE SODIUM PUMP IN HUMAN RED CELLS

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REVERSAL OF THE SODIUM PUMP IN HUMAN RED CELLS

This is to certify that the work on which this thesis is based results from my own observations.

Signed .....

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SYNOPSIS

A study has been made of some chemical reactions and ion movements related to the sodium pump in the membrane of the human red cell. Part One describes chemical studies with erythrocyte ghosts to test whether incorporation of radioactive orthophosphate ($^{32}P_i$) into adenosine triphosphate (ATP) was associated with concentration gradients of sodium and potassium across the membrane. Some incorporation was always found irrespective of the ionic distribution. Additional labelling depended on the ionic composition of ghosts and medium, and was prevented by ouabain. There was no ouabain-sensitive labelling with a gradient of only either sodium or potassium. The results show that a part of the incorporation of orthophosphate into ATP appeared to occur by reversal of the sodium pump.

In Part Two, movements of sodium and potassium down concentration gradients are described. A part of both sodium entry and potassium loss was sensitive to ouabain. Further, ouabain-sensitive potassium efflux was dependent on external sodium, and ouabain-sensitive sodium influx was abolished when potassium was added to the external medium. In the absence of

external potassium, cells lost potassium and gained sodium in a way partly sensitive to ouabain. A modified operation of the sodium pump seems to bring about exchange diffusion of sodium as well as a coupled movement of sodium entry and potassium loss.

The energetics of ion movements in relation to reactions of ATP were studied in Part Three. The number of ions moving downhill via the pump per mole orthophosphate incorporated into ATP was the same as the stoichiometry for active transport, indicating the same efficiency for ATP splitting and ATP labelling.

The general conclusion is that the reactions of ATP are closely linked to the migrations of ions across the membrane.

GENERAL INTRODUCTION

Processes of active transport denote cellular mechanisms whereby materials are transferred across membranes against an electrochemical gradient (Ussing, 1949a, 1954, 1960; Rosenberg, 1954; Wilbrandt and Rosenberg, 1961). There is now considerable evidence that the high concentration of potassium and low concentration of sodium in most living cells is maintained by active extrusion of sodium and uptake of potassium through the activity of a pump located in the cell membrane (Baker, 1966; Glynn, 1966; Albers, 1967; Heinz, 1967; Whittam, 1967). Maintenance of the normal gradients of sodium and potassium across the cell membrane depends on either anaerobic or aerobic metabolism according to the type of cell. Thus, for example, in nerve, muscle and nucleated red cells, the energy for driving coupled movements is derived from respiration (Maizels, 1954; Clarkson and Maizels, 1955; Hodgkin and Keynes, 1955; Caldwell, 1968), whilst in the mature mammalian red cell, energy is provided by anaerobic glycolysis (Harris, 1941; Danowski, 1941; Maizels, 1951). The only chemical reactions which

both anaerobic and aerobic metabolism share in common are the cyclical oxidation and reduction of nicotinamide adenine dinucleotide (NAD) and the synthesis of ATP from ADP. There is strong experimental evidence that availability of ATP is the common denominator in the dependence of active cation transport upon metabolism; this concept does not, however, exclude the possibility that an oxido-reduction reaction involving turnover of NAD might also be connected directly with the molecular mechanisms energising the pump (see Whittam, 1964b; Whittam and Wiley, 1967). One of the characteristic features of the coupled Na-K pump is that it is inhibited by low concentrations of cardiac glycosides such as ouabain, scillaren or digoxin, acting on the external surface of the cell membrane (Schatzmann, 1953; Edwards and Harris, 1957; Glynn, 1964).

In 1957, Skou found that the microsomal fraction prepared from minced crab nerve contained an adenosinetriphosphatase (ATPase) which, in the presence of magnesium, was activated synergistically by both sodium and potassium ions. He suggested that such an enzyme might be involved in the mechanism of active transport

of sodium and potassium across the cell membrane. This important work was rapidly confirmed and membranous fragments prepared from a variety of tissues were found to possess cation-activated ATPase activity (Post, Merritt, Kinsolving and Albright, 1960; Dunham and Glynn, 1961; Bonting, Simon and Hawkins, 1961; Wheeler and Whittam, 1962; Bonting and Caravaggio, 1963; Gibbs, Roddy and Titus, 1965; Stahl, Sattin and McIlwain, 1966). Over the subsequent decade, much study has been devoted to the characterisation and features of the Na-K ATPase in fragmented and intact cell membranes in the hope of finding a molecular basis for the action of the sodium pump. The experimental findings have revealed a remarkable parallelism between the properties of membrane ATPase and those of the cation transporting system (Glynn, 1968). Among the outstanding features shared by both processes are first, sensitivity to low concentrations of cardiac glycosides; second, a characteristic spatial asymmetry in possessing internal Na^+ and external K^+-sensitive sites of activation, the activating ion being competitively inhibited by its partner at each site (Priestland and Whittam, 1968).

However, despite the strong support for an intimate

link between active cation movements across cell membranes and membrane ATPase activity, the precise nature of the linkage and the way in which the Na,K-ATPase represents or functions as an integral part of the pump mechanism, remain obscure. On the basis of available experimental evidence, it seems valid to consider the overall process of active transport of Na and K as an example of energy transduction in which the chemical energy of metabolism is being transformed in part into osmotic work by a "chemi-osmotic coupling reaction". The term "chemi-osmotic coupling" was introduced by Mitchell (1961) to describe a situation in which the driving force on a given chemical reaction was due to "the spatially directed channelling of the diffusion of a chemical component or group along a pathway specified in space by the physical organisation of the system".

The question arises: can the energy liberated by downhill movements of ions be utilised to synthesise ATP? The theoretical possibility of such a type of energy transformation was discussed by Davies and Krebs (1952). Experimental support for its occurrence at the plasma membrane was wanting until Garrahan and

Glynn (1967d) demonstrated that labelled phosphate ($^{32}P_i$) could be incorporated into ATP red cell ghosts when there were large concentration gradients of Na and K between the ghosts and surrounding media. These authors suggested that the sodium pump could be made to run backwards and synthesize ATP with energy derived from dissipation of ionic concentration gradients. Studies of the Na, K-ATPase system in membrane preparations derived from nerve (Skou, 1960) or electric organ (Fahn, Koval and Albers, 1966) have not revealed significant labelling of ATP by $^{32}P_i$. However, the tissue preparations used in these studies were of disrupted cells and although rich in ATPase activity, the cation concentrations must have been the same on both sides of the cell membrane. Such conditions would favour ATP breakdown, and hence no incorporation of phosphate into ATP could be expected. Experiments with fragmented membranes have proved useful in helping to clarify certain aspects of the mechanism of the ATPase reaction. For instance, when microsomal preparations derived from kidney cortex, electric organ or brain are incubated with $^{32}P_i$ - labelled ATP, it appears that turnover of a high energy phosphorylated intermediate may be involved

(Hokin and Hokin, 1963; Albers, 1967; Kahlenberg, Galsworthy and Hokin, 1968). The exact nature of this labile intermediate is unknown though there are indications that it is an acyl phosphate ($E\text{-}CO\text{-}P_i$) which undergoes alternate phosphorylation and dephosphorylation. Phosphorylation requires the presence of Mg^{2+} and Na^{+} whilst the liberation of bound phosphate is activated by potassium. If such a phosphorylated intermediate is indeed an integral component of the Na pump, then it follows that ATP hydrolysis may also involve a two-step reaction. At the present time, the details of the precise reaction sequence leading to the splitting of ATP are still not fully understood. It is clear, however, that the cation-activated ATPase system located in the cell membrane plays a key role in the chain of events through which chemical energy from intracellular metabolism becomes utilised in transporting Na and K against an electrochemical gradient.

The central problem underlying the work described in this thesis has been whether the two forms of energy - chemical and osmotic - which feature in the active cation transport system, are interconvertible. Does the transformation of chemical to osmotic energy which characteri-

ses normal operation of the Na pump represent an exclusively unidirectional process, or can there be a demonstrable increase in chemical energy at the expense of ions moving down a concentration gradient?

Part One describes chemical studies undertaken in human red cell ghosts to determine whether, under the influence of suitable ionic gradients, the sodium pump could be driven in reverse at a measurable rate. The ionic composition on either side of the cell membrane was varied independently so as to define the optimum ionic requirements for reversing the ATPase reaction and at the same time find out whether incorporation of phosphate into ATP could occur when the pump was operating normally. In Part Two, the kinetics of the downhill movements of Na and K across the red cell membrane have been examined with the help of radioactive tracers. Experiments were undertaken to see whether there were components of downhill movements of these cations which were connected with reversal of the pump, and at the same time displayed the features of coupling which characterise the uphill movements associated with forward operation of the pump. In Part Three, consideration is given to the energetics of reversing the ATPase

reaction associated with the Na pump. The results allowed assessment of the relative efficiencies of the reverse and forward reactions, and taken in conjunction with the quantitative measurements made in Part Two, an estimate was obtained of the approximate stoichiometry of the reverse reaction - namely, the number of ions moving downhill via the pump mechanism per mole of phosphate incorporated into ATP.

Abbreviations of chemical substances

ADP	adenosine diphosphate
AMP	adenosine 5' -phosphate
ATP	adenosine triphosphate
EDTA	ethylenediaminetetra-acetate
Hb	haemoglobin
IMP	inosine 5' -phosphate
IAA	iodoacetic acid
NAD^+ and NADH	oxidised and reduced forms respectively of nicotinamide-adenine-dinucleotide (diphosphopyridine nucleotide)
$NADP^+$ and NADPH	oxidised and reduced forms respectively of nicotinamide-adenine-dinucleotide phosphate (triphosphopyridine nucleotide)
Na, K-ATPase	the component of adenosine triphosphatase activity that requires Mg^{2+}, K^+ and Na^+ and is inhibited by ouabain.
P_i	orthophosphate (o-phosphate)
Tris	tris (hydroxymethyl) aminomethane

Special symbols

b	regression coefficient
Δ	difference
ε	molar extinction coefficient (OD of molar solution at 10 mm light path)
N.S.	not significant

Special Symbols (continued)

OD	optical density
P	probability
t	student t test

Statistical methods

Statistical analyses of the experimental data in this thesis have been performed according to the methods of Snedecor (1956), Ezekiel and Fox, (1965) and Lambe (1967). A P value of 0.05 or less was considered statistically significant

PART ONE

THE INFLUENCE OF IONIC GRADIENTS ON THE INCORPORATION OF LABELLED P_i INTO ATP

INTRODUCTION

Following the classical experiments of Skou (1957, 1960) on the Na, K-ATPase of crab nerve, much work has been devoted to study of the effects of ions on the activity of ATPases in different tissues. It has become clear that in almost all cells and tissues in which there is a coupled active transport of Na and K, there is an ATP- hydrolysing enzyme which, like other phosphatases requires Mg^{2+}, but which is also synergistically activated by Na and K. Although the use of broken or fragmented membrane preparations has yielded much information on the nature and properties of the Na,K-ATPase system, this technique does not allow one to test for certain spatial features of the cation pump.

The pump normally operates in vivo with different solutions on the two sides of the cell membrane. The characteristic directionality of the transport system in preferring Na on the inside and K on the outside cannot be evaluated in a preparation of broken cells where both sides of the membrane are inevitably exposed to the same concentrations of whatever constituents - activators or inhibitors - are present in the surrounding fluid. Similarly, the key role of intracellular ATP as a source of energy for the cation pump was for some years based largely on indirect evidence because there was no ready means of altering the internal constitution of the cell at will, whilst leaving the integrity of the cell membrane intact. The critical test of exposing the inner surface of the membrane to ATP as the sole source of energy was successfully applied to nerve tissue by Caldwell, Hodgkin, Keynes and Shaw (1960). Using a perfused nerve fibre preparation, ATP or one of its precursors such as arginine phosphate or phosphoenolpyruvate was introduced by injection into giant squid axons which had been poisoned with cyanide; and found to restore partially the active efflux of Na. Further evidence in nerve for the dependence of active

Na efflux on internal ATP has come from work with dialysed squid axons (Brinley and Mullins, 1968). In the case of the red cell, advantage was taken of the technique of "reversed haemolysis" or "membrane reconstitution after haemolysis" which allows emptied cells or ghosts to be loaded with various concentrations of cations as well as with materials like ATP to which the intact cell is normally impervious. Substrate-depleted red cell ghosts loaded with ATP actively transport Na (Hoffman, 1962 a and b) and K (Gárdos, 1954); there is a specific requirement for ATP since other nucleotide phosphates such as inosine or guanosine triphosphate cannot replace ATP as an energy source (Hoffman, 1960). Glynn (1962) and Whittam (1962), using the reconstituted ghost system, showed separately that the membrane ATPase of the red cell shares the spatial asymmetry of the cation transport system in requiring Na on the inside and K on the outside of the cell - the locations from which these ions are actively moved across the membrane.

These findings are of considerable importance. First, they provide further evidence for an intimate connexion between a part of the membrane-bound ATPase

and the coupled cation pump. Second, they draw attention to the potential usefulness of the reconstituted ghost preparation as an experimental tool for investigating the molecular mechanisms of active transport. The possibilities for metabolic manipulation of the ghost are boundless. Study of isolated reactions is possible by elimination of interference either by dilution or removal of substrate whilst at the same time a cytoplasm can be created whose composition is essentially of one's own choice. The fact that the structural organisation of the membrane is maintained allows the behaviour of the membrane ATPase and cation pump to be examined under different conditions as a single functional unit. Such qualities rendered the resealed ghost eminently suited to the particular purpose of the present work where it was desired to investigate the possibility of driving the ATPase reaction in reverse with energy derived from ion gradients. However, it is technically much easier to prepare a leaky red cell ghost preparation than one which will allow a substantial cation gradient to be maintained.

It has been known for over 45 years that after

hypotonic haemolysis, the red cell membrane can regain its natural impermeability to haemoglobin (Bayliss, 1924; Adair, Barcroft and Bock, 1926), but for some time it was considered that in the resulting ghosts, the selective impermeability to cations which features in the intact cell was lost irretrievably (Davson and Ponder, 1938). Subsequent experience has shown that this is not necessarily so. It is recognised that during the course of hypotonic haemolysis the cells pass through a phase in which they become highly permeable to cations, haemoglobin and various substrates (Gárdos, 1954; Gourley, 1957; Hoffman, 1958a). Partial recovery of low permeability to cations may occur spontaneously in a substantial proportion of cells (Teorell, 1952). The reconstitution process can be encouraged and rendered more complete by various refinements in methodology such as the inclusion of small concentrations of Mg^{2+} in the lysing fluid (Hoffman, 1962b), restoration of isotonicity by addition of sufficient concentrated salt (reversal) (Székely, Mányai and Straub, 1952; Hoffman, 1958, 1962a and b), a period of incubation at 37°C (Hoffman, Tosteson and Whittam,

1960).

Little is known of the detailed mechanisms involved in osmotic haemolysis and its reversal. Attention has naturally been directed towards stretch phenomena such as the reversible opening of pores in the membrane. Katchalsky, Kedem, Klibansky and de Vries (1960) have studied the process of 'gradual haemolysis' and claimed that exposure of erythrocyte to mild haemolytic conditions was gentler to the membrane and less likely to jeopardise recovery of low permeability properties, especially to small molecules such as cations. However, studies with the electron microscope have shown that during both gradual and rapid hypotonic haemolysis, transient membrane defects or holes develop (Seeman, 1967; Baker, 1967a and b). By following the time course of development and closure of these holes with the aid of ferritin and colloidal gold markers, it was found that for most cells, the permeable state exists for only 15-25 sec after the onset of haemolysis (Seeman, 1967). If the preparative conditions are carefully controlled, it is possible to make ghosts with membranes which have maintained many

of the structural characteristics (Hillier and Hoffman, 1953; Hoffman, 1956) and recovered also the low permeability to cations (Hoffman, 1962a and b; Whittam, 1962; Glynn, 1962) characteristic of the intact erythrocyte. Although certain of the properties of ghost systems are a function of the membranes of the parent erythrocytes from which they are derived (Ponder and Barreto, 1957) it is clear that one of the major determining factors is the particular technique of preparation.

Because of the critical role in the present work of the state of the cell membrane vis a vis cation permeability, experiments were first undertaken to define the physico-chemical characteristics of ghost populations prepared by a standardised method. The conditions at the time of haemolysis were studied in order to determine the optimal procedure for producing ghost membranes which were sufficiently "tight" to allow steep gradients of cations to be maintained. The incorporation of ^{32}P-labelled orthophosphate ($^{32}P_i$) into ATP in such ghosts was then studied when the Na pump was operating normally and when downhill movements were occurring of both Na and K or of only one ion.

METHODS

Procedure

Preparation of ghosts

The procedure employed was based on methods described previously (Hoffman, 1958a; Hoffman, Tosteson and 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^{o}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, ousbain 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 18,000g for 1 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 μ-moles of unlabelled ATP, 5 μ-moles of unlabelled P_i to act as carriers for the labelled material. In other experiments, the ghosts were lysed in 10 ml. water.

Deproteinisation 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 neutralised 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 neutralised 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 (1959a and 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 typical experiments are shown in Figs. 1 and 8. 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

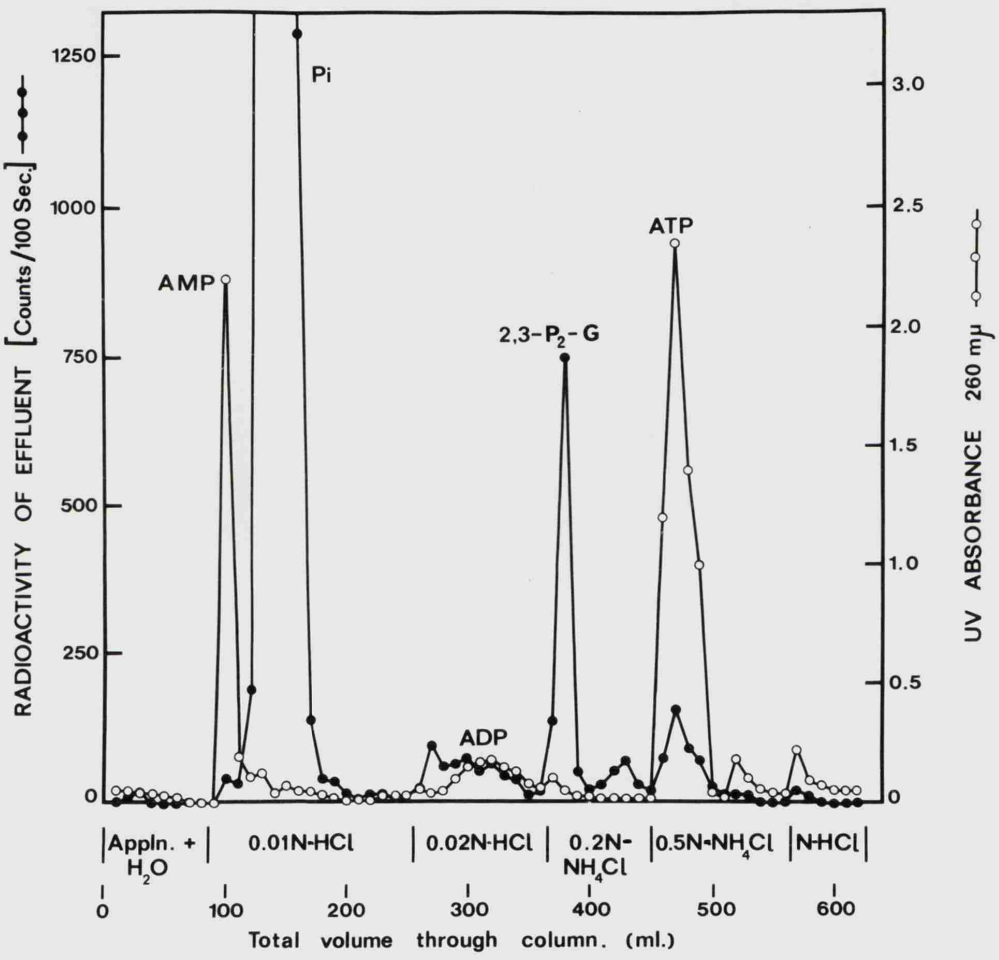

FIG. 1. Elution chart from Expt.II[2] showing separation of phosphate compounds in red cells on a 0.8 cm x 10 cm column of Dowex 1-X8 chloride. A neutralised $HClO_4$ extract was prepared from low Na, high K ghosts after incubation for 30 min at 37°C in 140 mM-NaCl; 10 mM-Tris phosphate (pH 7.4). The ghosts were sealed in a medium containing (mM): ATP, 4; adenosine, 5; K, 135; Na, 8; Mg, 2; Cl, 140; P_i, 5 (pH 7.4). The absorbed compounds were eluted with the indicated eluents in 10 ml. fractions at a rate of 0.5 ml./min.

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, although in four preliminary experiments where the red cells were lysed in hypotonic media containing adenosine, a ^{32}P-labelled adenylate compound, probably AMP, was found in the 0.01 N-HCl fraction, whilst a ^{32}P-phosphate ester giving little or no ultraviolet absorbance at 260 mμ appeared in the 0.2 - 0.5 N-NH_4Cl fraction; the latter was most probably 2, 3-diphosphoglycerate (2,3 - P_2 - G) (Bartlett, 1959a and b; 1968) [Fig. 1.]. 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 neutralised $HClO_4$ extracts, was used to give a measure of how much of the original phosphate had been esteri-

fied and become incorporated into 'high-energy phosphate'.

