The Connexion between Active Cation Transport and Metabolism in Erythrocytes

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1. A study has been made of the dependence on the concentrations of internal Na^+ and external K^+ of lactate and phosphate production in human erythrocytes. 2. Lactate production was stimulated by Na^+ and K^+ but only when they were internal and external respectively. The stimulation was counteracted by ouabain. The production of phosphate was affected in the same way. 3. There is a quantitative correlation between these effects and those previously found for cation movements and the membrane adenosine triphosphatase. 4. It is concluded that the rate of energy production in glycolysis is partly controlled by the magnitude of active transport; the extent of this regulation is shown to vary from 25 to 75% of a basal rate that is independent of active transport. 5. The activity of the membrane adenosine triphosphatase was also compared with rates of Na^+ and K^+ transport. The latter were varied by altering the concentrations of internal Na^+ and external K^+ , and by inhibiting with ouabain. 6. A threefold variation of active transport rate was accompanied by a parallel change in the membrane adenosine-triphosphatase activity. The results show a constant stoichiometry for the number of ions moved/mol. of ATP hydrolysed, independent of the electrochemical gradient against which the ions were moved. 7. Calculations show that the amount of ATP hydrolysed would provide enough energy for the osmotic work. The results are discussed in relation to possible mechanisms for active transport.

Schatzmann (1953) concluded that cardiac glycosides do not affect glucose metabolism in human erythrocytes in spite of active cation transport being inhibited. This view has been widely accepted, although it presents the difficulty of explaining why erythrocytes should produce the same amount of energy when their energy requirement has been decreased. Erythrocytes do not respire, and in respiring cells, in contrast, a decrease in the amount of energy required for active transport appears to be accompanied by a fall in energy production. Thus, with slices of brain and kidney cortex and of liver, direct interference with the transport mechanism, so as to stop or initiate active transport, elicits a decrease and increase respectively in the rate of respiration. Active transport is stopped either by deprivation of one of the ions (Na^+ and K^+) that is transported, or by the addition of ouabain in the presence of both ions. The magnitude of the decrease in respiration is the same with each procedure, and in the absence of one of the ions ouabain has no effect. Stimulation of the rates of Na^+ and K^+ transport causes a proportional increase in the rate of respiration (Whittam, 1961, 1962a; Elshove & Van Rossum, 1963; Whittam & Willis, 1963; Bilodeau & Elliott, 1963).

These effects of cations on respiration are related to the fact that in kidney, brain and liver slices, as in erythrocytes, one ion is transported only when the other ion is also present. The significance of cardiac glycosides is that they inhibit this coupled ion transport, and the part of the ATPase* activity that is connected with it (see Glynn, 1964). This enzymic activity is a measure of the rate at which cells utilize ATP for active transport, and it is the products of such hydrolysis in respiring cells that appear to regulate the rate of oxygen uptake. The question arises therefore whether metabolic control by active transport applies also to erythrocytes.

Further questions are whether the rate of ATP hydrolysis changes in direct proportion to variations in rates of transport, and whether the rate at which energy is liberated from ATP is sufficient to satisfy the energy required for transport. It is necessary to work with intact cells or erythrocyte 'ghosts' in studying these questions, in order to retain spatial asymmetry of the membrane. A previous study of ATP hydrolysis in relation to K^+ uptake in human erythrocytes showed that both processes fell in a parallel manner, as if there was a constant stoichi-

* Abbreviation: ATPase, adenosine triphosphatase.

ometry despite differences in the absolute rates; the energy supply was also sufficient for the energy required (Whittam, 1958). The reaction involved may be written:

The subscripts 'e' and 'i' denote external and internal respectively. The value of m , referred to below as the K^+ /ATP ratio, represents the number of K^+ ions actively taken up during the $(Na^+ + K^+)$ -sensitive hydrolysis of 1mol. of ATP; similarly n , referred to below as the Na^+ /ATP ratio, is equal to the number of Na^+ ions extruded/mol. of ATP that is hydrolysed. Estimates of the K^+ /ATP and Na^+ /ATP ratios have been made previously for a single condition of incubation (Glynn, 1962; Gardos, 1964; Sen & Post, 1964).

The object of the present work was to see whether the rates of lactate production and of ATPase activity changed when the rates of transport were varied by changes in the concentrations of external K^+ and internal Na^+ , and of ouabain. Measurements on the same cells show that metabolic activity is partly controlled by the active transport process; there are parallel changes over a threefold variation. A change in the rate of active transport always elicited the same proportional change in the rate of ATP hydrolysis, and, as a consequence, in the rate of metabolism.