Extraction technique

The extraction technique employed was based on the method of P_i determination of Berenblum and Chain (1938a and b) as modified by Martin and Doty (1949), Weil-Malherbe and Green (1951) and Nielsen and 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 neutralised 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 5mM-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 ± S.E. of mean for this labelling was 0.116±0.008% (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 deproteinised samples derived from parallel incubations. The precision of the values for bound ^{32}P, based on S.D., was within the range of ± 3.5% 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 and Guyver, 1965; Garrahan and 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 deproteinised extracts by the methods of either Fiske and Subbarow (1925) or Weil-Malherbe and 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.

Lactate

This was measured enzymatically on non-neutralised perchloric acid supernatants using Bio-chemica 'test kits' supplied by The Boehringer Corp. (London) Ltd. The procedure was similar to that described in detail by Hohorst (1963).

MATERIALS

ATP was obtained from Sigma, London, Ltd., as the crystalline disodium salt. Inosine and adenosine were also obtained from Sigma, London, Ltd. ^{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 neutralised 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 neutralised 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

Characteristics of Ghosts

Tonicity at reversal

Variations in the preparative procedure have profound effects on the permeability to cations of erythrocyte ghosts (Hoffman, 1962a and b). 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, Tosteson and Whittam, 1960), the ghosts were washed and then incubated for 15 min in an isotonic Na medium. (Fig. 2). The degree of sealing to haemoglobin and K was then assessed (Fig. 3).

The amount of K and haemoglobin found in the ghosts depended on the 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, 1962a). These

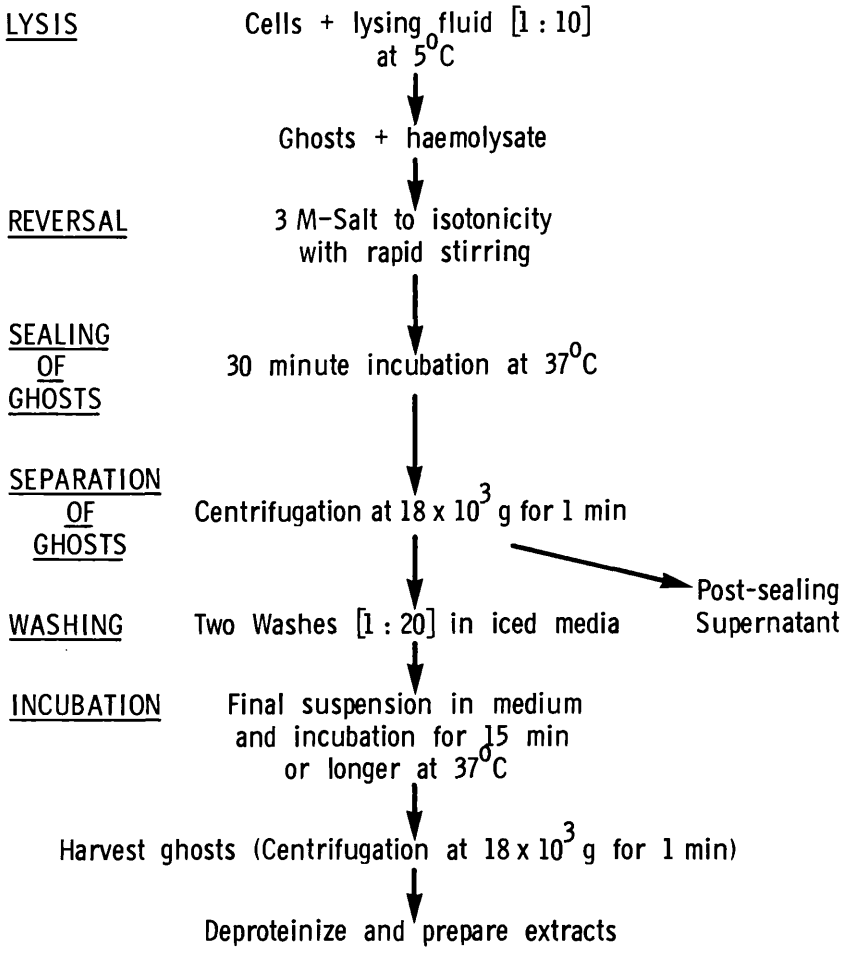

FIG. 2. Procedural steps in the preparation of isotonically resealed ghosts.

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, 1958a), 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

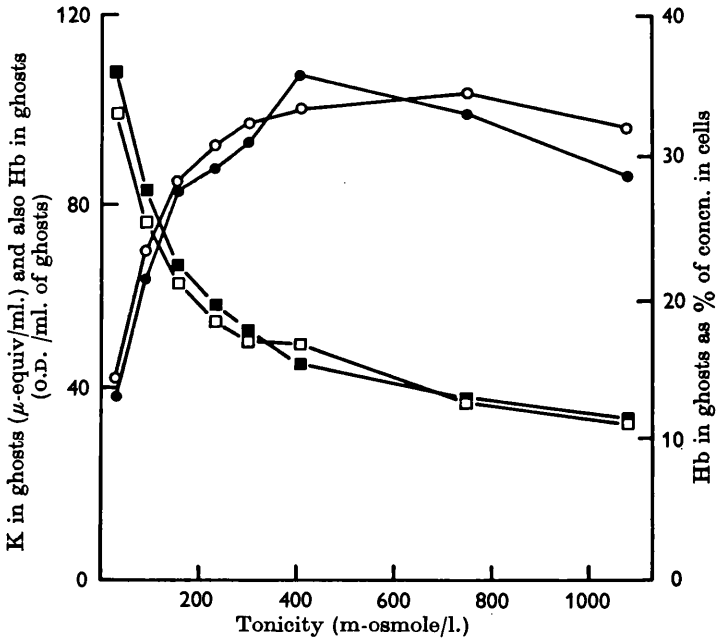

Fig. 3. The relationship between tonicity of the lysing medium at reversal, and cation and haemoglobin content of resealed ghosts. 150 mM-KCl-washed red cells were lysed (1:10 vol.) in a solution of the following composition (mM): ATP, 1; P_i, 1; Na, 2; Mg, 7; K, 4; iodoacetate, 0·2; fluoride, 2. Lysate 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.

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 μ-equiv/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 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 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, but 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. 4). 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

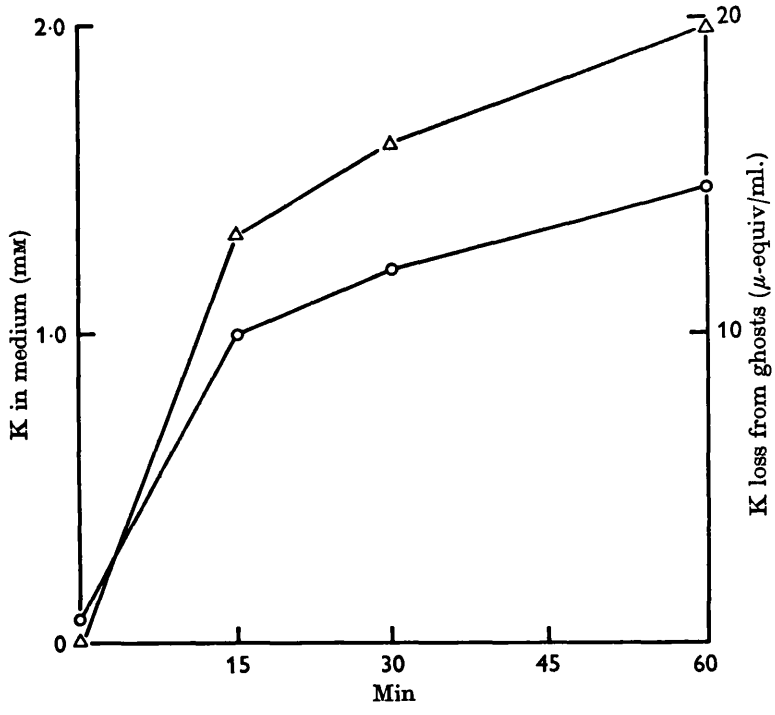

Fig. 4. The net loss of K from low Na, high K, isotonically resealed ghosts incubated in Na medium. The solution in which the ghosts were sealed contained (mM): ATP, 1; $^{32}P_i$, 1; Mg, 7; K, 147; Cl, 149; Na, 2; iodoacetate, 0·2; fluoride, 2. The sodium incubation medium contained (mM): Na, 142; Mg, 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.

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 connection it is relevant that K efflux from intact cells is not inhibited but stimulated by external K (see p. 128).

In five experiments where low Na, high K ghosts were washed and incubated in isotonic Na or choline medium, the concentration of K in the media after 15 min were compared. The mean values and S.E. obtained (mM-K) were 0.81 ± 0.13 in the Na medium and 0.39 ± 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 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.

Osmotic fragility

Osmotic fragility was assessed in three experiments

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.

by measuring the extent of haemolysis when isotonically resealed ghosts were added to a series of hypotonic saline solutions, When the percentage haemolysis was plotted against the concentration of external NaCl, a typical sigmoid curve was obtained indicating that the ghosts in the population possessed different resistances to osmotic haemolysis (Fig. 5). Since in the intact erythrocyte, one of the most important factors influencing osmotic fragility is cell shape (Prankerd, 1960), the lack of uniformity in osmotic behaviour observed with ghosts may similarly reflect differences in cell volume. This view is supported by the findings of Ponder and Barreto (1957) who showed that there was considerable variation in the shape and volume of reconstituted ghosts prepared under standard conditions. A single population of ghosts was found to contain an entire spectrum of cell shapes ranging from biconcave discs through spheres to crenated forms.

Homogeneity

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

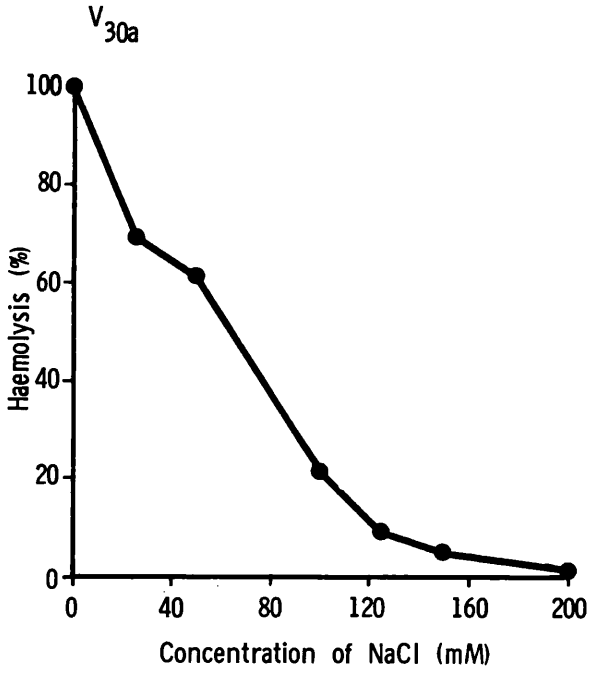

FIG.5 . Osmotic fragility of resealed ghosts. Low Na, high K ghosts were prepared as described in Fig. 4. Resealing was completed by incubating for 30 min at 37°C. The harvested ghosts were washed twice in iced medium containing (mM): Na, 142; Mg, 7; Cl, 161; Tris (pH 7.4), 5; iodoacetate, 0.2; fluoride, 2. 0.5 ml. packed ghosts were added to a series of stoppered glass tubes containing 10 ml. of suitable dilutions of medium containing (mM): Na, 140; Cl, 140; Tris phosphate (pH 7.4), 10. The tubes were inverted and allowed to stand for 10 min at 20°C. They were then remixed gently and centrifuged for 1 min at 18,000 g. The amount of haemolysis in each tube was compared with 100% lysis tube using OD at 540 mμ.

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 richest in K and haemoglobin (Fig. 6). 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 are smaller. This latter explanation agrees with some of the observations obtained from differential centrifu-

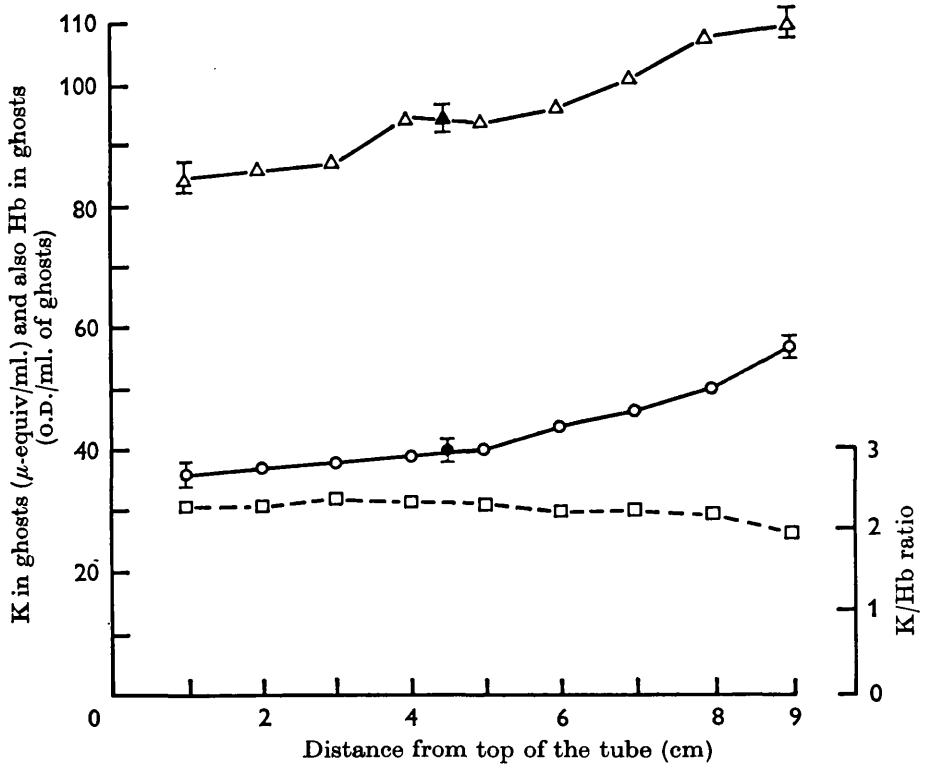

Fig. 6. K and Hb concentrations at different heights of columns of packed ghosts. Low Na, high K, isotonically resealed ghosts, sealed in a medium containing (mM): ATP, 1; P_i, 1; K, 140; Mg, 7; Cl, 149; Na, 2; iodoacetate, 0·2; fluoride, 2. The resealed ghosts were incubated at 37° C for 30 min and 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.

gation of intact red cells (Prentice and Bishop, 1965; Piomelli, Lurinsky and Wasserman, 1967) (see p. 105). A similar pattern of inhomogeneity in reconstituted ghosts to that described here was noted by Hoffman (1958a), 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.

Inhibition of Glycolytic Pathways

The red cell membrane contains a complement of tightly bound glycolytic enzymes of which triose phosphate dehydrogenase and phosphoglycerol kinase are present in highest concentration (Schrier and Doak, 1963; Schrier, 1966). In order to prevent any

phosphate incorporation into ATP by glycolytic pathways independent of the Na pump, low concentrations of iodoacetate and fluoride were included in the lysing, washing and incubating fluids. The respective sites of action of these inhibitors are upon the triose phosphate dehydrogenase and enolase reactions of glycolysis (London, 1961; Bishop, 1964) (Fig. 7). Initially, a concentration of 2 mM-iodoacetate was used, but this led to leakiness of ghosts. A concentration of 0.2 mM-iodoacetate was finally used together with 2 mM-fluoride as this combination was found to block the production of lactate from inosine (Table 2) whilst leaving membrane permeability to cations unaffected (Garrahan and Glynn, 1967d; Whittam, 1968).

Incorporation of $^{32}P_i$ into ATP

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 was investigated was whether such labelling was related to the amount of Na and K in the external

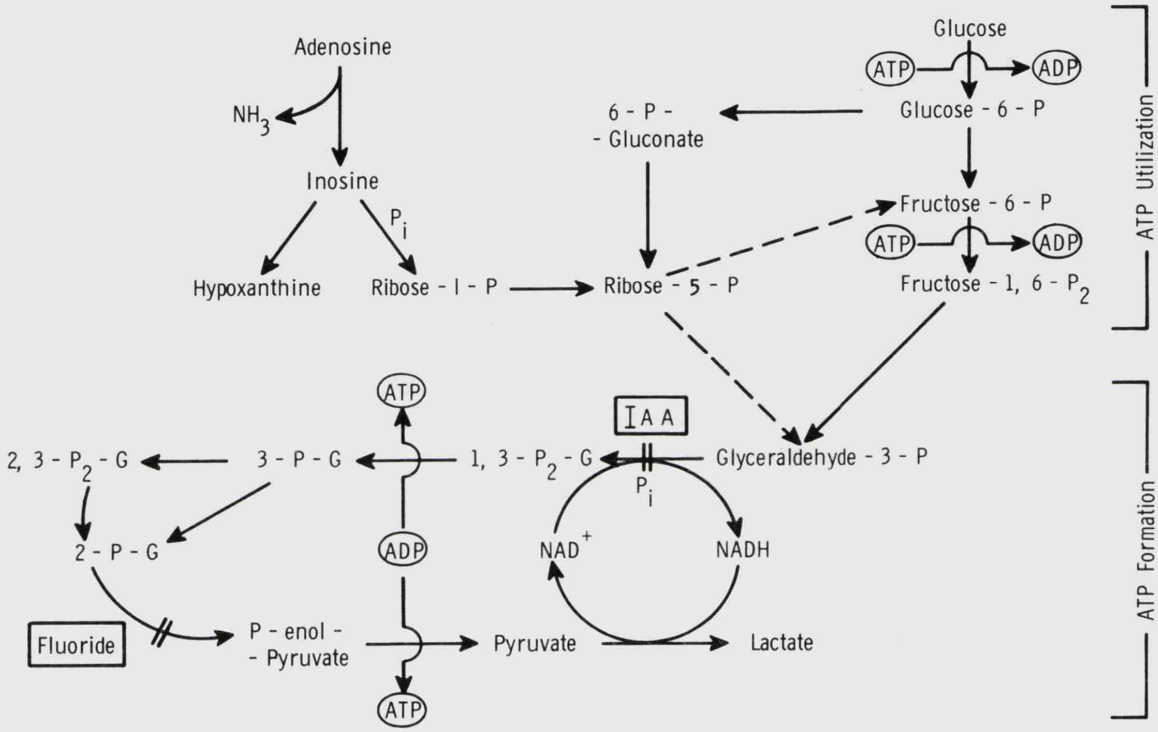

FIG. 7. Pathways of glucose and purine nucleoside utilisation by the red cell. For each two molecules of lactate produced, four molecules of ATP are produced from ADP. However, since two are needed for phosphorylation of glucose and fructose 6-phosphate, there is a net synthesis of only two molecules of ATP. The points of inhibitory action of IAA upon triose phosphate dehydrogenase, and of fluoride upon enolase are also shown.

1,3 - P_2 - G = 1,3 diphosphoglycerate; 3 - P - G = 3 - phosphoglycerate;

2,3 - P_2 - G = 2,3 diphosphoglycerate; 2 - P - G = 2 - phosphoglycerate.

TABLE 2. Effect of iodoacetate and fluoride on production of lactate from inosine

Composition of medium				Lactate concentration		Lactate production
Main cation	inosine	IAA	F	before incubation	after incubation	
	mM			μ-mole/ml.		μ-mole/ml.cells/hr
	10	0.2	2			
	-	-	-	6.5	9.9	3.4
Na	+	-	-	7.5	12.6	5.1
	-	+	+	6.9	6.7	-
Na	+	+	+	7.1	7.1	-

Expt. V^{34}. 3 week old cells were washed 3 times in an iced medium containing (mM): Na, 140; Cl, 154; Tris (pH 7.60 at 24°C) 10; Mg, 2. The washed cells were then spun at 2700 g for 8 min at 24°C. A series of flasks were prepared containing 10 ml. of incubation medium at 37°C. 2 ml. packed cells were added and the flasks shaken gently in a water bath. The incubation medium contained (mM): Na, 135; Mg, 2; Cl, 139; Tris phosphate (pH 7.40 at 37°C), 10. Where required, 10 mM-inosine, 0.2 mM-iodoacetate and 2 mM -fluoride were included. Incubation time: 1 hr. Samples were deproteinised with $HClO_4$ for estimation of lactate at the start and end of incubation.

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 enzyme 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. 8). 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 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. 8). In three experiments, with the same type of low Na, high K ghosts, both techniques of analysis were employed simultaneously (Table 3). 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

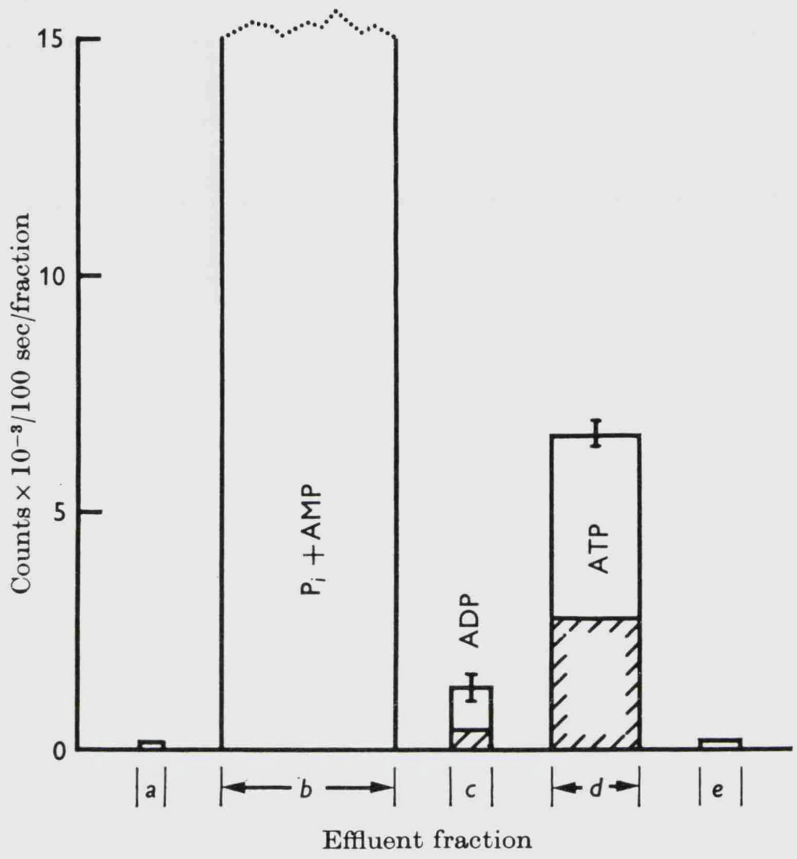

Fig. 8. Elution chart of P_i, ADP and ATP on Dowex columns. Extracts were from an experiment (II[8]) where low Na, high K ghosts were incubated for 15 min in a high Na, K-free medium and in an all K medium.

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 %.

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 Exp. III[3] of Table 3, 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

TABLE 3. 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 High K	Column	Na	85,000	1532	1·802	1·95
			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 High K	Column	Na	63,000	1075	1·706	1·44
			K	63,600	756	1·188	
		Extraction	Na	66,600	1430	2·147	1·33
			K	67,200	1084	1·613	
	High Na Low K	Column	Na	78,000	765	0·980	1·11
			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 High K	Column	Na	89,000	925	1·039	1·41
			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.

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 4). 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 4, 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 operation of the Na pump affected the labelling, ouabain (10-50 μM) was added. The incorporation was not sufficiently changed with either 10 or 150 mM-K in the medium (Table 4). Ouabain was also added to the high Na, K-free medium to see if it decreased the labelling as described by Garrahan and Glynn (1967d). Addition of

TABLE 4. 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[5]	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 3.

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 4).

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 4), 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 and 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, p. 79).

Absolute magnitude of labelling

In some experiments 1 mM P_i was added instead of the usual 5 mM (Table 5). 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 5).

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 hypotonic 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 5). The results suggest

TABLE 5. 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			
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	Activity of Na pump: Forwards	Activity of Na pump: 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 3). 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.

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, p.41). 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 choline ghosts, low in both Na and K (see Table 1, p.41), 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 6). 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 7 summarises 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,~~

TABLE 6. 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	Na+K	337,000	7952	2·359	95	15·5
	High choline						
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	Na+K	241,900	2915 (621)	1·205	73	7·8
	½ choline						
	Low Na	Na	203,700	2994	1·469	100	8·0
	Low K	Na+K	207,600	2899	1·396	95	7·7
	High choline						

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.

TABLE 7. 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 %)		Ionic gradients present
	Medium		
	Na + ouabain	K	
Low Na	−30	—	$[Na]_e > [Na]_i$; $[K]_i > [K]_e$, pump inhibited
High K	—	−39	Na and K gradients abolished
Low Na	+6	—	$[Na]_e > [Na]_i$; no K gradient, pump inhibited
Low K High choline	—	+6	No Na gradient; $[K] > [K]_i$
High Na	−3	—	Na and K gradients abolished, pump inhibited
Low K	—	−6	$[Na]_i > [Na]_e$; $[K]_e > [K]_i$

Ghosts were prepared and incubated as in Table 6. 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.

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.

Use of Mg as replacement for Na or K

In five experiments, a K-free isotonic Mg medium was used as a replacement for Na Ringer. The total radioactivity in high K, low Na ghosts at the end of 15 min incubation in Mg medium was found to be decreased by a variable amount ranging from 40 - 65% of the counts remaining in the same ghosts after incubation in Na medium. This may have been due to an increased leakiness of the ghosts in the presence of external Mg, an explanation supported by measurements of net K loss in the two situations. K loss (μ-equiv/ml. ghost/min)

from the same batch of low Na, high K ghosts amounted to 2.52 as compared with 1.06 after incubation for 15 min in Mg and Na Ringers respectively (Expt. III[17]). There was no evidence of rehaemolysis in the Mg medium. The variable loss of total radioactivity from the ghosts prevented valid assessment of the extent of $^{32}P_i$ incorporation compared with Na media, since it was conceivable that the observed values for nucleotide-bound counts might also be spuriously low. A similar difficulty was encountered when attempts were made to use Mg as a substitute for internal K by producing Mg ghosts. In a paired experiment, the behaviour of low Na, high K ghosts was compared with that of low Na, low K (high Mg) ghosts made from the same lot of cells (Expt.III[20]). The Na and K contents (μ-equiv/ml.) of the two types of ghost were 8 and 103 as compared with 9 and 6 respectively. However, after incubation in Na Ringer for 15 min, there was a variable decrease in total radioactivity in the Mg-containing ghosts in some cases to as low as 36% of the initial counts. This made it impossible to compare satisfactorily the extent of $^{32}P_i$ incorporation under different conditions of incubation.

It appeared from these findings that under the particular experimental conditions used, the red cell membrane was behaving in an anomalous manner when exposed to either external or internal Mg in high concentration, thereby rendering this cation unsuitable as an alternative replacement for external Na or internal K. Isotonic Mg solutions have been used extensively in studies with intact red cells where Mg^{2+} was required to replace external Na or K.(Ponder, 1953; Tosteson and Hoffman, 1960; Hoffman, 1966). No harmful effects on cell behaviour of such substitutes have been noted. However, whereas Mg does not penetrate the intact cell membrane, this may not hold true for the more permeable ghost membrane. Entry of Mg may be associated with formation of a complex with P_i inside the ghost. Changes in membrane permeability are known to be produced in resealed ghosts by alkaline earth metals especially in the presence of fluoride (Passow, 1964; Lepke and Passow, 1968). However, concentration levels of fluoride below 5 mM, though effective in blocking glycolysis, do not usually affect membrane permeability to cations. (Maizels, 1954; Gárdos and Straub, 1957; Whittam, 1968). The

effects of Mg on the cell membrane may also be concentration dependent. Hoffman (1962b) has presented evidence that Mg in small concentration (2-3mM) acts as an internal cohesive stabilising the membrane and regulating its permeability to monovalent cations. The present results suggest that at concentration levels of 100 mM, and in the presence of 10 mM-P_i 2 mM-fluoride and 0.2 mM-iodoacetate, Mg may have a deleterious effect on the membrane.

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 the 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 8 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

TABLE 8. 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 3.

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. 9). 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 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

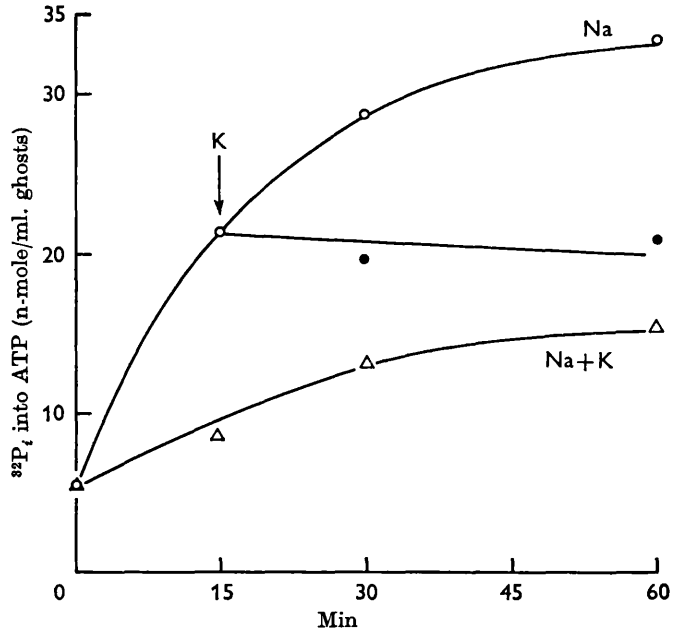

Fig. 9. The incorporation of $^{32}P_i$ into energy-rich phosphate by low Na, high K ghosts as a function of time. The effect of adding 10 mM-K to the all Na, K-free medium, during the course of incubation, was also investigated. The ghosts were sealed in a medium containing (mM): ATP 1; $^{32}P_i$, 1; K, 142; Na, 2; Mg, 7; Cl, 154; iodoacetate, 0·2; fluoride, 2. The medium contained (mM): Na, 142; Mg, 7 ; 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.

rate of 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 $\pm$ S.E. of mean) was 0.72$\pm$ 0.11 (6) with 1 mM $^{32}P_i$ in the lysing fluid, and 1.00$\pm$ 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.

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

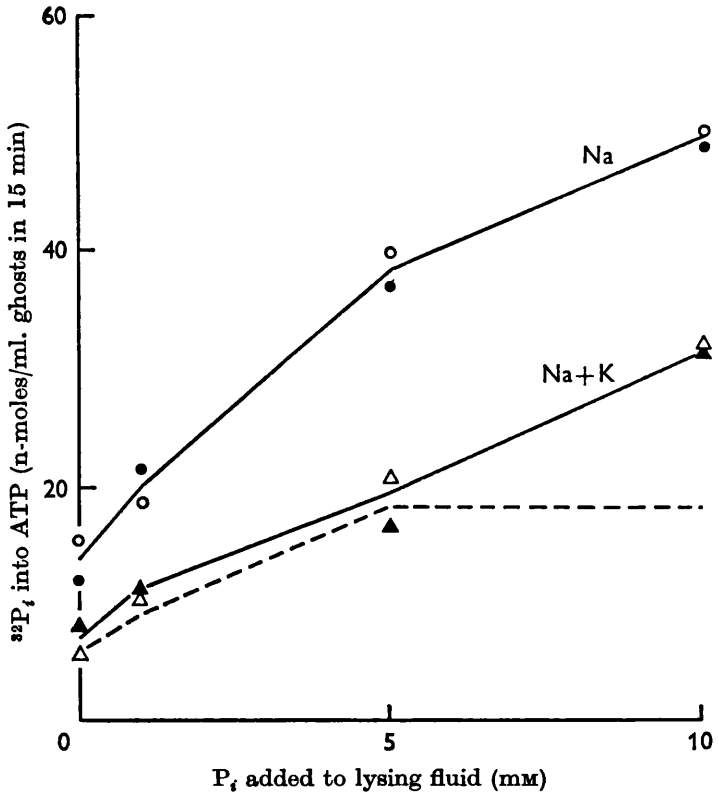

Fig. 10. The effect of increasing internal P_i concentration on $^{32}P_i$ incorporation into energy-rich phosphate by low Na, high K ghosts.

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.

Na medium in the absence and presence of external potassium is shown in Fig. 10. 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 $^{32}P_i$ into ADP and ATP, whilst 10 mM P_i did not lead to a further increase.

DISCUSSION

The results of the present study are in agreement with the finding of Garrahan and Glynn (1967d) that when suitable gradients are imposed across the membranes of red cell ghosts so as to permit downhill movements of Na and K, there is an ouabain-sensitive incorporation of $^{32}P_i$ into energy-rich phosphate. These observations have been extended by independently varying the ionic composition of the internal and external fluids bathing the membrane. 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 the system cannot work optimally in both directions at once. The overall reaction involving transport ATPase may be shown as in Fig. 11. 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,

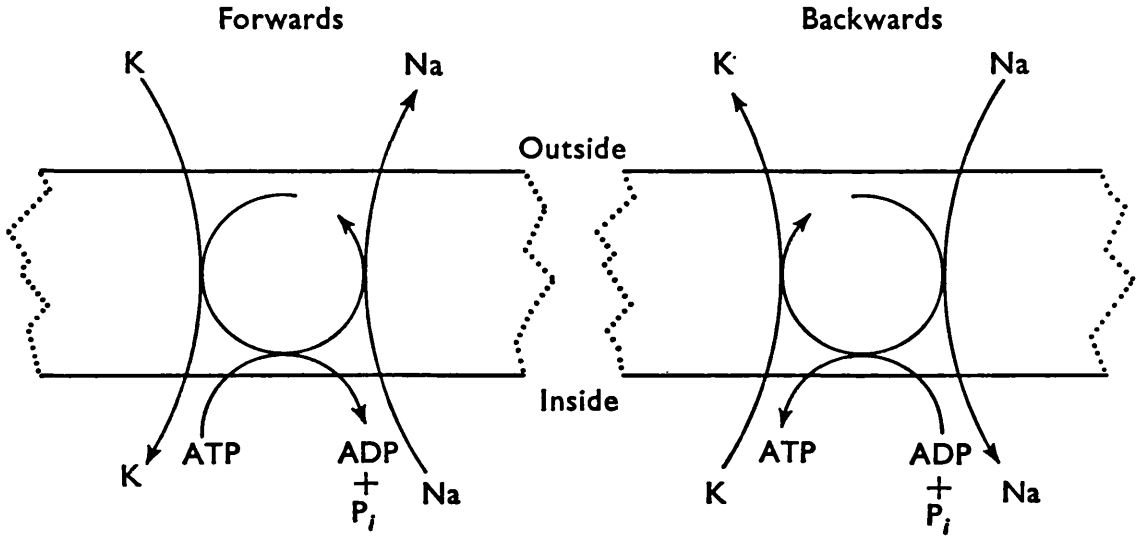

Fig.11. Model to illustrate forward and backward reactions of the Na pump.

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 and Glynn (1967d).

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 and Ager, 1964). The 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.

A drawback of the present incorporation studies is that net synthesis of ATP was not measured. It is not likely to be great, first, in view of the activity of adenylate kinase (Overgaard-Hansen, 1957; Kashket and Denstedt, 1958) which catalyzes the reaction:-

$$AMP + ATP \overset{Mg^{2+}}{\rightleftharpoons} 2ADP$$

and second, because of the low rate of labelling of ATP in relation to simultaneous ATP splitting (see p.150). 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 small amounts of iodoacetate and fluoride were included in the lysing fluid, washing and incubating media throughout all experiments. The low concentration of inhibitors did not affect membrane permeability to cations, but did block the production of lactate from inosine.

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 persisting activity of glycolytic enzymes known to be bound firmly within the red cell membrane. These enzymes catalyze 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 and 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 and Williams, 1966; Whittam and Wiley, 1968). Exchange reactions are known in which a complex molecule becomes labelled by a component, and the enzyme needed is the one which catalyzes net synthesis (Webster and Varner, 1954; Snoke and Bloch, 1955; de Verdier, 1963). 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 (Skou, 1960, 1965; see General Discussion, p.162). 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 breakdown must both be reversible. Energy made available by the hydrolysis of ATP supports ion

movements. From the evidence presented in this section, the opposite form of energy transformation now appears likely - namely that an increase in chemical energy can be derived from the dissipation of cation concentration gradients. It is concluded that a chemical reaction or series of reactions which result in the incorporation of P_i into ATP can be driven by a coupled flow of Na and K downhill. Studies of the relative magnitude and other characteristics of these ion movements are described in the following section.

SUMMARY

1. A study has been made of the labelling of ATP with [^{32}P] o-phosphate ($^{32}P_i$) in ghosts of human red cells. The ionic compositions of ghosts and suspending Ringer solutions have been varied independently. Measurements were made of the incorporation of $^{32}P_i$ 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.

PART TWO

DOWNHILL ION MOVEMENTS AND THEIR RELATION TO BACKWARD RUNNING OF THE Na PUMP

INTRODUCTION

The finding that ouabain sensitive incorporation of P_i into ATP required the simultaneous presence of gradients of Na and K across the red cell membrane raises the question whether there might not also be a coupling of downhill movements of these ions (Na and K) involved in backward running of the Na pump. It is well established that there is a stoichiometric dependence between the extrusion of Na and influx of K which is characteristic of the forward running of the Na pump (Sen and Post, 1964; Whittam and Ager, 1965; Garrahan and Glynn, 1967c). Based upon the original hypotheses of Ussing (1949a and b), cations can be considered to traverse the cell membrane by

three pathways, a) "uphill" against a gradient of electro-chemical potential (active transport through the Na pump) b) "downhill" with the gradient (passive diffusion or "leak") and c) exchange diffusion where there is a 1:1 exchange of Na for Na and K for K. Net movements of ions can only occur through the pump and leak pathways. The interrelationship between the activity of "pump" and the size of "leak" determines the control of ion composition of the cell in the steady state and regulates its volume (Tosteson and Hoffman, 1960; Post, Albright and Dayani, 1967; Robinson, 1968). Ussing (1949b) derived the following mathematical relationship to describe the independent movements of ions across a membrane under the sole influence of the concentration gradient and electro-chemical potential gradient:-

$$\frac{i_{M_A}}{o_{M_A}} = \frac{[A_e]}{[A_i]} \cdot \exp^{zFE/RT}$$

where the left hand function represents the ratio of the inward to outward fluxes, $[A_e]$ and $[A_i]$ are the concentrations of the ion in question in the

outside and inside solutions, respectively, z equals the valency and the remaining symbols have their usual meaning (see glossary, p. 89). The activity coefficients on the inside and outside are assumed to be equal.

The leak pathway for K efflux and Na influx would be expected to obey this relationship for the ratio of tracer fluxes across the membrane. However, Shaw, (1955) with horse, and Glynn (1956) with human red cells, found that although there was a component of K influx which showed a linear relationship between flux and external K concentration as would be expected of a diffusional flux, its magnitude was less than half as large as the value predicted from measurements of K efflux. It thus seemed likely that part of the passive (downhill) movement of cations was more complicated than could be accounted for by a simple diffusion pathway. Further indications that this was so came from the observations that cardiac glycosides exert an inhibitory effect both on K efflux and Na influx in human red cells (Glynn, 1957). Inhibition of K efflux by glycosides has also been reported in cardiac (Tuttle,

Witt and Farah, 1962) and skeletal (Schatzmann and Witt, 1954) muscle.

In this section kinetic studies of sodium uptake and potassium loss have been undertaken with the aid of radioactive tracers to see whether there is an interdependence of downhill ion movements sensitive to ouabain and comparable to the coupling (see Harris, 1954) which is characteristic of active transport through the Na pump.

GLOSSARY OF SYMBOLS USED IN PART TWO

A cation

E electrical potential across the membrane

F the Faraday constant

H haematocrit

k rate constant

M unidirectional flux

superscripts to left i = influx; o = efflux

to right c = cells; s = supernatant

subscripts to right indicate:

1. ion concerned, Na or K
2. time relations, o or t
3. internal (i) or external (e)

e.g. ${}^{o}M_{K}$ = K efflux; ${}^{i}M_{Na}$ = Na influx

N radioactivity or counts /100 sec

R the gas constant [1.987 cal/mole/degree]

sp. a. specific activity

T absolute temperature (^{o}K)

t time

z valency

METHODS

Procedure

3-4 week old human blood in acid-citrate-dextrose was kindly supplied in part by the Sheffield Regional Transfusion Service. Fresh blood was obtained under sterile conditions from the antecubital vein of volunteers and heparinized immediately (approx. 2000 units heparin/ml. blood). The erythrocytes were separated from plasma and the buffy coat by centrifugation at 1500g for 8 min., and were then washed 3-4 times by repeated suspension (1:10) in isotonic media at 5°C. In some experiments, the cells were initially washed once in a hypotonic medium (0.1 M-NaCl or KCl) to remove excessively fragile cells. Subsequent washes in isotonic medium were continued until the supernatant was free of haemoglobin.

Isotonically resealed ghosts

Erythrocyte ghosts, rich in K and low in Na, were prepared as described in Part One.

Flux measurements with tracers

K efflux was measured by loading ghosts or cells with ^{42}K and following the time course of loss of isotope

to non-radioactive incubation media. Isotope loading of ghosts was achieved by including ^{42}K in the lysing solution at the time when the washed packed cells were added. The ghosts were then sealed, washed and harvested as outlined previously. In cells, a pre-incubation (at a haematocrit of 3-4%) for 7-12 hr at 37°C was undertaken in a Na-free, K Ringer solution (concentrations (mM) : K, 140; Tris phosphate, (pH 7.6), 10; Cl, 140; inosine, 10; adenine 5), in order to lower internal Na and raise internal K content of the cells. The cells were then centrifuged at 2700g for 5 min., washed twice (1:20) in ice-cold K-Ringer without substrate and resuspended in an equal volume of 140 mM-KCl; 10 mM Tris-phosphate (pH 7.6) to which ^{42}K had been added. After incubating at 37°C for 6 hr with gentle shaking, the cells were centrifuged and washed 4 times (1:20) in the same non-radioactive medium (ice-cold) in which they were subsequently to be incubated.