METHODS

Source of erythrocytes

Blood that had been stored for about a week at 4° in 'acid-citrate dextrose' was obtained from The Blood Transfusion Centre, The Churchill Hospital, Oxford. When fresh blood was required, it was drawn from the antecubital vein with a syringe moistened with heparin (5000 units/ml.; Weddell Pharmaceuticals, The Union International Co.).

Treatment of cold-stored erythrocytes with adenosine

Erythrocytes were required in which the rate of production of P_i could be measured accurately. Cold-stored cells are unsuitable for this purpose on account of their high P_i content, but the latter can be conveniently lowered by incubation with adenosine. This treatment restores cell phosphate esters that have broken down during storage (Pranker, 1956; Gabrio, Hennessey, Thomasson & Finch, 1955; Rubinstein, Kashket & Denstedt, 1956). Blood (3 vol.) was added to 1 vol. of a suspension of adenosine ($40 \mu\text{moles/ml.}$) in $0.15M$ -NaCl. The mixture was incubated for 2 hr. at 37° and then stored for 1–3 days at 4° . Cells for the final incubation were required free of adenosine and of the inosine formed from it by deamination; they were therefore washed two to four times in about 4 times their volume of $0.15M$ -NaCl, and finally one to three times in medium *A* of composition: NaCl (150 mm); $MgCl_2$ (2 mm); imidazole-HCl

buffer, pH 7.5 (10 or 20 mm). The suspensions were left to stand for 10 min. each time before centrifugation. Examination of the absorption at 240–290 $m\mu$ of the fluid from the final wash and of the $HClO_4$ extract of the washed cells indicated that nucleosides had been removed, as would be expected from the high permeability of erythrocytes to these compounds (Whittam, 1960; Kübler & Bretschneider, 1963). The P_i concentration in cells aged about 1 week fell from 6–7 (three values) to 0.8 ± 0.05 (25 values) $\mu\text{moles/ml.}$ of cells after treatment with adenosine.

Final incubation

Erythrocytes were suspended in 4–9 times their volume of the appropriate incubation medium, obtained by modification of medium *A*, and maintained at 37° for 60 min. The pH of the cell suspensions (7.2–7.5) did not change during incubation. The low haemoglobin concentration in the medium after incubation showed that not more than 0.4–0.6% of the cells were haemolysed. The exact volume of cells/ml. of cell suspension was either determined directly, by centrifuging a sample for 30 min. at 2000g in a 'haematocrit tube' of narrow bore, or calculated from the ratio of the haemoglobin concentration in the suspension to that of cells packed for 30 min. at 2000g.

For estimation of P_i and lactate production, 5 ml. samples of suspension were deproteinized, either initially or after incubation, by the addition of 0.5 ml. of 50% (w/v) trichloroacetic acid or 0.4 ml. of 60% (w/w) $HClO_4$. The precipitated material was removed by centrifugation, and P_i was determined on samples of the clear extract. For enzymic estimations, 2 ml. samples of $HClO_4$ extract were transferred to graduated test tubes containing 1 drop of universal indicator, and cooled in ice. Then 2N-KOH was added dropwise to bring the pH to about 7, and after a further 10 min. at 0° the precipitate of $KClO_4$ was spun down and the total volume noted.

Variation of erythrocyte cation composition by the 'lactose' procedure

Previous workers have shown that the cation content of erythrocytes may be varied by storing them for different periods at 4° in media containing a range of Na^+ and K^+ concentrations (Post & Jolly, 1957; Post, Merritt, Kinsolving & Albright, 1960). This procedure has the disadvantage that, after storage for different periods, cells vary in their metabolic state as well as in Na^+ concentration. A more convenient approach makes use of the increase in permeability that takes place when cells are incubated in a solution of non-electrolyte (Maizels, 1935; Davson, 1939; Bolingbroke & Maizels, 1959). Incubation in lactose solution results in a fall of 85% in the total Na^+ and K^+ content of the cells, and of 30% in cell volume (Bolingbroke & Maizels, 1959). The shrunken cells are highly permeable to cations, and when incubated in mixtures of $0.3M$ -NaCl and KCl they regain their original salt content; on resuspension in a medium iso-osmotic with plasma their volume is restored to that of normal cells (McConaghey & Maizels, 1962). The cation permeability may be lowered after this loading procedure by adding $CaCl_2$ to the cell suspension and to the media in which the cells are later washed and incubated (Bolingbroke & Maizels, 1959). Cells treated in this way

are able to transport ions actively only if their phosphate ester content is restored, e.g. by including adenosine in the loading and incubation media (McConaghey & Maizels, 1962).