Flasks containing suitable media were pre-warmed to 37°C in a water bath and at the start of the experiment, 1-2 ml. of radioactive washed ghosts or cells were added to each flask. Ouabain was sometimes added

to give a final concentration of 50 μM. Immediately after mixing, a portion of the suspension was withdrawn (zero time sample) and the remainder was incubated with shaking. At appropriate time intervals, usually after 15, 30, 60, 90 and in some cases 120 min., approx. 3ml. samples were withdrawn from the flasks through holes in the stoppers.

Samples were rapidly transferred to small Pyrex centrifuge tubes immersed in an ice-bath. After cooling for 2 min., the tubes were spun at 2700g for 3 min., with ghosts, and for 1 min with cells. The radioactivity (N^s), optical density (540mμ) and potassium concentration were measured in 2 ml. samples of the supernatants. Total radioactivity $[(N^c)_o]$ and the haematocrit (H) were determined on the cell suspensions at zero time.

The radioactivity initially present in the volume of ghosts or cells associated with 2 ml. of supernatant $[(N^c)_o{}^{corr}]$ was calculated from the relationship:

$$(N^c)_o{}^{corr} = (N^c)_o\left[\frac{100}{100 - H}\right]$$

The fraction of counts which remained inside the

ghosts or cells at each time interval $\left[1 - \frac{N^s}{(N^c)_o}\text{corr}\right]$ was plotted on a logarithmic scale against time, and the best fitting straight lines were obtained by the method of least squares. The slope, b, (in hr^{-1}) was multiplied by the K content of ghosts or cells to obtain the K efflux (in μ-equiv/ml./hr). The ghost or cell K concentrations were determined at the start and end of the incubation and the mean value used.

Na influx in cells

Cells were prepared as for efflux experiments and ^{24}Na was added at the start of the incubation at 37°C for 1 hr. The cell suspensions were centrifuged for 2 min at 2700g and the cells then washed quickly 3 times with at least 50 volumes of ice-cold unlabelled K-free, Na Ringer and lysed in a small volume of 7 mM-NH_4OH. Radioactivity and absorbance at 540 mμ were determined. Na influx (in μ-equiv/ml.cells/hr) was calculated from the relationship:

$$i_{M_{Na}} = \frac{N^c}{N^s} \cdot \frac{[Na_e]}{t}$$

where N^c and N^s are the relative radioactivities in the cells and suspending medium respectively, $[Na_e]$ is the external sodium concentration and t is the duration of the experiment.

Na influx into ghosts

In two experiments (V^{28} and V^{29}), the method was modified so as to measure Na influx into resealed ghosts, rich in K, low in Na. Isotonically resealed ghosts were prepared as described on p. 20 . Packed ghosts were suspended in K-free, ^{24}Na Ringer at a haematocrit of approx. 1%. Flasks were incubated at 37°C for 30 min., at the end of which the ghosts were spun down, washed twice (1:50) in iced unlabelled medium and lysed in a convenient volume of 7 mM-NH_4OH for measurement of radioactivity and absorbance at 540 mμ. The results of both experiments were similar in showing high influx values ranging from 22.47 to 34.72 μ-equiv/ml.ghosts/hr; no significant inhibition by ouabain was detected. In Expt. V^{29}, Na influx (μ-equiv/ml.ghosts/hr) equalled 27.77 ± 2.94 (Na medium) and 25.97 ± 1.08 (Na medium with 50 μM-ouabain) (mean ± S.E. of mean; n = 3). Employing the observed values of Na influx, it was

possible to calculate the half-time ($t_{\frac{1}{2}}$) for isotopic exchange between medium and the ghosts, assuming that ${}^{i}M_{Na} = {}^{o}M_{Na}$

Since ${}^{i}M_{Na} = k_i [Na_e]$ where k_i = inward rate constant

$$\text{Range of } k_i = \frac{22.47}{142}(0.158) \text{ to } \frac{34.72}{142}(0.245)$$

For efflux, $k_o = {}^{o}M_{Na} / [Na_i]$

$N_t = N_o \exp^{-k_o t}$ At $t_{\frac{1}{2}}$, $N_t = \frac{N_o}{2}$

$$\therefore \quad t_{\frac{1}{2}} = \frac{\ln 2}{k_o} = \frac{2.30 \cdot \log_{10} 2}{k_o} = 2.8 - 4.3 \text{ hr}$$

This short half-time indicates a rapid rate of exchange of ^{24}Na across the ghost membrane and ~~stands in marked contrast to~~ agrees with values of the order of 20-28 hr obtained by Harris and Maizels (1951) and Solomon (1952) in intact cells. With Na influx of such magnitude in ghosts, downhill ion movements via "leak" pathways would inevitably mask any smaller changes occurring through the pump mechanism and prevent satisfactory characterisation of the latter.

Measurement of radioactivity

^{42}K and ^{24}Na were measured with a transistorised Ecko well-scintillated counter (M5402), used in conjunction with an automatic sample changer (N723) (Ecko Electronics, Ltd., Southend-on-Sea). At least 10,000 counts were usually measured.

Haematocrit determinations were made by spinning cell suspensions for 30 min at 2700g in Wintrobe tubes. With ghost suspensions, centrifuging was done at 5°C for 1 min., at 15,000g in an MSE 'High Speed 17' centrifuge (Measuring and Scientific Equipment, Ltd., London. S.W.1). Parallel determination of the optical density at 540 mμ of suspensions of ghosts and of packed ghosts were always made to obtain an independent measure of the haematocrit of ghost suspensions.

Net Na and K movements

Measurements were made of the net movements of Na into and K out of red cells incubated in a K-free, Na Ringer. The cells were prepared and usually pre-incubated in an all-K Ringer as for tracer flux experiments. The cells were then washed twice in 140 mM-NaCl; 10 mM Tris-phosphate (pH 7.6) and resuspended

in K-free, Na Ringer at a haematocrit of approx. 0.3 - 0.4% (0.5 ml. cells in 150 ml. medium). Suspensions were gently shaken in a water bath at 37°C. At specified times, usually, 1, 2 and 4 hr., 50 ml. samples of suspension were removed, and were replaced by 50 ml. pre-warmed medium to dilute external K. In early experiments, the cells were then separated and washed twice in iced 140 mM-choline chloride; 10 mM-Tris-phosphate (pH 7.6) prior to lysing in 7 mM-NH_4OH and analysis. Using this procedure, the Na content of the cells was found to fall with time (Fig. 12). In the absence of significant cell lysis, this anomalous effect could be explained by the entry of choline during the time taken for washing the cells. The procedure was therefore modified so as to eliminate the washing stage. Samples of suspension were centrifuged directly in Wintrobe tubes at 2700g for 25 min. Residual medium was sucked off carefully down to the upper face of the column of packed cells. With a long Pasteur pipette, the entire column of cells was then removed, mixed thoroughly, lysed in 7 mM-NH_4OH and the haemolysate diluted further with distilled water for analysis. Using this technique, the precision

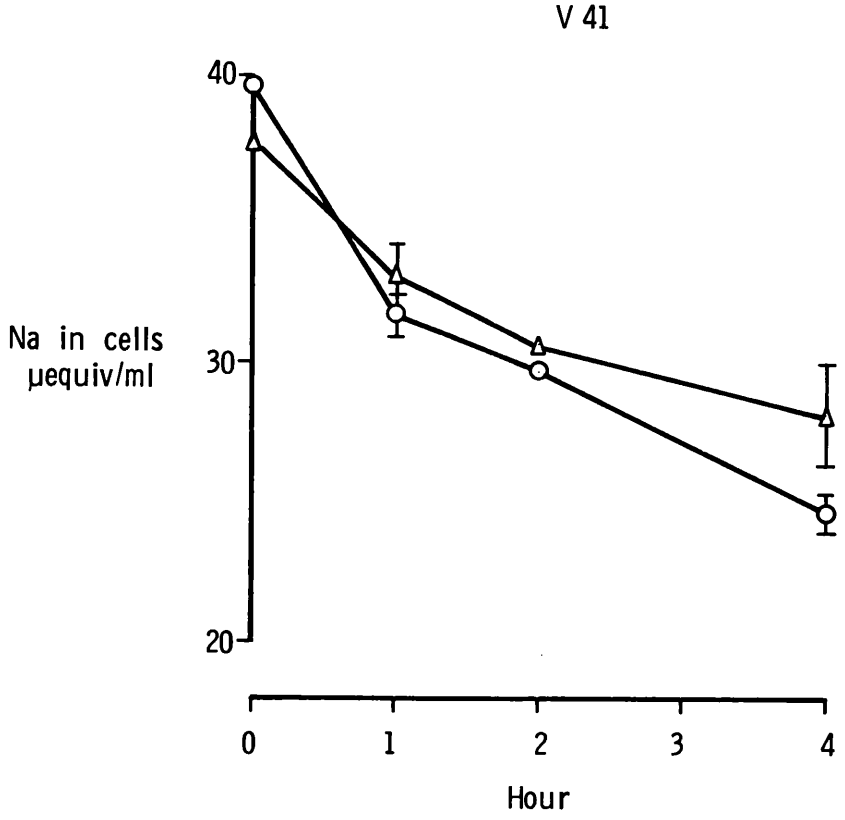

FIG. 12. The Na content of intact red cells incubated for 4 hr (haematocrit approx. 1% at start) in a K-free Na Ringer containing (mM): Na, 142; Mg, 7; Cl, 156; Tris phosphate (pH 7.4), 5; iodoacetate, 0.2; fluoride, 2. The results with two different batches of red cells are shown (O , 23 day old; Δ , 39 day old). At 1, 2 and 4 hr, 50 ml. of suspension was removed and centrifuged at 2700 g for 2 min. 50 ml. Na Ringer (pre-warmed to 37°C) was replaced at 1 and 2 hr. The cells were washed twice in iced 140 mM-choline chloride; 10 mM-Tris phosphate (pH 7.6 at 5°C) prior to sampling for cation and Hb content. The K concentration in the originally K-free medium at the end of 4 hr incubation was similar in both experiments and amounted to 80-86μM. Mean values of incubations carried out in triplicate are shown; the vertical bars at 1 and 4 hr represent the S.E. of the mean.

of values for cell cation content based on the S.D. of 7 duplicates taken at zero time in one experiment (V^{46}) was within $\pm$0.54% (for Na) and $\pm$0.82% (for K) of the mean.

Chemical analytical methods

Haemoglobin (Hb) was estimated as oxyhaemoglobin from the extraction at 540 mμ of suitably diluted samples clarified with 7 mM-NH_4OH as described in Part One.

Na and K These elements were measured either by an EEL flame photometer (Evans Electro-Selenium, Ltd., Halstead) or by a Unicam atomic absorption spectrophotometer (SP 90) (Unicam Instruments, Ltd.,Cambridge). Samples were prepared and estimated as outlined in Part One.

MATERIALS

^{24}Na and ^{42}K were obtained as sterile isotonic solutions of NaCl (ref. SGS.1P) or KCl (ref. PES.1P) from the Radiochemical Centre, Amersham. Heparin injection, BP was obtained from Boots Pure Drug Co., Nottingham. Remaining chemicals were of Analar grade, wherever possible, and all solutions were made up in glass-distilled water.

Theoretical consideration of cation fluxes and their calculation

Derivation of efflux formula

The rate of change of the internal concentration of ion A as a function of time:

$$\frac{d[A_i]}{dt} = k_i [A_e] - k_2 [A_i]$$

where k_i = inward, k_2 = outward } rate constants

Since the cells are incubated at very low haematocrit the backflux of label can be neglected.

$$\therefore \quad \frac{d[A_i]}{dt} = -k_2 [A_i]$$

At time t, the concentration of $[A_i]$ will equal $[A_i]_t$

$$\text{and} \quad \frac{d[A_i]_t}{dt} = -k_2 [A_i]_t$$

Rearranging:

$$dt = \frac{d[A_i]_t}{-k_2 [A_i]_t}$$

Integrating,

$$t = \int -\frac{d[A_i]_t}{k_2 [A_i]_t} = -\frac{1}{k_2} \ln [A_i]_t + \text{constant } C$$

The constant in this equation can be obtained by applying the equation to the situation at the start of the experiment when $t = o$, $[A_i]_t = [A_i]_o$

$$\therefore \quad k_2 \cdot \text{constant } C = k_2 t + \ln [A_i]_o$$

$$\therefore \quad C = \frac{\ln [A_i]_o}{k_2}$$

$$t = -\frac{1}{k_2} \ln [A_i]_t + \frac{1}{k_2} \ln [A_i]_o$$

$$k_2 t = \ln \left[\frac{[A_i]_o}{[A_i]_t} \right] = -\ln \left[\frac{[A_i]_t}{[A_i]_o} \right]$$

If we substitute radioactivity for concentrations:

$(N^c)_o$ = radioactivity in cells/ghosts at beginning of incubation (zero time)

$(N^c)_t$ = radioactivity remaining in cells/ghosts at various times

$$k_2 t = -\ln \left[\frac{[N^c]_t}{[N^c]_o} \right]$$

A measure of loss of isotope from the cells/ghosts is obtained from the supernatant at various times.

$(N^s)_t$ = radioactivity in 2 ml. of cell-free supernatants at various times.

In a two compartment system where the exchange of radioactivity between intra- and extra-cellular compartments is following exponential kinetics, the total radioactivity at any given time must be made up of the constituent cellular and supernatant fractions. In order to relate the radioactivity in the supernatant to a fixed volume of cells, the initial cell radioactivity, $\left[(N^c)_o\right]$, is corrected for a volume of cells associated with 2 ml. supernatant, $\left[(N^c)_o{}^{corr}\right]$.

$$k_2 t = -\ln\left[\frac{\left[N^c\right]_o^{corr} - \left[N^s\right]_t}{\left[N^c\right]_o^{corr}}\right] = -\ln\left[1-\frac{\left[N^s\right]_t}{\left[N^c\right]_o^{corr}}\right]$$

where the logarithmic function equals the fraction of original radioactivity remaining in the cells, and

$$\left[N^c\right]_o^{corr} = \left[N^c\right]_o \times \frac{100}{100 - H}$$

Where H = haematocrit of flasks

Derivation of influx formula

Influx was calculated from the following relationship:-

$$i_{M_A} = \frac{N^c}{\overline{sp.a.}^s \times t} = \frac{N^c \times [\bar{A}^s]}{\bar{N}^s \times t}$$

Where A = cation in question, in this case Na

N^c = radioactivity of unit volume of cells (counts/100 sec/ml.cells)

$\overline{sp.a.}^s$ = mean specific activity of supernatant or medium (counts/100 sec/μ-equiv.A)

This was calculated from $\frac{\bar{N}^s}{\bar{A}^s}$ where

$\bar{N}^s$ = mean radioactivity of supernatant (counts/100sec/ml.)

$\bar{A}^s$ = Mean cation content of supernatant (μ-equiv/ml.)

Use of this relationship was based on the following main assumptions:

a) the specific activity of the medium remained constant during the period of measurement

b) the effect of backflux of isotope from cells to medium could be ignored

c) the value of N^c was of negligible magnitude compared with the total counts originally in the (extracellular fluid) medium

These assumptions are justifiable only for experiments of short duration and only at the very low haematocrit levels used where the extracellular compartment was over 20 times larger than the intracellular compartment. In order to minimise errors due to small variations in extracellular specific activity the means of at least ten values of A^s and N^s were used to obtain the mean specific activity of the medium ($\overline{sp.a.}^s$) in each experiment.

RESULTS

Homogeneity of cells

Because of its importance in relation to radioactive tracer uptake and release of cations, the homogeneity of populations of red cells from the same individual was studied in two experiments. Samples were taken at descending levels from columns of packed red cells in haematocrit tubes. It was found that haemoglobin, Na and K were not uniformly distributed. Cells in the upper third of each column contained less Hb but were richest in Na and K (Fig. 13). The mean haemoglobin concentration increased from top to bottom of the column by 12%; Na and K concentrations fell from top to bottom by 42 and 14% respectively. The large difference in Na content may be anomalous and due to the unusually high Na value marked ↙ in Fig. 13. The latter may represent contamination by residual incubation medium, since these cells appear to have lost Na after incubation for 2 hr in an all-Na medium and the mean value for unmixed cells in this case does not reflect a component of cell Na of such a high order. The pattern of distribution of Hb and cations persisted

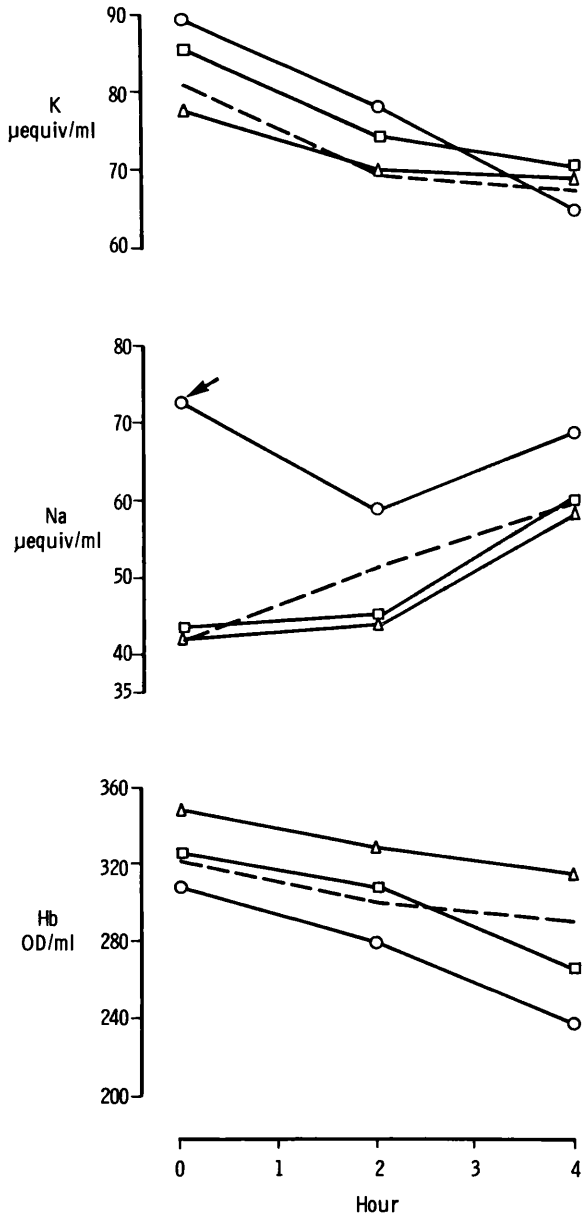

FIG. 13. K, Na and Hb concentrations at top (○), middle (□) and bottom (△) thirds of columns of packed cells. 17 day old red cells were washed four times in iced 140 mM-NaCl; 10 mM-Tris phosphate (pH 7.6 at 5°C). 0.5 ml. packed cells were added to flasks containing 100 ml. K-free, Na Ringer of composition (mM): Na, 142; Mg, 7; Tris phosphate (pH 7.4 at 37°C), 5; Cl, 156; IAA, 0.2; fluoride, 2. At the start of incubation and after 2 and 4 hr at 37°C, samples of suspension were removed, transferred to five haematocrit tubes and spun for 20 min at 2700 *g*. The clear supernatant was sucked off down to the upper level of cells. The contents of two haematocrit tubes served as sources of unseparated samples. The remaining three tubes were divided by eye into thirds and 20 µl. samples taken from each for analysis. Plotted figures are means of three. The dotted line represents the mean values for mixed but unseparated samples at each time.

on incubation though there was an absolute fall in Hb with time coupled with a decrease in K and increase in Na content. The inhomogeneity can be explained by differences in cell volume, larger cells containing more Na and K tending to collect in the upper part of the column, whilst smaller cells showing an increase in the apparent concentration of Hb and containing less Na and K accumulated in the middle and bottom portions of the column. Such an interpretation agrees with the findings of a number of studies where it has been shown that red cells do not behave on differential centrifugation as a homogenous population (Chalfin, 1956; Hoffman, 1958b; Prentice and Bishop, 1965; Piomelli, Lurinsky and Wasserman, 1967). On centrifugation, younger, larger cells such as reticulocytes tend to predominate in the top layers whilst older smaller cells accumulate in the bottom layers. A similar gradation in Na and K content in columns of packed red cells to that noted here was also found by Joyce (1958); however, despite the difference in cation content, this author found no significant difference in Hb concentrations of the separate portions analysed.

Mixed samples of packed cells gave values for haemoglobin, Na and K which were close to the arithmetical mean of the concentrations at top and bottom of the packed columns. In the subsequent experiments on unidirectional ion fluxes and net movements, cells were not partitioned by differential centrifugation. Well-mixed but unseparated samples of packed cells were used for sampling.

Preincubation

1. Effect on cation content

Preincubation in a Na-free, balanced K solution was undertaken in order to lower internal Na and raise internal K of red cells preparatory to their use in studies of ion movement under the same conditions of steep Na and K gradients needed for $^{32}P_i$ incorporation into ATP. Inosine (10mM) and adenine (5mM) were usually added as sources of energy, and preincubation at 37°C continued for 5-12 hr. This procedure resulted in a substantial reduction in internal Na by 33-80% and a rise in internal K of between 17 and 57% (Table 9).

2. Effect on osmotic fragility

The effect of preincubation on the osmotic

TABLE 9. **Preincubation of cells in a K-rich, Na-free medium to lower internal Na and raise internal K content**

Expt.	Age of cells (day)	Length of preincubation (hr.)	Concentration in cells before preincubation Na (μ-equiv/ml.)	Concentration in cells before preincubation K (μ-equiv/ml.)	Concentration in cells after preincubation Na (μ-equiv/ml.)	Concentration in cells after preincubation K (μ-equiv/ml.)
v^{19}	25	7 *	42	51	12	80
v^{20}	16	8 *	29	62	10	80
v^{21}	11	12	27	64	15	75
v^{22}	22	12	39	53	20	66
v^{24}	20	6 **	54	66	10	91
v^{25}	19	5 **	42	72	19	96
v^{38}	21	8 *	30	78	20	104

2-3 week old cells were washed four times in 140 mM-KCl, 10 mM-Tris phosphate (pH 7.6 at 26°C) and then suspended at haematocrit of 4% in more of the potassium medium to which was added 10 mM inosine (*) and in some cases also 5 mM adenine (**). Preincubation at 37°C was continued with gentle shaking in a water bath for 5-12 hr when the cells were spun down. For measurement of Na and K content, portions of cells were set aside and washed 2-3 times in iced 150 mM-KCl or 150 mM-NaCl respectively. The remainder of the cells were washed 3 times in the same Ringer solution (iced) that was to be used in the subsequent incubation.

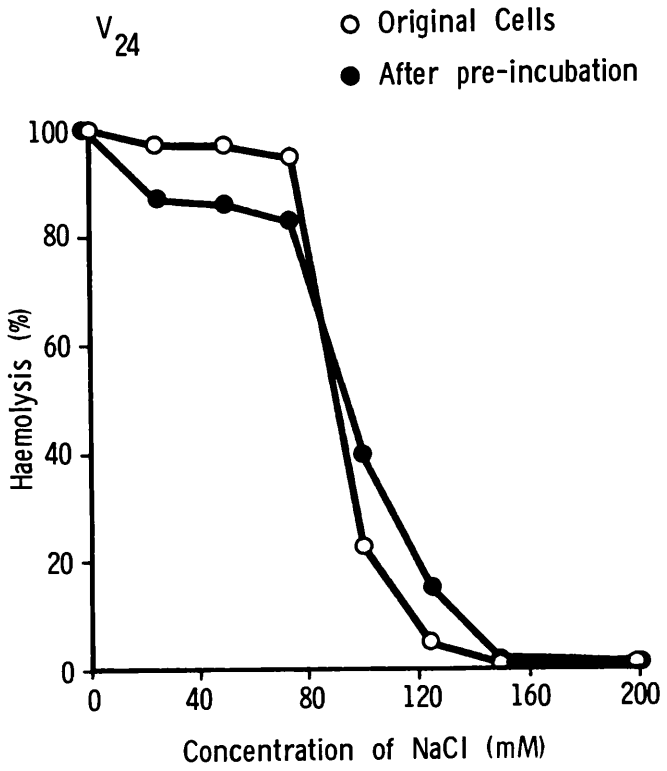

FIG. 14. Osmotic fragility of intact cells. 3 week old cells were washed four times in iced medium containing 140 mM-KCl; 10 mM-Tris phosphate (pH 7.4). The cells were then divided into two lots. One lot was pre-incubated for 6 hr at 37°C in a medium containing (mM): K, 130; Cl, 130; Tris phosphate (pH 7.4), 10; inosine, 10; adenine, 5. The other was left in more of the buffered KCl solution without substrate for 6 hr at 5°C. Both lots of cells were then washed 4 times (1:20) in iced medium containing (mM): Na, 142; Mg, 7; Cl, 156; Tris phosphate (pH 7.4), 5; IAA, 0.2; fluoride, 2. 0.1 ml. packed cells were added to 10 ml. of suitable dilutions of medium containing 140 mM-NaCl; 10 mM-Tris phosphate (pH 7.4) in stoppered glass tubes at 20°C. The tubes were inverted and allowed to stand at 20°C for 20 min. They were then remixed gently and centrifuged for 2 min at 2700 g. The amount of haemolysis in each tube was compared with 100% lysis tube using OD at 540 mμ.

behaviour of cells was investigated in two experiments (V^{24}; V^{25}) by assessing the osmotic fragility of the same batch of cells before and after incubation in the Na-free, K Ringer (Fig. 14). The characteristic S-shaped fragility curves obtained were a further reflection of underlying inhomogeneity of the cell population. There were minimal differences in the fragility curves before and after preincubation indicating that the procedure had not resulted in any significant deterioration in the membrane.

K efflux in the absence of external K

K efflux from high K, low Na ghosts

Since the labelling of ATP from $^{32}P_i$ was studied in ghosts, K efflux was also measured in ghosts under optimal conditions for phosphate incorporation. Ouabain was added to see if it decreased K efflux. High K, low Na ghosts containing 10mM P_i were loaded with ^{42}K and the migration of isotope followed into Na-rich, K-free Ringer solution. Preliminary experiments showed that the appearance of ^{42}K was approximately linear with time during $1\frac{1}{2}$ to 2 hr indicating first order

kinetics (Fig. 15). Values for K efflux varied considerably from experiment to experiment and ranged from 5.2 to 11.7 μ-equiv K/ml. ghost/hr (Table 10). Despite the considerable scatter of results, addition of ouabain caused a reduction in K efflux in 4 out of 5 experiments, the mean decrease being 1.2 μ-equiv/ml. ghost/hr. The decrease with ouabain was somewhat less consistent when ghosts contained less phosphate (1 or 5mM). (Fig. 15).

The scatter of results is not surprising in view of Hoffman's (1958b; 1962a) work on inhomogeneity of ghost behaviour with respect to tracer uptake and release. In order to overcome the variability, subsequent experiments were made with intact red cells rendered low in internal Na and high internal K by preincubation in a high K medium (see above, p.108).

K efflux from cells

K efflux was determined in cells incubated in a K-free, Na Ringer. Loss of the isotope followed first order kinetics over a period of $1\frac{1}{2}$ to 2 hr (Fig. 16). Absolute values for K efflux were more uniform than in resealed ghosts and averaged 2.12 ± 0.03 μ-equiv/ml.

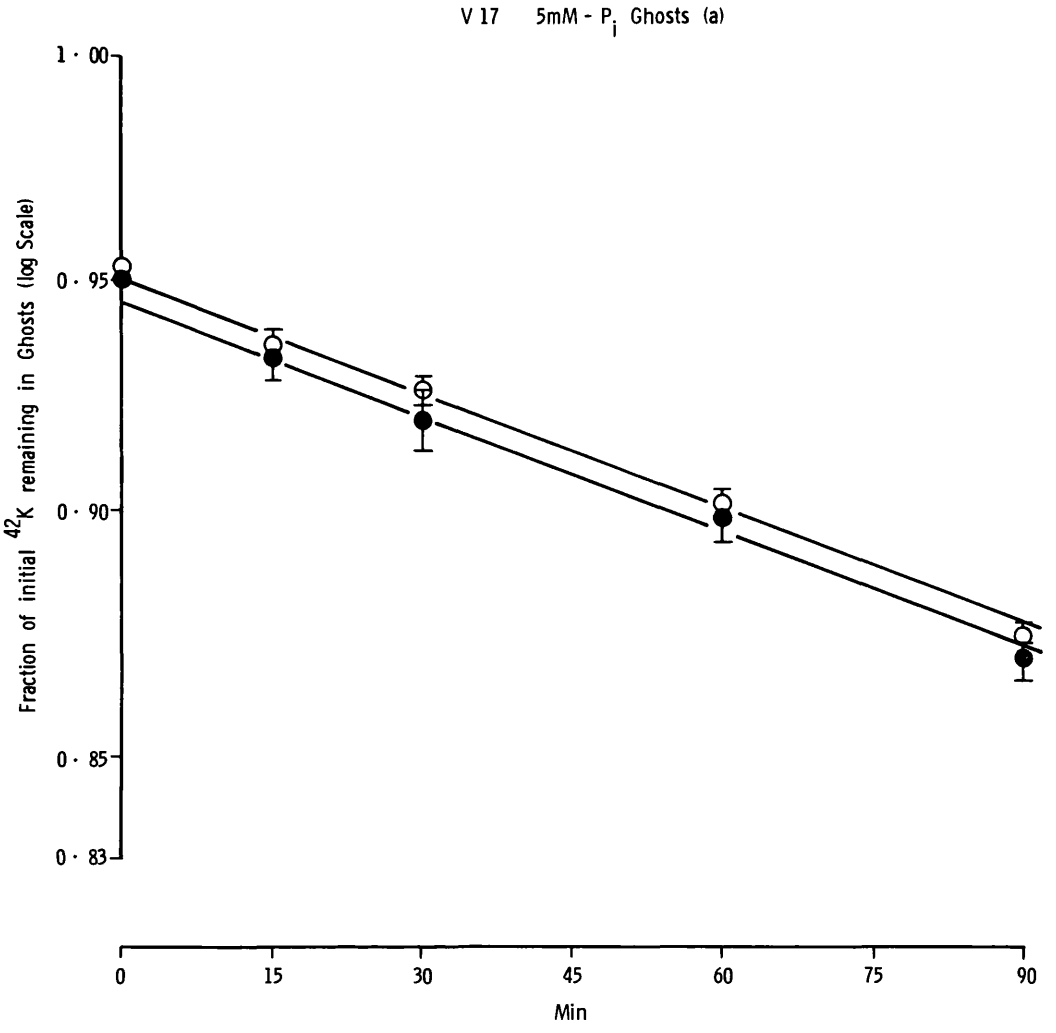

FIG. 15. The efflux of K from isotonically resealed ghosts, rich in K, low in Na, containing different concentrations of P_i. Two lots of ghosts were prepared from the same batch of red cells. The lysing solutions contained (mM): ^{42}K, 5 or 10; Na, 2; Mg, 7; Cl, 19; P_i, 5 or 10; Tris (pH 7.4), 5; IAA, 0.2; fluoride, 2. Isotonicity was restored with 3M-KCl. After sealing, the ghosts were washed and incubated in a K-free, Na Ringer of the same composition as in Table 10, with 5 or 10 mM-Tris phosphate (pH 7.4). The ouabain concentration was 50 μM. The vertical bars represent the S.E. of the means from incubations done in quadruplicate. The equations for each regression line were derived by the method of least squares and relate to the common ordinate:

$$y = -\ln\left[1 - \frac{(N^e)_t}{(N^c)_{o\,corr}}\right]$$

The respective equations are:

(a) ○, Na medium: $y = 0.949 - 0.049\ t$

●, Na medium plus ouabain: $y = 0.945 - 0.052\ t$

$\Delta\ b = 0.003\ hr^{-1}$; $P > 0.05$. N.S.

(b) see next page

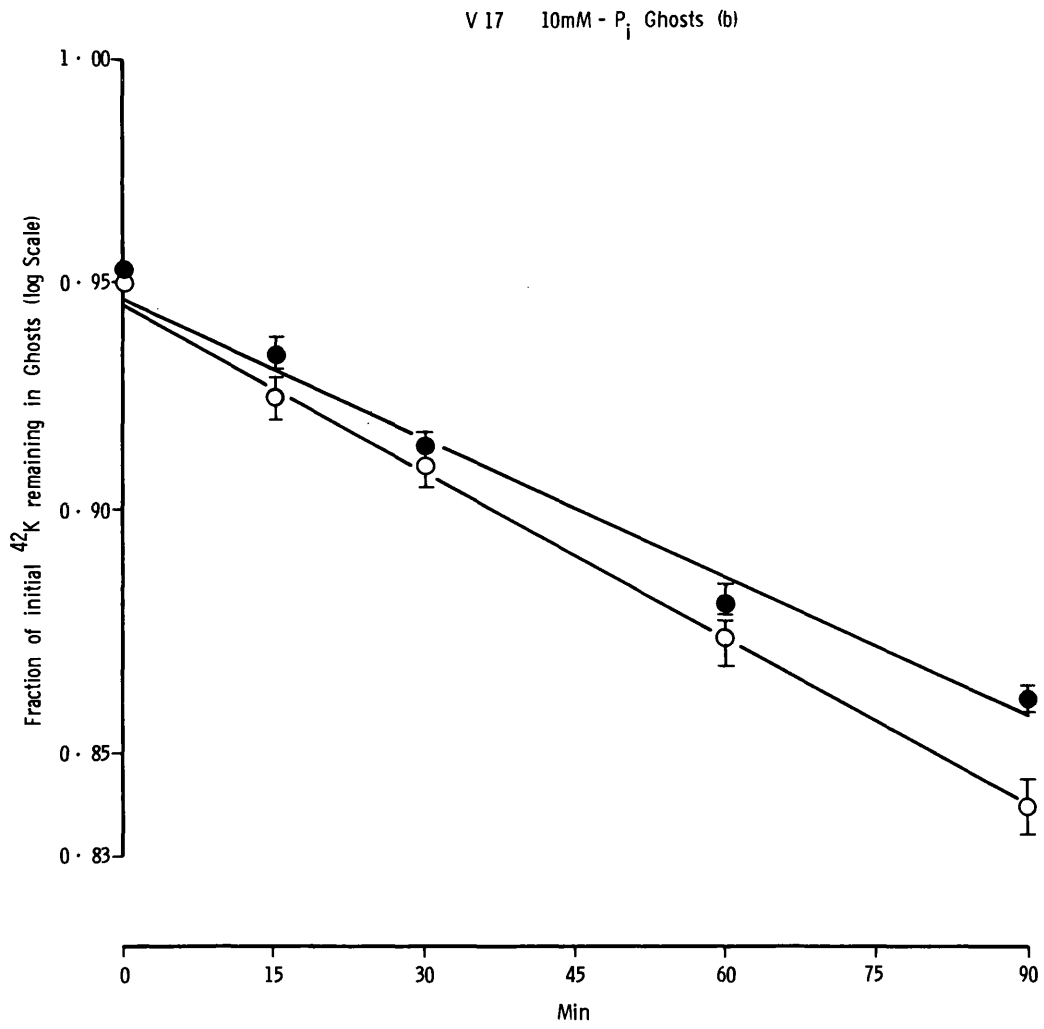

FIG. 15. (contd.)

(b) ○ Na medium: $y = 0.945 - 0.071\ t$

● Na medium plus ouabain: $y = 0.946 - 0.059\ t$

$\Delta\ b = 0.012\ hr^{-1}$; $0.025 < P < 0.05$

TABLE 10. The efflux of potassium from isotonically resealed ghosts to a K-free, Na Ringer medium in the presence and absence of ouabain

Potassium efflux (μ-equiv/ml.ghosts/hr.)

Incubation Medium (mM)	144 Na, 0 K								
P_i in lysing fluid (mM)	1			5			10		
ouabain	-	+	Difference	-	+	Difference	-	+	Difference
	Expt.			Expt.			Expt.		
	v^1 6.81	6.81	0	v^6 8.65	8.65	0	v^7 10.43	7.25	-3.18
	v^2 9.91	7.98	-1.93	v^7 11.40	9.85	-1.55	v^9 5.24	4.77	-0.47
	v^3 11.86	10.71	-1.15	v^{17} 4.53	4.53	0	v^{10} 8.80	7.42	-1.38
				v^{36} 12.32	12.32	0	v^{14} 11.74	11.74	0
							v^{17} 6.95	6.00	-0.95
Mean difference			-1.03			-0.39			-1.20
S.E. of mean			±0.56			±0.39			±0.55

Resealed high K, low Na ghosts were prepared as described in Methods Section and were incubated in K-free, Na Ringer with and without ouabain (50 μM). The solution in which the ghosts were sealed contained (mM): ATP, 1; ^{42}K, 147; Mg, 7; Na, 2; P_i (pH 7.4) 1 - 10; Cl, 149; iodoacetate, 0.2; fluoride, 2. The washing and incubation medium contained (mM): Na, 144, Mg, 7; Cl, 158; Tris phosphate (pH 7.4) 1 - 10; iodoacetate, 0.2; fluoride, 2. Incubation time: 60-90 min. Each value for K efflux is the mean of incubations undertaken in triplicate.

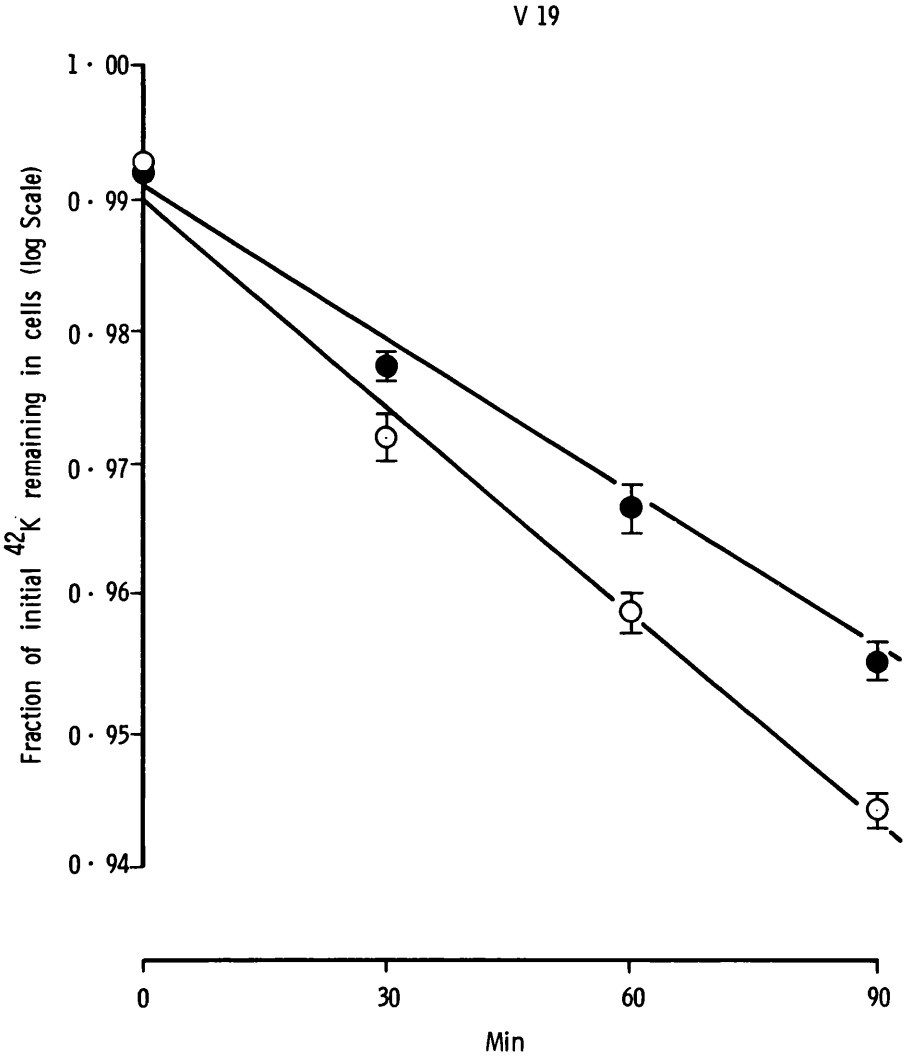

FIG. 16. Effect of ouabain on the efflux of K from red cells incubated in a K-free, Na Ringer of the same composition as in Table 11. The haematocrit averaged 4%. The ouabain concentration was 50 μM. The vertical bars represent the S.E. of the means from incubations done in quadruplicate. The equation for each regression line was derived as in Fig. 15. The respective equations are:

○ , Na medium: $y = 0.990 - 0.031\ t$

● , Na medium plus ouabain: $y = 0.991 - 0.024\ t$

$\Delta b = 0.007\ hr^{-1}$ $P < 0.01$.

cells/hr (mean ± S.E. of mean; n = 10) (Table 11). Glynn and Lüthi (1968) have described a fall in K efflux with ouabain when cells are incubated in K-free, Na Ringer. The same order of decrease was found in the present studies. Table 11 shows that ouabain caused a small but significant reduction of approximately 25% in K efflux amounting to 0.48 ± 0.07 μ-equiv/ml. cells/hr (mean ± S.E. of mean; n = 10) when external Na was replaced by choline, ouabain no longer inhibited K efflux (Table 12). Whereas in the presence of external Na, ouabain decreased K efflux by 0.54 μ-equiv/ml. cells/hr, in the choline Ringer there was a small increase of 0.17. These results indicate that under the same conditions in which there was ouabain-sensitive incorporation of P_i into ATP, about 25% of the efflux was inhibited by ouabain and depended on the availability of external Na. The possibility therefore arises that ouabain-sensitive K efflux might be balanced by Na influx.