The procedure of Bolingbroke & Maizels (1959) and McConaghey & Maizels (1962) has been adapted to allow measurement of P_i and lactate production in erythrocytes of varied cation composition. The experimental procedure falls into three stages.

Stage 1: cation depletion. Erythrocytes aged about 1 week were washed twice with lactose solution (6%, w/v), suspended in about 10 times their volume of the same solution and incubated for 3 hr. at 37°.

Stage 2: cation loading. The erythrocytes were next washed twice in medium of composition: NaCl (300 mm); Na_2HPO_4 (0.25 or 0.5 mm); imidazole-HCl buffer, pH 7.5 (40 mm). Where appropriate the NaCl of the medium was replaced partially or completely by KCl or LiCl, and Na_2HPO_4 by K_2HPO_4 . The washed cells (1 vol.) were suspended in 4 vol. of this medium, modified by the addition of adenosine (10 $\mu\text{moles/ml.}$), and were incubated at 37° for 2.5 hr. Sufficient M-CaCl_2 was then added to give a final concentration of 2.5 mm, and after a further 30 min. at 37° the suspension was left overnight at 4°. The cells were then washed four times with medium B, which had the composition: NaCl (150 mm); MgCl_2 (2 mm); CaCl_2 (2.5 mm); imidazole-HCl buffer, pH 7.5 (20 mm); glucose (10 mm). The suspensions were allowed to stand for 10 min. each time before centrifugation, so that unused adenosine and inosine could leak out of the cells. After four washes the extinction at 250 $\mu\mu$ of the supernatant fluid obtained on centrifugation corresponded to a concentration of hypoxanthine-containing material of only 0.35 $\mu\text{mole/ml.}$ However, examination of the extinction of neutralized cell extracts, by the procedure of Szentkiralyi (1957), indicated that, in addition to 1.6 ± 0.1 (7) μmoles of adenine-containing compounds/ml. of cells, hypoxanthine-containing material [2.6 ± 0.3 (7) $\mu\text{moles/ml.}$ of cells] was also present. This may have included IMP, of which 1.2 μmoles are formed/ml. of cells when cold-stored erythrocytes are incubated with inosine (J. S. Wiley & R. Whittam, unpublished work; see Bartlett & Schafer, 1961). Paper chromatography in the isobutyric acid-aq. NH_3 solvent of Krebs & Hems (1953), in 0.1M-borate buffer, pH 10 (Whittam, 1960), and in aq. NH_3 , pH 10 (Wyatt, 1955), and paper electrophoresis in 0.1M-citrate buffer, pH 3.5, indicated the presence of material moving at the same rate as inosine and possibly of IMP, but not of hypoxanthine. It seems possible that the final addition of CaCl_2 to cells prepared by the 'lactose' procedure renders them less permeable than normal cells to nucleosides. However, the results indicate that the concentration of nucleoside in the final incubation medium would be less than 0.5 mm after equilibration between cells and medium, and could not account for the lactate produced during incubation with glucose for up to 3.5 hr. Moreover, net P_i liberation occurred during the final incubation with glucose, in contrast with the fall in P_i concentration associated with nucleoside metabolism.

Stage 3: final incubation. After four washes in medium B, the erythrocytes were finally suspended in 4-9 times their volume of this medium, modified as required for each particular experiment, and incubated at 37° for periods of up to 3.5 hr. The pH of the suspensions (7.5) did not change during incubation. The haemoglobin concentration in the

Table 1. *Variation of erythrocyte cation composition by the 'lactose' procedure*

Erythrocytes aged 1 week were washed twice with 6% (w/v) lactose solution and then incubated under the following conditions. Stage 1: for 3 hr. at 37° in 6% (w/v) lactose. Stage 2: for 3 hr. at 37° in 300 mm-(NaCl+KCl)-40 mm-imidazole-HCl buffer (pH 7.5)-0.5 mm- Na_2HPO_4 -10 mm-adenosine, plus CaCl_2 (2.5 mm) after 2.5 hr., and then overnight at 4° on the same medium. Stage 3: the cells were finally washed four times with medium B and twice with 0.15M-choline chloride, and were then haemolysed for flame photometry.

Concn. of cation in loading medium (mm)		Final cation content of cells ($\mu\text{equiv./ml.}$ of cells)		
Na ⁺	K ⁺	Na ⁺	K ⁺	Na ⁺ +K ⁺
40	260	12	108	120
50	250	16	101	117
75	225	24	89	113
100	200	31	78	109
150	150	50	65	115
300	0	101	12	113

medium after incubation showed that only 0.8-1.4% haemolysis had occurred. Lactate and P_i production were determined in the way described above. For measurements of cell cation composition, cells were washed twice in 15-30 times their volume of cold 0.15M-choline chloride, and then haemolysed in distilled water for flame photometry.

Composition of cells prepared by the 'lactose' procedure. In several experiments cells were required with a range of internal Na^+ concentrations, and for this purpose erythrocytes were incubated first with lactose solution and then with 'loading media' containing various proportions of NaCl and KCl, with a total concentration of 0.3M. Table 1 shows the final Na^+ and K^+ contents of cells, expressed in $\mu\text{equiv./ml.}$ of initial cells; the unit here, and in other experiments with 'lactose-treated' cells, refers to the number of cells present in 1 ml. of cold-stored erythrocytes, packed for 30 min. at 2000g without any pretreatment. Since the cells return to their normal volume in stage 3 of the 'lactose' procedure (McConaghey & Maizels, 1962), this unit gives the actual cation concentration in the final cells.

The cell Na^+ content was proportional to the concentration in the loading medium rising from 12 to 101 $\mu\text{equiv./ml.}$ as the Na^+ concentration used for loading increased from 40 to 300 mm. Similarly, the K^+ content of the erythrocytes increased in linear fashion as the K^+ concentration in the loading medium was raised to 260 mm. A remnant of the original cell K^+ was retained throughout the preparative procedure, however, since cells prepared in a K^+ -free loading medium had a final K^+ content of 12 $\mu\text{equiv./ml.}$ The total amount of cation taken up by the cells was independent of the proportions of Na^+ and K^+ present; it was similar in magnitude (109-120 $\mu\text{equiv./ml.}$) to the cation content of normal erythrocytes, confirming that the cells regain their original volume.

Enzymic estimation showed that the concentrations of ATP, ADP and AMP in cells treated by the 'lactose'

procedure were 1.0, 0.5 and 0.3 $\mu\text{mole/ml.}$ of cells respectively. The addition of adenosine to the loading medium thus restored the intracellular ATP concentration to a value similar to that in fresh erythrocytes. The cells contained rather lower concentrations of phosphate esters than are found in normal cells, however, since in five experiments the total acid-soluble phosphate content was only 8–12 $\mu\text{moles/ml.}$ of cells.

Estimation procedures

K⁺ influx. This was measured according to the method of Glynn (1956), and net changes in Na⁺ and K⁺ contents of the cells were determined by flame photometry.

Orthophosphate. This was estimated in deproteinized extracts by the method of Fiske & Subbarow (1925), in which there is no appreciable hydrolysis of phosphate esters (Bartlett, 1959). The s.e.m. for a mean of 1.58 $\mu\text{moles/ml.}$ of cells for four estimations was 0.22 (range 1.53–1.61). Total acid-soluble phosphate was determined as P_i after digesting 0.1ml. samples of extract with 0.1ml. of 70% (w/w) HClO₄.

Haemoglobin. This was estimated as oxyhaemoglobin, from the extinction at 540 m μ of suitably diluted samples, clarified with aq. NH₃ as described by King (1951).

Na⁺ and K⁺. These were estimated with an EEL flame photometer. Samples were diluted to contain less than 0.2mm-Na⁺ or -K⁺, freed of cell debris by centrifugation, and read three times in alternation with standard solutions of NaCl or KCl. The cation concentration in erythrocytes was calculated by multiplying the concentration in the haemolysate by the ratio of the haemoglobin concentration in packed cells to that in the haemolysate.

Lactate. This was estimated enzymically, by a procedure similar to that used by Hohorst, Kreutz & Bücher (1959) and described in more detail by Hohorst (1963).

Adenine nucleotides. These were estimated enzymically with 'test kits' supplied by C. F. Boehringer und Soehne G.m.b.h., Mannheim, Germany. The methods were similar to those described by Adam (1963).

RESULTS

Stimulation of phosphate and lactate production by external K⁺. Table 2 shows that omission of K⁺ from the medium, or the addition of ouabain in

the presence of K⁺, caused a fall in the amounts of P_i and lactate produced in fresh cells. Lactate production fell from 1.9 to 1.4 $\mu\text{moles/ml.}$ of cells/hr., and P_i production from 0.62 to about 0.45 $\mu\text{mole/ml.}$ of cells/hr. Moreover, in the K⁺-free medium ouabain had no effect. This combination of results suggests that the effects arose through changes in the activity of the membrane ATPase that is involved in active transport. The extent of this activity will be given by the sum of the P_i and lactate production that is sensitive to external K⁺ and to ouabain. This enzymic activity in the absence of lactate production would simply be shown by P_i production. However, in cells forming lactate, the K⁺-dependent lactate production has to be taken into account, because each lactate mol. produced necessitates the esterification of 1mol. of P_i when glyceraldehyde 3-phosphate is oxidized to 1,3-diphosphoglycerate.