Na influx in cells

Na influx was measured in cells from the same blood used for measuring K efflux. Na influx from K-free

TABLE 11. The efflux of potassium from cells incubated in a K-free, Na Ringer medium in the presence and absence of ouabain

Expt.	K concentration in cells (μ-equiv/ml.)	ouabain	Potassium efflux (μ-equiv/ml.cells/hr.) (a) -	(b) +	(a)-(b) Difference
V^5	60		1.82	1.45	0.37
V^8	75		2.68	1.77	0.91
V^{12}	69		1.58	1.42	0.16
V^{15}	78		2.08	1.79	0.29
V^{18}	64		1.52	1.13	0.39
V^{19}	74		2.45	1.88	0.57
V^{20}	74		1.87	1.49	0.38
V^{37}	91		1.55	1.16	0.39
V^{38}	78		2.85	2.17	0.68
V^{39}	98		2.84	2.16	0.68
			Mean difference		0.48
			S.E.		±0.07

Cells were incubated at 37°C for 90-120 min. The K-free washing and incubation medium contained (mM): Na, 144; Mg, 7; Cl, 158; Tris phosphate (pH 7.4), 10; iodoacetate 0.2; fluoride, 2. The concentration of ouabain was 50μM. Each flux value is the mean of incubations undertaken in triplicate.

TABLE 12. The effects of ouabain on efflux of potassium from cells incubated in K-free, Na or choline Ringer medium

Incubation medium (mM)	Potassium efflux (μ-equiv/ml.cells/hr.)					
	144 Na, 0 K			144 Choline, 0 K		
	(a)	(b)	(a)-(b)	(c)	(d)	(c)-(d)
ouabain	-	+	Difference	-	+	Difference
Expt.						
V^{37}	1.55	1.16	0.39	1.72	1.89	-0.17
V^{39}	2.84	2.16	0.68	2.85	3.02	-0.17
Mean difference			0.54			-0.17

Cells were incubated at 37°C for 60-90 min. Ouabain concentration was 50μM. The K-free choline Ringer was of the same composition as the K-free Na Ringer of Table 11 except that choline chloride replaced NaCl.

Na Ringer solution was inhibited about 50% when ouabain was added. The mean value (μ-equiv/ml. cells/hr) for ouabain-sensitive Na influx from an incubation medium containing 10mM P_i was 1.05 ± 0.15 (mean ± S.E. of mean; n = 3) (Table 13). The fall with ouabain was the same in Ringer without P_i and also when 0.2mM - IAA and 2mM-fluoride were added to inhibit metabolism. The results are consistent in showing in each case a fall in Na influx with ouabain.

Effect of external K

In order to test whether external K decreased the Na influx sensitive to ouabain, cells were incubated in Na Ringer containing ^{24}Na, with and without K. Addition of 10mM-K caused a fall in Na influx in the control of 0.75 ± 0.17 μ-equiv/ml./hr (mean ± S.E. of mean; n = 3), but there was no further fall when ouabain was added. In the presence of 10mM-K, the ouabain-sensitive component of Na influx was only 0.14 ± 0.03 as compared to 1.07 ± 0.25 μ-equiv/ml./hr (means ± S.E. of means; n = 3) in the same cells incubated in a K-free medium (Table 14).

These experiments show that under the same conditions

TABLE 13. **The sodium flux into red cells in the presence and absence of ouabain from K-free Na Ringer containing differing concentrations of P_i and/or inhibitors of glycolysis**

Sodium influx μ-equiv/ml./hr.

Expt.	Cell cation content μ-equiv/ml.		Incubation medium	145 Na, 0 K, 7 Mg											
										IAA 0.2; F,2					
				no P_i			10mM P_i			no P_i			10mM P_i		
	Na	K	ouabain	−	+	difference	−	+	difference	−	+	difference	−	+	difference
V^{24}	50	61								1.91 ±0.16	1.09 ±0.02	−0.82	2.37	1.20 ±0.02	−1.17
V^{25}	36	70		1.97	1.31	−0.66	2.73	1.50	−1.23	1.84 ±0.20	1.54 ±0.08	−0.30	2.04 ±0.06	1.34 ±0.12	−0.70
V^{27}	46	82											1.84 ±0.13	1.25 ±0.03	−0.59
V^{30}	43	65		2.70 ±0.31	1.35	−1.35	2.46 ±0.34	1.30	−1.16	2.24 ±0.35	1.06	−1.18	2.29 ±0.10	1.10	−1.19
V^{31}	19	94		2.22	0.99	−1.23	2.12 ±0.12	1.37	−0.75				2.43 ±0.03	1.01	−1.42
			Mean difference			−1.08			−1.05			−0.77			−1.01
			S.E. of mean			±0.21			±0.15			±0.26			±0.16

Cells were incubated at 37°C for 1 hr. The K-free, Na Ringer contained (mM): Na, 145; Mg, 7; Tris (pH 7.4),5; Cl, 164. In some experiments the incubation medium was modified by inclusion of 10mM -Tris phosphate (pH 7.4) instead of Tris chloride, 0.2 mM -iodoacetate and 2mM -fluoride, 50 μM -ouabain, where required.

Single values represent the means of duplicate incubations.
Other values are means ± S.E. of mean of results from incubations done in triplicate.

TABLE 14. The effects of external potassium on the influx of sodium into red cells incubated in a high Na Ringer medium in the absence and presence of ouabain

		Sodium influx (μ-equiv/ml. cells/hr.)							
	Incubation medium	140 Na; 0K			140 Na; 10K			Change in Na influx with 10K in Ringer μ-equiv/ml./hr.	
		(a)	(b)	(a)-(b)	(c)	(d)	(c)-(d)	(c)-(a)	(d)-(b)
Expt.	ouabain	-	+	Difference	-	+	Difference	-	+
V27		1.84±0.13	1.25±0.03	0.59	1.33±0.04	1.25±0.03	0.08	-0.51	0
V30		2.29±0.10	1.10	1.19	1.63	1.44	0.19	-0.66	+0.34
V31		2.43±0.03	1.01	1.42	1.36	1.22	0.14	-1.07	+0.21
	Mean difference			1.07			0.14	-0.75	0.18
	S.E. of mean			±0.25			±0.03	±0.17	±0.10

Cells were incubated at 37°C for 1 hr. The K-free, Na Ringer contained (mM): Na, 140; Mg, 7; Tris phosphate (pH 7.4 at 37°C) 10; Cl, 154; iodoacetate 0.2; fluoride, 2. In the 10 mM-K medium, K replaced an equivalent amount of Na. The concentration of ouabain was 50 μM.

Single values represent the means of duplicate incubations. Other values are means ± S.E. of mean of results from incubations done in triplicate.

which permit optimal incorporation of P_i into ATP ghosts, there is a component of both K and Na movements in cells which is sensitive to ouabain. Moreover, ouabain-sensitive K efflux is dependent on the presence of external Na; ouabain-sensitive Na influx only occurs when external K is zero.

Garrahan and Glynn (1967a and b) have shown that there is an ouabain-sensitive portion of Na influx which is linked and balanced by an equal ouabain-sensitive portion of Na efflux when red cells are incubated in K-free, Na Ringer. Now, if part of ouabain-sensitive Na influx is also involved in backward running of the Na pump, then Na influx should exceed Na efflux to an extent equal to the ouabain-sensitive K efflux. If this is so, the implication is that ouabain-sensitive Na influx is balanced by two ion movements:

1) the Na efflux associated with Na : Na exchange and, 2) the K efflux associated with reversal of the Na pump. This can be represented by the following equation:

ouabain-sensitive $\left[{}^{i}M_{Na}\right] = {}^{o}M_{Na} + {}^{o}M_{K}$

Since net movement of ions results from a difference in the rates of unidirectional fluxes, a net gain of cell Na, sensitive to ouabain, should be demonstrable if $^{i}M_{Na} > {}^{o}M_{Na}$.

On similar grounds, there should be a net loss of K which is sensitive to ouabain, since, in the absence of external K, this would reflect the inhibitory effect upon unidirectional K efflux. To test these points, measurements were made of net changes in Na and K content of cells incubated in K-free, Na Ringer.

Net cation movements

Net K loss

Measurement of net K loss was made in some of the same cells and under identical conditions as used in the experiments on ^{42}K efflux. In most cases, the net changes were determined from the potassium content of the cell-free supernatants after incubation, allowance being made for K released from lysed cells. Where net K movements were determined from measurements of changes in cell cation content, special precautions were taken in sampling of the cells after packing in haematocrit tubes as outlined in Methods (p. 97).

The net losses of potassium to a K-free, Na Ringer were found to be significantly smaller in the presence of ouabain (Table 15). The mean difference (μ-equiv/ml./hr) was 0.51 ± 0.13 (mean ± S.E. of mean; n = 7). The mean concentration of potassium in the supernatants at the end of 2 hr incubation ranged from 180 - 250μM. In order to keep the level of extracellular K as low as possible when longer periods of incubation were undertaken, an equal volume of K-free, Na Ringer (pre-warmed to 37°C and containing ouabain where necessary) was added to each flask after 1 hr and at each sampling time. In Expt. V^{47} the final concentration of K in the medium at the end of 4 hr incubation ranged between 170 - 190 μM.

Net Na uptake

Net Na uptake was studied in experiments of similar design to those employed for studying K loss. The changes in Na content were determined from the unwashed packed cells after centrifugation in haematocrit tubes. Net Na uptake levels ranged from 0.40 to 2.10 μ-equiv/ml./hr; ouabain caused a small reduction (μ-equiv/ml./hr) of 0.38 ± 0.16 (mean ± S.E. of mean; n = 7) (Table 16).

TABLE 15. Net loss of potassium from red cells incubated in a high Na, K-free Ringer medium in the absence and presence of ouabain

Expt.	Time of Incubation (hr.)	Initial cell potassium μ-equiv/ml.	Net potassium loss μ-equiv/ml./hr.		Difference due to ouabain μ-equiv/ml./hr.
			control	+ ouabain	
V^{4}	2	62	1.10	1.10	0
V^{12}	2	69	2.59	1.74	0.85
V^{15}	2	78	1.31	1.00	0.31
V^{19}	2.	74	2.34	1.38	0.96
V^{20}	2.	74	2.92	2.16	0.76
V^{47}	4	64	0.49	0.12	0.37
	4	73	2.72	2.41	0.31
				Mean difference	0.51
				S.E. of mean	±0.13

In Expt. V^{47}, net movements were measured from the changes in unwashed cell K content before and after incubation, adopting the procedure described in Methods section. In the remaining experiments, the net changes were derived from the losses of K to the medium, allowance being made for loss due to lysis during incubation. The K-free, Na Ringer was of the same composition as in Table 14. The concentration of ouabain was 50μM. The results in the Table are means of incubations done in triplicate.

TABLE 16. Net uptake of sodium into red cells incubated in a high Na Ringer medium in the absence and presence of ouabain

Expt.	Time of Incubation (hr.)	Cell sodium μ-equiv/ml.			Net sodium uptake μ-equiv/ml.		Differences due to ouabain μ-equiv/ml./hr.
		Initial	After incubation				
			control	+ouabain	control	+ouabain	
v^{40}	5	18.5*	20.5	19.0	2.0	0.5	0.30
v^{44}	3	41.0	44.0	41.5	3.0	0.5	0.83
v^{46}	4	53.5	58.0	54.0	4.5	0.5	1.00
v^{47}	4	41.5	50.0	50.0	8.5	8.5	0
	4	34.0	42.5	40.5	8.5	6.5	0.50
	4	41.0	46.0	46.0	5.0	5.0	0
	4	36.0	44.0	44.0	8.0	8.0	0
					Mean difference		0.38
					S.E. of mean		±0.16

* cells preincubated in 140 mM-KCl: 10 mM-Tris phosphate (pH 7.4) for 42 hours at 7°C

Cell Na content was measured before and after incubation at 37°C for 3-5 hr in a K-free, Na Ringer of the same composition as in Table 14. The concentration of ouabain was 50 μM. The results in the Table are means of incubations done in triplicate.

These results lend support to the view that in the absence of external K, ouabain-sensitive Na influx may be coupled to two cation effluxes which are also ouabain-sensitive. First, the 1:1 Na - Na exchange described by Garrahan and Glynn (1967a and b) and second, the K efflux which is associated with backward running of the sodium pump.

Effect of external K on K efflux

In two experiments, cells derived from the same batch were incubated in Na Ringer with and without potassium and with and without ouabain. Addition of 10mM-K to the Na Ringer caused an increase of approximately 25% in K efflux (Fig. 17). Part of this increase was ouabain-sensitive, since with 10mM-K outside, ouabain decreased K efflux to a similar extent as in the K-free medium (0.38 and 0.34 μ-equiv/ml./hr respectively, Table 17). As it has already been shown (p. 120) that there is no significant ouabain-sensitive Na influx in the presence of 10mM-K outside, the ouabain-sensitive K efflux under these circumstances can be seen as a 1:1 K-K exchange as has been suggested by Glynn and Lüthi (1968). On comparing columns (b)

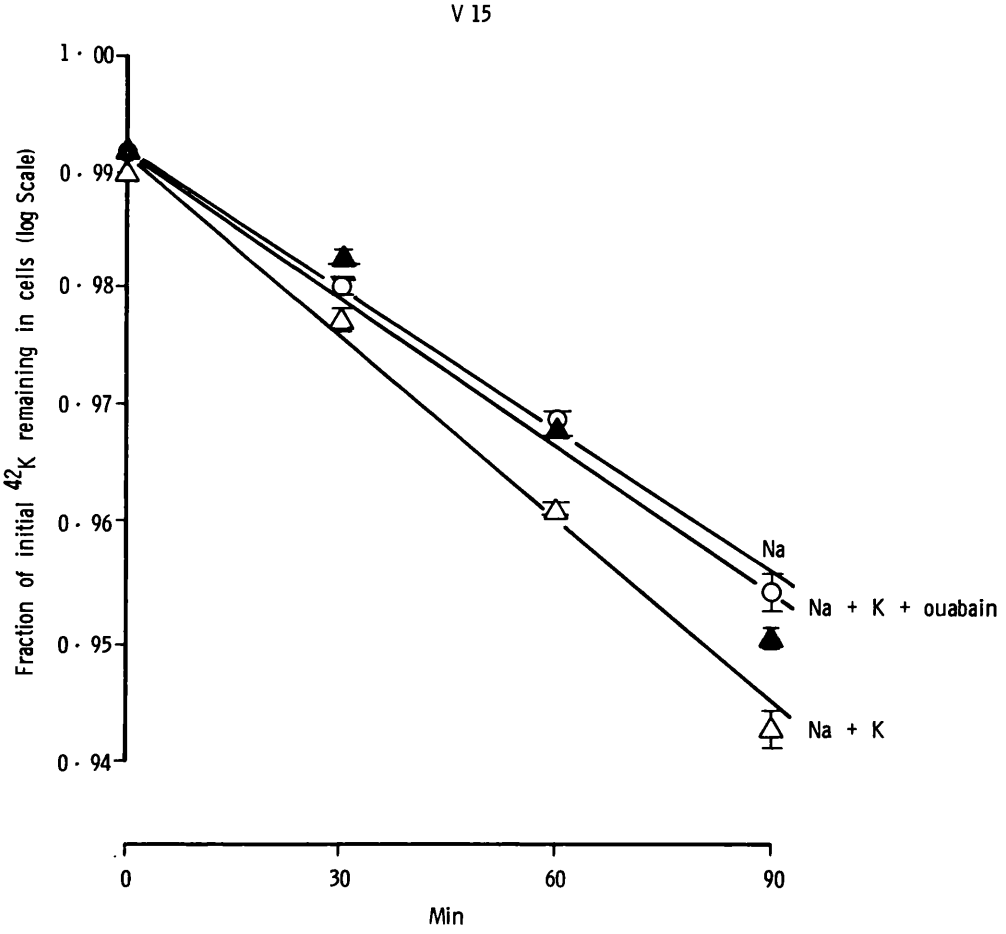

FIG. 17. The effect of external K on K efflux from intact red cells incubated in the presence and absence of ouabain. The K-free, Na Ringer was the same as described in Table 11. In the 10 mM-K medium, K replaced an equivalent quantity of Na. The haematocrit averaged 4%. The ouabain concentration was 50 μM. The vertical bars represent the S.E. of the means from incubations done in quadruplicate. At the end of 1 hr incubation, the K-free medium contained 268 μM-K. The equation for each regression line was derived as in Figs. 15 and 16. The respective equations are:

1. ○ , K-free medium: $y = 0.992 - 0.026\ t$
2. △ , 10 mM-K medium: $y = 0.992 - 0.032\ t$
3. ▲ , 10 mM-K medium plus ouabain: $y = 0.993 - 0.028\ t$

$\Delta\ b_{1,2} = 0.006\ hr^{-1}$; $0.01 < P < 0.02$; $\Delta\ b_{1,3} = 0.002\ hr^{-1}$; N.S.
$\Delta\ b_{2,3} = 0.004\ hr^{-1}$; $0.02 < P < 0.03$;

and (d) in Table 17, it is clear that external K also stimulated K efflux in the presence of ouabain to a similar extent as in the absence of glycoside (0.53 and 0.56 μ-equiv/ml./hr respectively). This stimulation of ouabain-resistant K efflux by external K is of interest since it raises the possibility of a parallelism with the findings of Hoffman (1966) and Lubowitz and Whittam (1969) that there is a component of K influx which is ouabain-insensitive and which depends on external Na.

TABLE 17. The effects of external potassium on the efflux of potassium from red cells incubated in a high Na Ringer medium in the absence and presence of ouabain

Expt.	Incubation medium (mM)	Potassium efflux (μ-equiv/ml./hr.) 144 Na, 0 K			144 Na, 10 K			Increase in K efflux with 10K in Ringer	
		(a)	(b)	(a)-(b)	(c)	(d)	(c)-(d)		
	ouabain	-	+	Difference	-	+	Difference	-	+
V^{15}		2.08	1.79	0.29	2.62	2.40	0.22	0.54	0.61
V^{18}		1.52	1.13	0.39	2.10	1.57	0.53	0.58	0.44
				———			———	———	———
			Mean	0.34			0.38	0.56	0.53

2 week old cells, loaded with ^{42}K, were incubated at 37°C for 90 - 120 min. The K-free washing and incubation medium was of the same composition as in Table 11. In the 10 mM-K medium, K replaced an equivalent quantity of Na. The concentration of ouabain was 50 μM. Each flux value is the mean of incubations undertaken in triplicate.

DISCUSSION

These results show that there is an interdependence of ouabain-sensitive downhill K and Na movements which parallels the requirements for incorporation of P_i into ATP described in Part One. In K-free Ringer, a part of both K efflux and Na influx is sensitive to ouabain. The ouabain-sensitive component of K efflux is dependent on external Na, whilst ouabain-sensitive Na influx is abolished by external K. A prerequisite for the conceptual view of a coupling between part of the downhill movements of K and Na is that in the absence of external K, ouabain-sensitive influx of Na should exceed the simultaneous ouabain-sensitive Na efflux. Indication that this was so came from measurements of net Na and K movements, though in view of the small changes involved conclusive demonstration of this was difficult. Further experiments are needed in which $^{i}M_{Na}$ and $^{o}M_{Na}$ are measured at the same time and in the same cells, to provide confirmation of this point. It is relevant in this connexion that under similar conditions of zero external potassium, Garrahan and Glynn (1967a and b) measured ouabain-sensitive

influx and efflux of Na and found the two fluxes equalled one another. However, they included glucose in the incubation medium. Addition of substrate is without effect on Na influx but, despite absence of external K, could cause a small increase in $^{o}M_{Na}$ sufficient to make it equal the simultaneous $^{i}M_{Na}$. Furthermore, the cells of Garrahan and Glynn (1967a and b) had a low P_i content.

Whereas ouabain-sensitive Na influx was abolished when 10mM-K was added to the external medium, ouabain-sensitive K efflux was stimulated by approximately 25%. This pattern fits with the occurrence of a 1:1 K-K exchange as described by Glynn and Lüthi (1968). The conclusion can be drawn that in the presence of external K, K-K exchange supervenes in place of the K efflux linked to Na influx which is associated with backward running of the Na pump. At the same time, by activating normal operation of the pump, external K permits occurrence of the coupled movements of Na efflux and K influx.

The spatial localisation in the cell membrane of the pathways for these various ion movements is of

interest. The question arises: do the processes of exchange diffusion and active transport occur by independent routes? The fact that K-K exchange is ouabain-sensitive as is also the Na-Na exchange which occurs in the absence of external K (Garrahan and Glynn (1967a and b) suggests that exchange diffusion may represent a modified mode of behaviour of the Na pump involving the same carrier mechanism.