In view of the nature of the stimulation of K⁺ influx by external K⁺, with half-maximum activation by 2mm-K⁺ (Glynn, 1956), it was decided to test whether the stimulation of ATPase activity showed the same features. When ouabain was present, the rates of P_i and lactate production were independent of the external K⁺ concentration, being 0.2–0.3 and 1.6–1.8 $\mu\text{moles/ml.}$ of cells respectively. Fig. 1, in contrast, shows that there was a gradual increase in ouabain-sensitive activity as the K⁺ concentration was raised to 10mm. The P_i and lactate production were alike in showing the same dependence on K⁺ concentration. The lines in Fig. 1 represent equations of the type:

$$V = \frac{\alpha[\text{K}^+]_e}{\beta + [\text{K}^+]_e}$$

The value of β is 1.8mm and values of α (in $\mu\text{moles/ml.}$ of cells/hr.) are 0.35 for P_i, 1.2 for lactate and 1.55 for the sum of P_i and lactate. The fact that more lactate than P_i was sensitive to external K⁺ indicates that some 80% (i.e. 1.2/1.55) of the P_i liberated by K⁺-dependent ATPase activity became

Table 2. Sensitivity of orthophosphate and lactate production by erythrocytes to external K⁺ and ouabain

Freshly drawn erythrocytes were washed three times with medium B (see the Methods section) and added (1ml.) to 9ml. of this medium, modified to contain KCl (10mm) and ouabain (40 μM) where indicated. A 0.8ml. portion of 60% (w/w) HClO₄ was added initially or after incubation for 60 min. at 37°, and lactate and P_i were determined.

	Lactate production ($\mu\text{moles/ml.}$ of cells/hr.)				P _i production ($\mu\text{mole/ml.}$ of cells/hr.)			
	+K ⁺	+K ⁺ + ouabain	−K ⁺	−K ⁺ + ouabain	+K ⁺	+K ⁺ + ouabain	−K ⁺	−K ⁺ + ouabain
Expt. 1	1.9	1.5	1.4	1.4	0.62	0.43	0.46	0.42
Expt. 2	1.7	1.4	—	—	0.86	0.61	—	—

Fig. 1. K^+ -dependence of ouabain-sensitive P_i and lactate production. Erythrocytes, loaded with Na^+ by the 'lactose' procedure, were incubated at 10% haematocrit in medium B, modified to contain KCl (0.5–20 mm) and ouabain (40 μM) where appropriate. A 0.8 ml. portion of 60% (w/w) $HClO_4$ was added to 10 ml. samples of suspension either initially, or after incubation for 1 hr. at 37°, and P_i and lactate were determined. The graph shows the K^+ -dependence of the ouabain-sensitive components of P_i (○) and lactate (Δ) production, and of their sum, which represents ouabain-sensitive ATPase activity (●). The curves are drawn to fit equations of the type: $V = \alpha[K^+]_e / (\beta + [K^+]_e)$, where V represents the reaction rate and $[K^+]_e$ the external K^+ concentration. The values of the constant α (in $\mu moles/ml.$ of cells/hr.) are: for P_i , 0.35; for lactate, 1.2; for P_i + lactate, 1.55. The value of β is 1.8 mm.

esterified. The quantitative similarity between the dependence of lactate and P_i production on external K^+ , and the comparable dependence of K^+ influx, is a further indication that the metabolic effects arise from changes in the activity of the transport system.

The above results do not agree with Schatzmann's (1953) conclusion, and it is therefore necessary to try to explain the different results. An important point is that Schatzmann (1953) measured, not lactate or glucose, but the evolution of carbon dioxide from a Ringer's solution buffered with bicarbonate. Moreover, his cells contained haemoglobin that had been converted into carbon monoxide-haemoglobin. A possible reason for the lack of effect of ouabain is low pH, although this was said to be approx. 7.2 with 5% carbon dioxide and 9.2 mm-sodium hydrogen carbonate. The haematocrit is not given, so it is impossible to refer $\mu l.$ of carbon dioxide evolved to unit volume of cells. Table 3 shows the dependence of ouabain-sensitive lactate production on pH. The sensitivity is greatest at pH 7.2 and 7.5, but falls to zero at pH 6.8. The total lactate production is markedly dependent on pH, as shown by the control values, in agreement with Clarkson & Maizels (1955). Although these results do not resolve the discrep-

Table 3. pH-dependence of lactate production by erythrocytes

Erythrocytes aged about 1 week were washed three times with 0.15 M-NaCl and twice with medium of composition: NaCl (120 mm); KCl (10 mm); $MgCl_2$ (2 mm); glucose (10 mm); tris-HCl buffer, pH 7.4 (30 mm). Portions of cell suspension (haematocrit 0.2 or 0.3) were adjusted to pH values from 6.5 to 9.0 by the addition of N-NaOH or N-HCl with constant stirring. Samples of suspension (2 ml.), with or without ouabain (25 μM), were maintained for 0 or 60 min. at 37°, and deproteinized by adding 2 ml. of 6% (w/w) $HClO_4$; lactate was determined on the neutralized extracts. The pH values remained unchanged during incubation, except at pH 8 and 9, when a fall of 0.2 pH unit occurred. Haemolysis increased from 0.5% at pH 6.5 to 1% at pH 7.5 and to 5% at pH 9.0. Two further experiments covering the same pH range gave similar results.

Initial pH	Lactate production ($\mu moles/ml.$ of cells/hr.)		
	Control	+ Ouabain	Inhibition by ouabain
6.5	0.2	0.2	0
6.8	0.5	0.5	0
7.2	1.8	1.4	0.4
7.5	3.2	2.7	0.5
8.0	4.2	4.1	0.1
9.0	2.5	2.4	0.1

ancy with Schatzmann's (1953), they confirm the importance of pH.

Control of lactate and phosphate production by internal Na^+ . The dependence of lactate and P_i production on internal Na^+ was measured in cells whose Na^+ content had been changed during preliminary treatment with lactose. A check was first made to test whether these cells had the same properties as fresh cells when their Na^+ content was the same. This was found to be so, for in fresh cells the sum of ouabain-sensitive lactate and P_i production was $(1.9 + 0.62) - (1.5 + 0.43) = 0.6 \mu mole/ml.$ of cells/hr. (Table 2), whereas in lactose-treated cells containing 12 m-equiv. of $Na^+/ml.$ of cells the value was also $0.6 \mu mole/ml.$ of cells/hr. A further preliminary check showed that lactate was produced at a constant rate throughout the 3.5 hr. period of incubation.

The cell Na^+ concentration was varied from 12 to 101 $\mu equiv./ml.$ of cells. In the presence of ouabain, lactate and P_i production were unaffected by the Na^+ concentration. In cells without ouabain, however, there was a marked effect of internal Na^+ . The P_i production for an incubation of 3.5 hr. was raised from 0.5 to 1.3 $\mu moles/ml.$ of cells as the Na^+ concentration was raised from 12 to 50 $\mu equiv./ml.$ of cells (Fig. 2). Little further increase occurred with higher Na^+ concentrations. Again, the rate of

lactate production was affected similarly, the comparable increase being from 1.6 to 3.5 $\mu\text{moles/ml.}$ of cells. The ouabain-sensitive components of lactate and P_i production each attained half their maximum rates with an internal Na^+ concentration of about 20 $\mu\text{equiv./ml.}$ of cells. This value is close

to that for half-maximum activation of Na^+ extrusion and of the membrane ATPase (Post & Jolly, 1957; Post *et al.* 1960).

Stimulation by external alkali-metal ions. Other alkali-metal ions can replace K^+ in being transported inwards and in activating the membrane ATPase (Solomon, 1952; Kahn, 1962; Whittam & Ager, 1964). Table 4 shows that they are also similar in regulating lactate and P_i production in intact cells. The alkali-metal ions K^+ , Rb^+ , Cs^+ and Li^+ caused the same increase in P_i production over that in the control in sodium chloride (Table 4). The stimulation was abolished by ouabain. There was no further increase on adding 10mm- K^+ , indicating that maximum stimulation was being achieved with 75mm- Rb^+ , 75mm- Cs^+ and 110mm- Li^+ . These ions also stimulated lactate production, again to the same extent as did K^+ . Further, the extent of the inhibition by ouabain for each of the alkali-metal ions Li^+ , K^+ , Rb^+ and Cs^+ was the same as the extent of the stimulation.