It is clear from the present findings and those of Glynn and Lüthi (1968), Lubowitz and Whittam (1969) that a substantial part of the downhill fluxes of both Na and K are insensitive to ouabain and appear to occur by some kind of diffusion process which does not involve transient complex of the ion with the membrane. Little is known of the mechanisms controlling ouabain-insensitive downhill movements, though an inter-relationship with the metabolic state of the cell and also ionic calcium appears to be implicated (see Passow, 1964; Whittam, 1968). The present results indicate that in the presence of external Na there is an ouabain-insensitive exchange diffusion of K. Its magnitude appears similar to the ouabain-sensitive K-K exchange;

dependence, if any, on external Na has not been investigated. The work of Hoffman (1966) and Lubowitz and Whittam (1969) has revealed a small ouabain-insensitive K influx which depends on external Na and could be the counterpart of the ouabain-insensitive K efflux measured here. Furthermore, Lubowitz and Whittam (1969) found evidence for the occurrence of ouabain-insensitive exchange diffusion of Na in presence of external K. These observations imply the existence in the membrane of at least one other pathway for exchange diffusion of Na and K by a mechanism which is independent of the Na pump. Fig. 18 is a schematic representation of the relationship between downhill movements of Na and K occurring independently of the active transport system and those movements which are associated with modified pump behaviour such as occurs in reversal or in the catalysis of exchange diffusion.

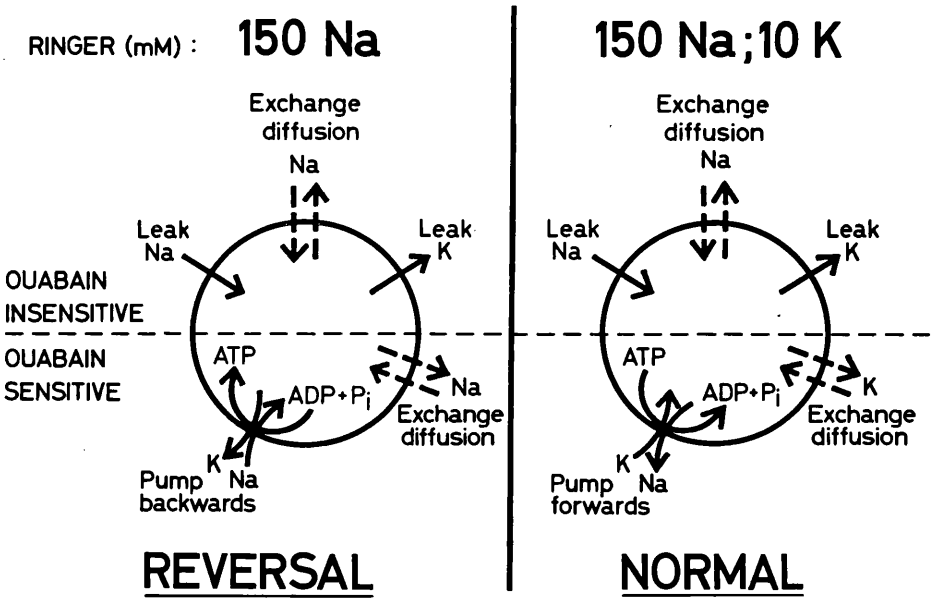

FIG.18. Ion movements, chemical reactions and the sodium pump.

SUMMARY

1. Isotopic tracers have been used to study the downhill movements of Na and K in resealed ghosts and intact red cells incubated in high Na, K-free Ringer under conditions which favour backward running of the Na pump.

2. There was considerable scatter in values of K efflux in resealed ghosts though ouabain produced a definite reduction at high levels of internal P_i. In intact cells, K efflux was more uniform in magnitude and was reduced by about one quarter when ouabain was added. The ouabain-sensitive K efflux was abolished when external Na was replaced by choline.

3. In identical batches of intact cells, Na influx was reduced by about one half with ouabain. The ouabain-sensitive Na influx was essentially abolished when 10mM-K was added to the external Ringer.

4. Measurements of net transport of Na and K showed that in the absence of external K, there was a small net loss of K and also net entry of Na that was ouabain-sensitive.

5. When 10mM-K was added to the high Na Ringer, K efflux was stimulated in a way which was ouabain-sensitive. There was also an ouabain-insensitive component of K efflux which was raised by external K.

6. These results suggest that in K-free, Na Ringer, there is a modified operation of the Na pump in two ways - first, coupling of a small Na entry and K loss associated with reversal, and second, 1:1 exchange diffusion of Na. When 10mM-K is included in the external medium two other sets of ouabain-sensitive fluxes supervene. First, coupling of Na efflux and K influx through normal operation of the pump, in place of Na:Na exchange diffusion; second, K:K exchange diffusion, in place of the K efflux linked to external Na.

7. There also appears to be an exchange diffusion of K which can take place when the pump is blocked by ouabain.

PART THREE

ENERGETICS OF REVERSAL OF THE SODIUM PUMP

INTRODUCTION

If osmotic energy from downhill ion movements is being transformed into chemical energy in the form of ATP, the question arises: what are the free energy changes involved? In order to consider this problem, quantitative information was needed of the absolute levels of the major reactants in the ATPase reaction $\left[ATP^{4-} + H_2O \overset{Mg^{2+}}{\rightleftharpoons} ADP^{3-} + P_i^{2-} + H^+ \right]$ both in the ghost and cell systems used for studying ouabain-sensitive downhill Na and K movements. As well as allowing an estimate to be obtained of the stoichiometry of the reversed ATPase reaction, the findings of the chemical studies undertaken in this section have a bearing on the general pattern of metabolic turnover of high energy phosphate and adenine nucleotide within the red cell.

METHODS

Isotonically resealed ghosts and intact red cells preincubated to lower internal Na and raise internal K were prepared as described in the Methods Sections of Parts One and Two (pp.20 and 108).

Adenine nucleotides

The neutralised supernatants obtained following deproteinisation with $HClO_4$ were used for assay (see p. 22). Measurements were performed in an Eppendorf-Howe photometer with an 100M fluorescence attachment (V.A. Howe & Co.,Ltd., London) using a final volume of 2.0 ml. in the reaction cuvette. The enzymatic techniques used were based on those of Adam (1963). ATP was determined by measuring the rate of conversion of $NADP^+$ to NADPH in the presence of known excesses of glucose, hexokinase, glucose-6-phosphate dehydrogenase, magnesium chloride and di-sodium-EDTA in a 0.1M- triethanolamine hydrochloride buffer, pH 8.0. ADP was assayed in a reaction mixture containing phosphoenol-pyruvate, pyruvate kinase, lactic dehydrogenase and NADH in a 0.05M -triethanolamine hydrochloride buffer, pH 7.5. The amount of NADH oxidised was measured

and was stoichiometrically related to the amount of ADP present. AMP was determined by the further addition of adenylate kinase (myokinase) at the conclusion of the ADP assay.

Orthophosphate (P_i) was estimated using the method of Fiske and Subbarow (1925) (see p. 28).

Na and K These elements were measured by a Unicam atomic absorption spectrophotometer (SP 90) (Unicam Instruments, Ltd.,Cambridge). Samples were prepared and estimated as described on p. 29.

RESULTS

Nucleotide and orthophosphate content of resealed ghosts

The adenine nucleotide and orthophosphate contents of isotonically resealed ghosts rich in K, and low in Na and prepared under standard conditions, were measured in two experiments (Table 18). In Expt. V^{23}, the parent cells were 2 weeks old and had lowered ATP but increased ADP, AMP and P_i levels; in Expt. V^{35}, cells were fresh and the ATP level was over twice that in V^{23} with negligible AMP and P_i content. The major source of nucleotide in the ghosts originated from the 1mM-ATP included in the lysing fluid, and although hardly any of this was hydrolysed in the ghost-free supernatant remaining after the 30 min incubation for sealing, the harvested ghosts at this time contained only 0.38 - 0.64 μ-mole/ml. ATP. This indicated that significant splitting of ATP was only occurring inside the ghosts. Although the lowered $\frac{[ATP]}{[ADP]}$ ratio would favour driving the ATPase reaction in reverse, the subsequent changes in nucleotide levels on incubation

for 15 min in an all Na, K-free Ringer were not significant and did not reveal a net formation of ATP which was ouabain-sensitive.

There was a disparity between the total nucleotide levels found by enzymatic assay and the total indicated by absorbance at 260mμ. In the cold-stored cells of V^{23}, total nucleotide assayed was only 59% of the amount expected from 260mμ extinction. This disparity was evident in both parent cells and sealed ghosts, but not in the lysate or post-sealing supernatant (Table 19). Its explanation may lie in the presence of an unknown adenine nucleotide not measured by the enzymatic assays used or by the formation of a non-adenylate metabolite with significant absorbance at 260 mμ. A possible compound fulfilling the latter requirement is inosine monophosphate (IMP), one of the immediate products of degradation of AMP formed through the activity of AMP deaminase (Askari and Franklin, 1965; Rao, Hara and Askari, 1968). In Expt. V^{35} where the ghosts were prepared from fresh cells, the measured and expected levels of adenine nucleotide showed close agreement (Table 19).

TABLE 18. Adenine nucleotide and orthophosphate content of intact red cells, haemolysate and derived isotonically resealed ghosts

Expt.		original cells	Post-reversal lysate	Post-sealing supernatant	Harvested ghosts	after 15 min. incubation	
						Na medium	Na medium plus ouabain
				µ-mole/ml.			
V^{23}	ATP	0.505±0.012	0.909±0.018	0.929±0.030	0.378±0.030	0.319±0.080	0.283±0.027
	ADP	0.270±0.026	0.081±0.006	0.175±0.018	0.257±0.029	0.183±0.051	0.164±0.027
	AMP	0.143±0.003	0.002±0.001	0.009±0.003	0.377	0.331	0.266
	P_i	6.90	4.22	4.42	4.45	3.96	3.34
V^{35}	ATP	1.275±0.022	0.913±0.027	1.013±0.032	0.636±0.032	0.602±0.013	0.548±0.012
	ADP	0.280±0.010	0.044	0.094	0.304±0.019	0.295±0.010	0.365±0.014
	AMP	0.043±0.001	0.004	0.007	0.063±0.003	0.086±0.004	0.073±0.005
	P_i	0.92	4.30	4.23	6.47	5.37	5.03

The solution in which the ghosts were sealed contained (mM): ATP, 1; P_i, 5; Mg,7; K, 147; Cl, 149; Na, 2; iodoacetate, 0.2; fluoride, 2. The ghosts were incubated for 15 min. in a high Na Ringer which contained (mM): Na, 142; Mg, 7; Cl, 161; Tris (pH 7.4 at 37°C), 5; iodoacetate, 0.2; fluoride, 2. Ouabain concentration was 50µM. The nucleotide figures are means of 4 estimates ±S.E. of mean. The orthophosphate and single AMP values are means of two estimates.

Nucleotide and orthophosphate content of intact cells

Two experiments were undertaken in which nucleotide and orthophosphate levels were measured in cells prepared and incubated exactly as in ion flux experiments except that no ^{42}K or ^{24}Na was added (Table 20). The cells in Expt. V^{21} were 16 days old and in V^{22}, 22 days old. Preincubation in a buffered K-Ringer medium resulted in a considerable fall in ATP to approximately 7-8% of the original levels. The concomitant fall in ADP and rise in AMP levels probably reflected the activity of adenylate kinase through whose action ADP formed from ATP hydrolysis is converted to AMP and ATP (Overgaard-Hansen, 1957; Kashket and Denstedt, 1958). During the 90 min incubation in Na medium without and with added ouabain, the levels of ATP, ADP and AMP continued to fall. The total nucleotide measured by enzymatic assay again revealed a marked disparity when compared to the expected total calculated from absorbance at 260 mμ (Table 19). The differences between levels attained in the presence and absence of ouabain were not significant.

TABLE 19. Total adenine nucleotide content of isotonically resealed ghosts and intact red cells

		Total nucleotide assayed	Total nucleotide by 260mμ absorbance	Total nucleotide assayed	Total nucleotide by 260mμ absorbance
		μ-mole/ml.			
Ghost expt.		V^{23}		V^{35}	
original cells		0.918	1.570	1.598	1.690
post-reversal lysate		0.992	0.890	0.961	1.000
post-sealing supernatant		1.113	0.926	1.114	1.035
harvested ghosts		1.012	1.294	1.003	1.028
ghosts after incubation	Na	0.883	1.192	0.983	0.995
	Na plus ouabain	0.713	1.005	0.986	0.958
Cell expt.		V^{21}		V^{22}	
original cells		1.595	-	1.497	1.992
after 12 hr preincubation		0.560	1.075	0.559	0.789
after 90 min incubation	Na	0.331	0.828	0.273	0.800
	Na plus ouabain	0.289	0.903	0.286	0.807

Comparison of the total quantities determined by enzymatic analysis with those derived from extinction values at 260 mμ. The molar extinction coefficient for adenine $\varepsilon = 15.0 \times 10^3$ at pH 7. Experimental conditions as in Table 18.

TABLE 20. Adenine nucleotide and orthophosphate content of intact red cells

Expt.		Control: original cells	Control: after 12 hr preincubation	after 90 min incubation: Na medium	after 90 min incubation: Na medium plus ouabain
			µ-mole/ml. cells		
V^{21}	ATP	1.081	0.091	0.023	0.010
	ADP	0.380 ±0.002	0.220 ±0.008	0.135 ±0.013	0.097 ±0.008
	AMP	0.134 ±0.009	0.249 ±0.003	0.173 ±0.005	0.182 ±0.007
	P_i	9.52	5.05	1.89	1.89
V^{22}	ATP	0.811 ±0.037	0.059 ±0.007	0.014 ±0.001	0.016 ±0.002
	ADP	0.402 ±0.020	0.143 ±0.024	0.059 ±0.008	0.059 ±0.011
	AMP	0.284	0.357	0.200	0.211
	P_i	8.71	3.78	1.82	1.79

After preincubation, the cells were incubated for 90 min in a high sodium Ringer medium of same composition as in Table 14. Ouabain concentration was 50 µM.
Nucleotide figures are means of 4 estimates ±S.E. of mean. The orthophosphate and single AMP values are means of two estimates.

Efficiency of conversion of osmotic work into phosphate bond energy

Resealed ghosts

Energy available from ouabain-sensitive downhill movements of Na and K

The mean ouabain-sensitive K efflux in ghosts containing 10 mM-P_i was 1.20 m-equiv/l./hr. If it is assumed that this flux is balanced by an equal ouabain-sensitive Na influx, and that the activity coefficients of Na and K are the same in the cell as in the medium,

see page 115

$$\Delta G = 1.20\ RT\ \ln\left[\frac{\lceil K^+_i \rfloor}{\lceil K^+_e \rfloor}\right] + 1.20\ RT\ \ln\left[\frac{\lceil Na^+_e \rfloor}{\lceil Na^+_i \rfloor}\right]$$

$$= 2.30 \times 1.20 \times 10^{-3} \times 1.99 \times 310 \cdot \log_{10}\left[\frac{120}{0.1}\right] + \log_{10}\left[\frac{140}{8}\right]$$

$$= 7.40\ \text{cal/l.ghost/hr.}$$

Where $\lceil K^+_i \rfloor$ $\lceil Na^+_i \rfloor$ = the mean K and Na concentrations in the ghosts (m-mole/l.ghost water)

$\lceil K^+_e \rfloor$ $\lceil Na^+_e \rfloor$ = the extracellular K and Na concentrations (m-mole/l.)

Energy needed to synthesize one mole of ATP under conditions prevailing in the ghost

Using the nucleotide and P_i contents determined in Expt. V^{23}, the energy available from ATP hydrolysis equals:

$$\Delta G = G^{o'} + RT \ln \left[\frac{[P_i] \cdot [ADP]}{[ATP]} \right]$$

$$= -7000 + 2.30\ RT \log_{10} \left[\frac{0.38 \times 10^{-3}}{4.45 \times 0.26} \times 1.43 \times 10^{-6} \right]$$

$$= -7000 - 3350 = -10,350 \text{ cal}$$

The standard free energy $\Delta G^{o'}$ at pH 7.0 and at 37°C in the presence of Mg^{2+} has been taken as -7.0 K cal. (Benzinger, Kitzinger, Hems and Burton, 1959)

$$\text{Efficiency of energy transformation} = \frac{\text{Phosphate bond energy formed}}{\text{Phosphate bond energy available from ion movements}}$$

An estimate of the ATP formed in ghosts under the optimal conditions for reversing the ATPase reaction was obtained experimentally in Part One and was of the order of 1 n-mole/ml.ghost/min or 60 μ-mole/l.ghost/hr.

This value was based on the observed rate of incorporation of P_i into ATP (m) and represents the net difference between the incorporation achieved through reversal of the pump (x) and the liberation of P_i through hydrolysis via the ATPase unconnected with the pump - non-specific ATPase or apyrase (a)

This may be shown as follows:

Net P_i Incorporation into ATP (observed labelling)	=	P_i incorporation into ATP by reversal of pump	-	P_i released from ATP by action of non-specific ATPase
(m)		(x)		(a)

Apyrase activity represents ATPase activity persisting in the presence of ouabain or in the absence of external K. Its magnitude can be assessed by deducting ouabain-sensitive ATPase from the total ATPase activity. In Expts. V^{23} and V^{35}, the acid-labile phosphate concentration is given by the term $(2 \times [\text{ATP}]) + [\text{ADP}]$ and lies in the range 1.02 to 1.58 μ-mole/ml.ghost. From the results of Whittam and Ager (1964), it is possible to define the relationship between the concentration

of acid-labile phosphate and apyrase activity in ghosts containing different amounts of ATP. Using this relationship, the apyrase activity associated with the above range of acid-labile phosphate is of the order of 0.30 - 0.50 μ-mole/ml.ghost/hr.

$$x = m + a = 60 + 400 = 460 \text{ μ-mole/l.ghost/hr}$$

If each mole of ATP provides an available 10,350 cal, 460 μ-moles would provide $460 \times 10{,}350 \times 10^{-6}$ cal.

$$\text{Efficiency} = \frac{460 \times 10{,}350 \times 10^{-6}}{7.40} \times 100\% = 64\%$$

Intact cells

The energy available for dissipation of ionic concentration gradients can be estimated from the following equation where the downhill movements of Na and K sensitive to ouabain are taken to equal one another:

ouabain sensitive $[\text{K efflux} = \text{Na influx}] = 0.50$ m-equiv/l.cell/hr.

$$\Delta G = 0.50 \times 10^{-3} \times 2.30\ RT \log_{10}\left[\frac{[K]_i}{[K]_e} \times \frac{[Na]_e}{[Na]_i}\right]$$

$$= 710 \times 10^{-3} \log_{10}\left[\frac{100}{0.10} \times \frac{140}{29}\right]$$

$$= 2.62 \text{ cal/l.cell/hr.}$$

The values employed are those of Expt. V^{21}, corrected for an approximate 70% water content of cells and expressed per litre of cell water.

The free energy available from ATP under the conditions prevailing in the cells of Expt. V^{21} is given by

$$\Delta G = -7000 + 2.30\ RT\ \log_{10}\left[\frac{5.05 \times 0.22 \times 1.43 \times 10^{-6}}{0.091 \times 10^{-3}}\right]$$

$$= -9500 \text{ cal/mole}$$

It has been shown in Part One that, under optimal conditions, the measured rate of the reverse reaction in resealed ghosts amounts to approximately 2% of the forward ATPase reaction. The optimal rate of ouabain-sensitive ATPase activity in intact cells is of the order of 1 μ-mole/ml.cell/hr or 1000 μ-mole/l.cell/hr (Whittam and Ager, 1965). If we assume that the same relationship between backward and forward reactions of the Na pump found in ghosts holds true also for intact cells, optimal functioning of the reverse reaction should lead to formation of $20\left[\frac{2}{100} \times 1000\right]$ μ-mole/l. cell/hr. However, as in ghosts, allowance has to be made for ATP hydrolysis by non-specific ATPase independent of the pump. In order to assess the magnitude of

the latter, use is made of the fact that in ghosts the ouabain-sensitive and insensitive components of the ATPase activity are about equal. (Hoffman, 1962b; Whittam and Ager, 1964). In intact cells with an acid-labile P_i content of 2.50 μ-mole/ml., non-specific ATPase activity is of the order of 1.00 μ-mole/ml. (Whittam and Ager, 1965). In the cells of Expt. V^{21}, acid-labile P_i, based on the term $(2 \times [ATP]) + [ADP]$ equals 0.40 μ-mole/ml. This would be associated with an apyrase activity of $0.16 \left[\frac{1.00 \times 0.40}{2.50}\right]$ μ-mole/ml.cell/hr.

The formation of ATP by reversal of the pump is, therefore, of the order of 180 (160 + 20) μ-mole/l.cell/hr.

$$\text{Efficiency} = \frac{180 \times 9500 \times 10^{-6}}{2.62} \times 100 = 65\%$$

Comparison of efficiencies of forward and backward reactions

The efficiency of the forward reaction of the Na pump can be calculated from the conditions prevailing in the fresh cells of Expt. V^{35}.

there, ΔG for ATP hydrolysis

$$= -7000 + 2.30\ RT \log_{10}\left[\frac{0.92 \times 0.28 \times 1.43 \times 10^{-6}}{1.28 \times 10^{-3}}\right]$$

$$= -7000 - 5023$$

$$\cong -12000 \text{ cal/mole.}$$

If the energy utilised in the extrusion of 3 Na and uptake of 2 K ions associated with hydrolysis of 1 mole of ATP is about 9000 cal (Whittam and Ager, 1965), it can readily be calculated that the efficiency of the forward reaction is approximately 75%.