Lack of effect of Ca^{2+} in the medium. Similar results for lactate and P_i production were obtained irrespective of whether the medium contained 2.5mm-calcium chloride. Thus in Table 2 the results are the same for fresh cells incubated in a medium containing Ca^{2+} as those in Tables 3 and 4 where the medium did not contain Ca^{2+} . Again, in Figs. 1 and 2 the medium contained Ca^{2+} , and the results (with lactose-treated cells) are similar to those for cells not exposed to Ca^{2+} .

Fig. 2. Dependence of P_i and lactate production on internal Na^+ concentration. Erythrocytes were loaded with different concentrations of Na^+ as described in Table 1, washed four times with medium B containing K^+ (10mm) and suspended at about 15% haematocrit in the same medium, modified to include ouabain (50 μM) where appropriate. A 0.4ml. portion of 60% (w/w) HClO_4 was added to 5ml. samples of suspension initially or after incubation for 3.5 hr. at 37° , and P_i and lactate were determined. The graph shows ouabain-sensitive P_i (○) and lactate (Δ) production and their sum (●), which represents ouabain-sensitive ATPase activity.

Table 4. *Effect of different alkali-metal ions on lactate and orthophosphate production in cold-stored erythrocytes*

For Expt. 1, washed erythrocytes aged 1 week were added (1ml.) to 4ml. of medium of type A, in which Na^+ was replaced where appropriate by K^+ , Rb^+ , Cs^+ or Li^+ , and containing glucose (10mm) with or without ouabain (20 μM). A 0.4ml. portion of 60% (w/w) HClO_4 was added initially or after incubation for 60 min. at 37° , and lactate was determined on neutralized extracts. In Expt. 2 the erythrocytes were pretreated with adenosine (see the Methods section), washed four times and added (1ml.) to 4ml. of medium A, modified by the addition of appropriate quantities of 150mm- KCl , - RbCl , - CsCl or - LiCl , with or without ouabain (60 μM). A 0.5ml. portion of 50% (w/v) trichloroacetic acid was added either initially or after incubation for 60 min. at 37° , and P_i was determined.

Cation composition of medium		Lactate or P_i production ($\mu\text{moles/ml. of cells/hr.}$)			
		Control	+ Ouabain	Inhibition by ouabain	Stimulation by K^+ , Rb^+ , Cs^+ or Li^+
Expt. 1 (lactate production)	Na^+ (150mm)	2.1	—	—	—
	K^+ (10mm) + Na^+ (140mm)	2.6	2.2	0.4	0.5
	Rb^+ (10mm) + Na^+ (140mm)	2.5	2.1	0.4	0.4
	Cs^+ (150mm)	2.5	1.9	0.5	0.4
	Li^+ (150mm)	2.4	2.0	0.4	0.3
Expt. 2 (P_i production)	Na^+ (150mm)	1.5	1.5	0	—
	K^+ (75mm) + Na^+ (75mm)	2.1	1.6	0.5	0.6
	Rb^+ (75mm) + Na^+ (75mm)	2.0	1.7	0.3	0.5
	Cs^+ (75mm) + Na^+ (75mm)	2.0	1.6	0.4	0.5
	Li^+ (110mm) + Na^+ (40mm)	2.0	—	—	0.5

Table 5. *Effect of ouabain on K⁺ influx in erythrocytes depleted of ATP*

In the first two experiments shown in this Table, erythrocytes were washed five times to remove glucose, left for 3 days at 4° in medium A (containing 10mm-K⁺) and incubated for 0-8 hr. at 37° in the same medium. They were then washed twice in medium A and samples were taken for the estimation of ATP. The remaining cells were suspended (at about 10% haematocrit) in medium A, modified to contain K⁺ (10mm, including 0.1μC of 42K/ml.), and ouabain (20μM), glucose (10mm) or adenosine (5mm) where appropriate. In the last two experiments shown washed cells were loaded with Na⁺ by the 'lactose' procedure, modified by omitting adenosine from the loading medium. In the last experiment, the cells were also preincubated for 2 hr. in glucose-free medium B after the loading procedure. Finally the cells were washed four times in glucose-free medium B, and suspended in the same medium, modified to include K⁺ (10mm), and ouabain (40μM), glucose (10mm) or adenosine (2.5 or 5mm) where appropriate. In each experiment K⁺ influx was determined after incubation for 60 min. at 37°.