Stoichiometry of the reverse reaction

Closely related to the problem of the energetics and efficiency of the reverse reaction of the Na pump is its stoichiometry, i.e., how many Na and K ions move per mole of ATP synthesized? Since net synthesis of ATP by reversal of the pump has not been demonstrated

conclusively as yet, the question centres strictly round the relationship between the number of ions moving downhill and the quantity of P_i incorporated into ATP.

Resealed ghosts

From the work on $^{32}P_i$ labelling of ATP in ghosts (Part One), a value of 60 μ-mole/l.ghost/hr was arrived at for the observed rate of the reverse reaction under optimal conditions. Allowing for simultaneous ATP hydrolysis by non-specific ATPase activity, this gave a corrected value for P_i incorporation by reversal of the pump of 360 to 560 μ-mole/l.ghost/hr. The mean value for ouabain-sensitive K efflux in identical batches of ghosts studied in Part Two was 1.20 μ-equiv/ml.ghost/hr or 1200 μ-equiv/l.ghost/hr. This yields a value for $^{K^+}/_{ATP}$ of 2.1 - 3.3.

Intact cells

We have seen that by extrapolating to cells the observed relationship between reverse and forward reactions of the transport ATPase found in ghosts and allowing for simultaneous ATP hydrolysis via non-specific ATPase activity, a value of 180 μ-mole/l.cell/hr

was obtained for the optimcl rate of the reverse reaction in cells. The mean value for ouabain-sensitive K efflux in cells was approximately 0.50 μ-equiv/ml.cell/hr or 500 μ-equiv/l.cell/hr. This gives a $^{K^+}/_{ATP}$ ratio of 2.8. The value of ouabain-sensitive influx obtained in identical batches of cells was about 1000 μ-equiv/l.cell/hr., but if we assume that the component of Na influx associated with reversal of the pump is coupled and balances an equal ouabain-sensitive K efflux, then a similar stoichiometric ratio for $^{Na^+}/_{ATP}$ would be arrived at.

DISCUSSION

The combination of a low ratio of $[ATP]$ to $[ADP]$ together with a high internal $[P_i]$ would be expected to stimulate reversal of the ATPase reaction. The chemical studies described in Part Three show that this set of conditions was achieved satisfactorily both in resealed ghosts and intact cells. Nevertheless, despite the presence of suitable cation gradients, net synthesis of ATP was not demonstrated. The reasons for this are mainly two. First, from the studies of $^{32}P_i$ incorporation described in Part One, the observed rate and absolute levels of labelling of ATP were very small even under optimal conditions. Second, although absence of external K prevents normal functioning of the Na, K-ATPase associated with the Na pump, at least half of the total membrane ATPase activity of ghosts and cells is unconnected with active cation transport (Hoffman, 1962b; Whittam and Ager, 1964; 1965) and persists independently of the state of the pump. The situation in ghosts and preincubated intact cells is therefore one in which the splitting of ATP is necessarily exceeding any trend toward net formation of ATP.

The turnover of ATP in the red cell depends on the balance between the capacity to generate adenine nucleotide and the reactions which consume ATP. In the metabolically competent cell with intact glycolysis, the former reactions predominate and the ratios of ADP to ATP and AMP to ATP are low (Whittam, 1958; Bartlett, 1959a and b; 1968). Throughout the studies in this work, generation of ATP by glycolytic mechanisms was blocked intentionally by the inclusion of small amounts of iodoacetate and fluoride in the incubation media (see p. 46). The adenylate kinase reaction ($ADP \rightleftharpoons AMP + ATP$) which is freely reversible, tends to restore ATP levels but can only do so with a stoichiometric formation of AMP. The experimental results, however, did not reveal any accumulation of AMP whilst at the same time, the total adenylate pool was decreased. The most likely explanation for this picture is that any AMP formed is rapidly degraded further to inosinate by the action of AMP deaminase (Askari and Franklin, 1965; Rao, Hara and Askari, 1968). Occurrence of an irreversible loss of adenylate by this pathway is supported by the finding of significant amounts of IMP

in red cells which have been stored in acid citrate dextrose (ACD) for several weeks at 4°C whereas no inosinate is detectable in fresh cells (Bishop, Rankine and Talbott, 1959; Bartlett and Shafer, 1961; Bishop, 1961).

The energetic considerations which have been based on the measurements of adenine nucleotide and P_i concentrations are necessarily subject to uncertainties. The values for the efficiency and stoichiometry of the reversed ATPase reaction are but approximations because of the assumptions adopted in their derivation. An added difficulty is the fact that the figure for the standard free energy of ATP in biological systems remains controversial (Philips, George and Rutman, 1963). Nevertheless, the findings show that from a thermodynamic point of view, a stoichiometric ratio of 2 to 3 Na or K : ATP for the reverse reaction is feasible. It is of particular interest that the stoichiometry of the reverse reaction is of the same order of magnitude as that which has been determined (Sen and Post, 1964; Whittam and Ager, 1965; Garrahan and Glynn, 1967c) for the ATP splitting linked to active transport of Na and K.

SUMMARY

1. Measurements have been made of the nucleotide and P_i content of resealed ghosts and cells prepared and incubated under conditions favourable for reversing the Na pump.

2. Between one- and two-thirds of the ATP trapped in the ghosts at reversal was split during the incubation necessary for resealing. Preincubation of intact cells in an all-K Ringer to lower internal Na and raise internal K resulted in a fall in ATP to about one tenth of the original levels.

3. The fall in ATP content was not accompanied by a parallel accumulation of AMP in the ghosts or cells. Incubation was accompanied by a progressive fall in total adenylate.

4. The efficiency of converting osmotic work into phosphate bond energy by reversal of the pump was found to be about 65%. This value was close to the calculated efficiency of the forward transport ATPase reaction.

5. The results showed that the stoichiometry for the

number of ions moved per mole of P_i incorporated into ATP was of the same order of magnitude as that which exists between ion movement and ATP hydrolysis during forward operation of the pump.

GENERAL DISCUSSION

The fact that the Na pump and the Na, K-ATPase share so many properties in common has made it clear that the enzyme is either wholly or substantially part of the pump mechanism. Work along a number of lines has indicated, however, that the transport ATPase system is complex and may involve an entire array of component reactions. Arising from the evidence presented in this thesis, the question may be posed: What is the functional significance of the reversibility of the transport system in terms of clarifying the molecular mechanism of the pump?

Reaction sequence

It is generally held that the liberation of P_i by the transport ATPase system represents only the terminal step of a sequence of reactions in which a phosphoryl radical is transferred from ATP to various acceptors which may or may not be involved in the process of energising osmotic work. Much work has been devoted to characterising reaction intermediates and as a result a number of partial reactions associated

with ATPase activity have been discovered in various tissues. The Na, K-ATPase from crab nerve microsomes has been found to catalyse an ATP-ADP but not an ATP-P_i exchange reaction (Skou, 1960), whilst a Na^+-activated ADP-ATP transphosphorylase reaction (apparent only at low Mg^{2+} concentrations or in the presence of N-ethyl-maleimide) has been found to be catalysed by microsomal ATPase preparations of Electrophorus electric organ (Fahn, Hurley, Koval and Albers, 1966; Fahn, Koval and Albers, 1966). A radioactive phosphorylated intermediate has been isolated when membrane ATPase preparations from a number of tissues such as kidney cortex, brain or electroplax of Electrophorus are incubated with $[\gamma\text{-}^{32}P]$ATP. In each tissue Na^+ increases, whilst K^+ decreases the amount of ^{32}P incorporated (Whittam, Wheeler and Blake, 1964; Post, Sen and Rosenthal, 1965; Nagano et al., 1967; Fahn, Koval and Albers, 1968; Lindenmayer, Laughter and Schwartz, 1968). K^+-dependent phosphatases have been isolated from microsomal preparations of several tissues displaying transport ATPase activity, though the precise relationship between these two enzymic entities is not

clear (Albers and Koval, 1966; Nagai, Izumi and Yoshida, 1966; Israel and Titus, 1967). Current opinion favours the view that K^+-dependent acylphosphatase activity represents the terminal step of the transport ATPase reaction (Glynn, 1968; Rega, Garrahan and Pouchan, 1968; Garrahan, Pouchan and Rega, 1969; Yoshida et al. 1969).

Taken as a whole, these observations have suggested that the pump mechanism involves a sequence of at least two stages: a) formation of a phosphorylated intermediate activated by outward passage of Na_i, b) a stage of dephosphorylation with liberation of inorganic phosphate to the cell interior, associated with entry of K_e. The experimental findings of Fahn, Koval and Albers (1968) and Post et al (1968) indicate that the phosphorylated intermediate itself probably exists in two distinct forms.

The occurrence in low-Na resealed red cell ghosts suspended in a high Na, K-free Ringer of a 1:1 exchange of Na which requires but does not utilise ATP, (Garrahan and Glynn, 1967a and b), has lent additional support for a Na^+-activated phosphorylation step in the ATPase

reaction. If the inward movement of K associated with the pump involves dephosphorylation, it might be expected that internal $[P_i]$ would enhance this process. Presence of internal P_i has been found necessary for the occurrence of ouabain-sensitive K efflux from Na-depleted red cells (Post and Sen, 1965; Glynn and Lüthi, 1968). The results in the present work have shown that, given the coupled gradients of Na and K, the rate of reversal of the entire pump system can be stimulated by increasing internal $[P_i]$.

However, although it would seem likely that the mechanism of the transport ATPase reaction was similar in most animal species and tissues, there have been difficulties in accepting the two-stage hypothesis involving a phosphorylated high-energy intermediate as the reaction sequence in erythrocyte membranes. Ouabain-sensitive incorporation of $^{32}P_i$ into membranes of intact red cells to an extent dependent on Na_i and K_e was described by Judah, Ahmed and McLean (1962). However, both Heinz and Hoffman (1965) and Blake, Leader and Whittam (1967) were unable to demonstrate the presence of a phosphorylated intermediate as a

component of the transport system. These failures have been particularly disappointing since the red cell is the one tissue in which it has proved possible to connect firmly the utilisation of ATP as substrate, through the action of Na,K-ATPase, to the active transport of Na and K. One reason that such an intermediate eluded detection in the erythrocyte may be the low order of enzymatic activity per unit weight in the red cell sompared to other tissues rich in ATPase. Also, occurrence of a high level of non-specific phosphorylation would tend to mask any small amount associated with the transport ATPase reaction. These obstacles have been overcome in two recent studies in which membrane-bound phosphate has been detected in erythrocyte membranes incubated with $[\gamma\text{-}^{32}P]$ATP (Bader, Post and Bond, 1968; Blostein, 1968). The parallelism between the properties of this phosphorylated material and that isolated from 14 other sources of Na,K-ATPase (Bader, Post and Bond, 1968) supports the view that the material is a functional intermediate in the transport ATPase system and points to a generally similar mechanism for the membrane ATPase reaction in

most actively transporting tissues. If, as it appears, phosphorylated intermediates are an integral part of the Na,K-ATPase reaction, then they must also participate in the reverse reaction leading to incorporation of $^{32}P_i$ into ATP which has been studied in this work. It follows that each step of the multicomponent reaction sequence should also be reversible.

Active sites and inhibitors

A number of hypothetical models have been formulated to explain the various properties of the Na pump. These are largely based on some form of shuttle or circulating carrier molecule with a high affinity for Na toward the inside of the cell and a high affinity for K toward the outside (Glynn, 1956; Whittam, 1964; 1967; Baker, 1966; Mitchell, 1967a and b; Pardee, 1968; Stone, 1968).

In order to account for the coupled pumping of Na and K, it has been proposed that a change in selectivity of the carrier occurs in the membrane with conversion of a Na- to a K-carrier (Post, Sen and Rosenthal, 1965; Baker and Connelly, 1966). What is not clear at first, is whether such a modification requires a preliminary phosphorylation step mediated by ATP

hydrolysis and, second, how the chemical transformation processes are interlinked with ion translocation.

It is quite conceivable that rather than there being two ion specific carrier sites, the system may consist merely of a single site which is vectorially orientated. A mechanism may be envisaged based on an allosteric effect of external K on the hydrolysis of ATP by the ionic attachment to a site of transport ATPase, distant from the active centre, but which somehow initiates a conformational change in a subunit of the enzyme protein (see Whittam, 1967).

Like Garrahan and Glynn (1967d), the present work has shown that ouabain blocks the reversal of the Na pump. It would appear that the inhibitor immobilises the Na pump for forward and backward running alike. The findings of Hoffman (1966) indicate that the relationship between $[K_e]$ and ouabain, as far as blocking the Na pump, is of a non-competitive nature. Since Na:Na exchange under conditions of zero $[K_e]$ is also blocked by ouabain (Garrahan and Glynn, 1967a), the mechanism of glycoside inhibition seems to involve both Na and K reaction steps of the coupled transport

ATPase system. The interaction between cardiac glycosides and Na,K-ATPase preparations derived from various tissues including the fragmented red cell membrane has been studied recently in greater detail using tritiated ouabain or digoxin (Albers, Koval and Siegel, 1968; Matsui and Schwartz, 1968; Ellory and Keynes, 1969; Hoffman and Ingram, 1969). In general, the findings have revealed an interesting parallelism between the conditions needed for binding of glycoside to the enzyme and those which characterise the phosphorylated intermediate. The precise nature of the linkages involved in formation of the membrane-bound complex and the changes which lead to inhibition of transport ATPase activity remain obscure. It is most probable that combination of glycoside with Na,K-ATPase induces a conformational alteration in the enzyme molecule connected with the state of phosphorylation of the catalytic site.

Oligomycin has attracted a lot of attention as a metabolic inhibitor through its capacity to block the phosphate transfer step of oxidative phosphorylation in mitochondria whilst leaving the electron transfer

of the respiratory chain unaffected (Lardy, Johnson and McMurray, 1958; Racker, 1965). The antibiotic also exerts direct inhibitory effects on the transport ATPase of the red cells which lack the enzymes needed for oxidative phosphorylation (Blake, Leader and Whittam, 1967).

A certain parallelism between the actions of ouabain and oligomycin is seen in their relationship to the phosphorylated intermediate of the microsomal transport ATPase system. Ouabain in low concentration (Charnock and Post, 1963; Whittam, Wheeler and Blake, 1964) and oligomycin (Whittam, Wheeler and Blake, 1964; Fahn, Koval and Albers, 1966) do not affect its Na^+-dependent formation. However, the K^+-activated dephosphorylation in kidney (Whittam, Wheeler and Blake, 1964) and also electric organ (Fahn, Koval and Albers, 1968) is inhibited by both drugs. Kinetic analysis of the inhibitory effects of oligomycin on a microsomal ATPase from beef brain has indicated that the enzyme acquires its affinity for the antibiotic only after interaction with Na and ATP (Inturrisi and Titus, 1968). Of interest, too, is the fact that inhibitory effects are exerted by oligomycin

and ouabain on the Na:Na exchange in red cells occurring in the absence of external K (Garrahan and Glynn, 1967a and b) whilst oligomycin causes partial inhibition of the reversed action of the pump (Garrahan and Glynn, 1967d).

It is hard to fit all these observations into a unitary concept of the workings of the transport ATPase system. A major problem has been the difficulty of extrapolating data from experiments on other tissues and different species to the detailed mechanisms of the transport system of red cells where, until recently, it proved difficult to isolate the phosphorylated intermediate with certainty. Although Skou (1960) was the first to propose a role for a high energy phosphorylated intermediate in the transport ATPase system, his later views (Skou, 1965) hold that phosphorylation may not be an obligatory feature of the system except for the abnormal situation when, in the absence of K_e, the K-sensitive external site becomes occupied by Na.

Relationship between pump reversal and oxidative phosphorylation

The type of energy transformation implicated in

the present study is that an increase in chemical energy might be achieved at the expense of ionic gradients. The question arises whether this is a phenomemon of general occurrence in other biological systems, notably in mitochondria.

The mammalian red cell lacks mitochondria and the cytochrome system; its energy is provided by glycolysis (Maizels, 1951), in particular, that for active transport comes from hydrolysis of ATP (see p. 15). There is evidence also in the red cell for interaction between the ouabain-sensitive ATP-utilising pump system and the generation of ATP by glycolysis (Whittam and Ager,1965). This mutual control of ATP utilisation and synthesis in relation to the pump may occur at the level of phospho-glycerate kinase action in the membrane (Parker and Hoffman, 1967).

On the other hand, the normal metabolic role of mitochondria is to carry out oxidative phosphorylation - the coupling of oxidative processes and synthesis of ATP. The precise mechanism of oxidative phosphorylation and its relation to active ion transport is one of the major problems occupying current thought in the field of

cellular metabolism. It seems clear that there is close coupling between release of respiratory energy, oxidative phosphorylation and cation transport (Lehninger, 1962; Lehninger and Wadkins, 1962).

The mechanisms underlying oxidative phosphorylation have been studied extensively. Two main mechanistic theories have been propounded:

First, the orthodox chemical coupling hypothesis where high energy intermediates are generated during electron transport via the respiratory chain and these are used to form ATP or transport ions by stoichiometrically coupled reactions which share a common intermediate (Racker, 1961, 1965; Lehninger and Wadkins, 1962).

Second, the H^+ pump hypothesis which holds that no high energy intermediates are formed, but there is instead a gradient of H^+ across the mitochondrial membrane resulting from vectorial reactions of electron transport (Mitchell, 1961; Mitchell and Moyle, 1965). This gradient is held to be the driving force for oxidative phosphorylation of ADP as well as for ion transport. ATP is formed by reversal of ATPase in the membrane

according to the general equation:-

$$ADP + P_i \rightleftharpoons ATP + H^+(inside) + OH^- (outside)$$

the reaction is essentially "pulled" to the right because the H^+ delivered to the inside compartment reacts with OH^- ions generated in excess by electron transport with the formation of water. As well as the pH gradient, the electrochemical potential difference across the membrane is a contributory factor. Both the chemical coupling and vectorial H^+ pump hypotheses agree with the general view that oxidative phosphorylation and active cation transport are alternative processes for dissipating the electron transfer energy associated with respiration.

The system responsible for oxidative phosphorylation appears to be reversible in that high rates of ATP hydrolysis, $^{32}P_i$ -ATP exchange and ATP-dependent reverse electron transport can be readily observed in mitochondria (Chance, 1961; Chance and Hollunger, 1961; Racker, 1961). Cation accumulation can be energised either by ATP via an oligomycin-sensitive reaction or by an oligomycin-insensitive reaction which depends on electron

transport (Brierley, Murer, Bachmann and Green, 1963; Lehninger, Carafoli and Rossi, 1967). A related question is whether the osmotic work associated with cation accumulation in mitochondria might also be harnessed for the synthesis of ATP. Net synthesis of ATP associated with enhanced K efflux in the presence of valinomycin has been reported in mitochondria when electron transfer was blocked with rotenone (Cockrell, Harris and Pressman, 1967). Whilst these authors have attributed their finding entirely to stimulation of an energy-linked cation transport system tightly coupled to ATP synthesis, the role of the generated membrane potential could be significant (Glynn, 1967). The resemblance between this demonstration of ATP synthesis driven by cation gradients to the findings in red cells described in this thesis, raises the question of whether a similar high energy intermediate might not be involved in the ion translocation across the membrane which features in both processes. There are, however, important differences between the transport ATPase of the red cell and the ATPase of mitochondrial membranes (see Mitchell, 1967a and b); of particular

relevance is the point of entry of P_i into the synthetic pathway leading to ATP generation. Viewed according to traditional concepts, osmotic energy from ion transport would be utilised in formation of a non-phosphorylated high energy intermediate which, on combination with P_i, would then serve as precursor of the high-energy bond of ATP. As already discussed above, synthesis and breakdown of a high energy phosphorylated intermediate may be an integral part of the mechanism involved in translocation of Na^+ and K^+ via the ATPase system of plasma membranes. However, if such is the case, P_i release from ATP breakdown would occur <u>after</u> and not before translocation of the ions.

It is thus difficult to reconcile the evidently important role of phosphorylation in the Na^+-K^+ translocator ATPase with the characteristics of H^+ translocation which features so prominently in mitochondria. Generation of a high energy intermediate in mitochondrial oxidative phosphorylation has still to be substantiated by sound experimental evidence. If, as the chemiosmotic coupling hypothesis (Mitchell, 1967a and b) maintains, the gradient of H^+ and transmembrane

potential emanating from electron transfer drives ATP synthesis directly, then it could be that no high energy intermediate at all features in the mitochondrial system.

The studies described in this thesis have not been concerned with the chemical nature of the intermediate steps involved in the hydrolysis of ATP which energises active transport of cations across the plasma membrane. By studying the behaviour of the Na, K-ATPase in its natural milieu - the intact membrane - the work has aimed at a greater understanding of the overall mechanism of the Na pump. Information has been obtained on the reversibility of the system - in particular, the utilisation of osmotic energy via the pump to produce an increase in chemical energy, but much remains to be learnt of the detailed sequence of events underlying these energy transformations. As Ussing (1965) so aptly comments: "Much more work, undoubtedly, is needed before we can settle the question of the formal nature of the (Na) pump, let alone solve the riddle of its molecular construction. So far, the search for the evasive active transport mechanism has been rather like peeling an onion. One removes

one layer after another, always hoping that removal of the next will reveal the core. We may not find any core, but in the process of peeling we may learn what the onion looks like."

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