'Lactose', treatment	Total period of preincubation at 37° (hr.)	Cell age (weeks)	Rate of K ⁺ influx (μequiv./ml. of cells/hr.)							Initial concn. of ATP (μmole/ml. of cells)	
			Additions to medium					Inhibition by ouabain in absence of glucose	Stimulation by glucose		
			None	Ouabain	Glucose		Adenosine + ouabain				
					Glucose	+ ouabain					
-	0	2	1.3	0.4	1.9	0.4	3.5	0.5	0.9	0.6	0.3
-	6		0.5	0.3	0.6	0.3	2.9	0.4	0.2	0.1	0.1
-	8	1	0.4	0.2	0.5	0.2	3.2	0.3	0.2	0.1	0.1
+	6	2	0.9	0.7	1.1	0.7	2.7	0.6	0.2	0.1	0.1
+	8	2	0.7	0.5	0.7	0.4	2.6	0.5	0.2	0	0.05

Ouabain-sensitive K⁺ influx as a measure of active K⁺ influx. Glynn (1957) found that the inhibition of K⁺ influx by cardiac glycosides was greater than the fall produced by glucose deprivation. One possible explanation is that glycosides retard passive tracer movements as well as active ones (see also Hoffman, 1964). Another explanation is that glucose deprivation did not completely deprive the cells of other sources of energy for active transport. To test these possibilities, the action of ouabain has been examined in cells with an ATP concentration so low that glucose caused no significant stimulation of K⁺ influx. Cells were depleted of glucose by washing, and then of ATP either by incubation for several days at 4° or for some hours at 37°. Table 5 shows that K⁺ influx depended on the ATP concentration in the cells. Cells that contained 0.3 μmole of ATP/ml. (about one-third of that in fresh cells) had a K⁺ influx of 1.3 μequiv./ml. of cells/hr. even without glucose, and of 1.9 μequiv./ml./hr. with glucose. Comparable values for cells with less than 0.1 μmole of ATP/ml. were 0.5 and 0.6 μequiv./ml./hr., showing that there was no response to glucose. In the latter cells, there was no irreversible damage to the membrane, but only deprivation of ATP, since the addition of adenosine (a precursor of ATP) raised the influx to 2.9 μequiv./ml./hr. In each of the three conditions in Table 5, the values for K⁺ influx after adding ouabain were 0.3-0.5 μequiv./ml./hr., which are indistinguishable from the control values in cells deprived of ATP. When cells are sufficiently deprived of ATP so that they have little response to the addition of glucose, there is then little inhibition of K⁺ influx by ouabain. Ouabain-sensitive K⁺ influx may therefore be taken to represent the magnitude of active transport. A similar conclusion may be drawn from the other experiments shown in Table 5.

Effect of external K⁺ concentration on active K⁺ influx. To discover whether active K⁺ influx depends on the external K⁺ concentration in the same way as ATPase activity, erythrocytes that had been loaded with Na⁺ by the 'lactose' procedure were incubated in media containing K⁺ (0.5-20mm) with or without ouabain (40 μM). Fig. 3 shows that the rate of K⁺ influx rose steeply from 0.4 to 3.2 μequiv./ml. of cells/hr., as the K⁺ concentration was increased from 0.5 to 4mm, and then flattened off to reach 4.4 μequiv./ml./hr. with 20mm-K⁺. In medium containing ouabain, however, influx was much lower, with a maximum value of 1.2 μequiv./ml./hr. Thus the component of influx sensitive to ouabain rose sharply as the K⁺ concentration was brought to 4mm, but approached a plateau with higher K⁺ concentrations. The curves drawn for K⁺ influx, *M*, in Fig. 3 each represent an equation of the following type, previously used by Shaw

Fig. 3. Dependence of K^+ influx on external K^+ concentration. Erythrocytes, loaded with Na^+ according to the 'lactose' procedure, were washed four times in medium *B* containing no K^+ and suspended at 1% haematocrit in the same medium, modified to include K^+ (0.5–20 mm), and ouabain (40 μM) where appropriate. The K^+ influx was determined after incubation for 60 min. at 37°. The curves relating influx, M , and external K^+ concentration $[K^+]_e$, are drawn to fit equations of the type: $M = \alpha[K^+]_e/(\beta + [K^+]_e)$. The values of the constants are as follows. Ouabain-sensitive influx (Δ): $\alpha = 3.5 \mu\text{moles/ml. of cells/hr.}$, $\beta = 1.8 \text{ mm}$; ouabain-insensitive influx ($\bullet$): $\alpha = 1.5 \mu\text{moles/ml./hr.}$, $\beta = 8 \text{ mm}$; total influx ($\circ$): $\alpha = 4.8 \mu\text{moles/ml./hr.}$, $\beta = 2.5 \text{ mm}$.

(1955) and Glynn (1956) to describe total K^+ influx:

$$M = \frac{\alpha[K^+]_e}{\beta + [K^+]_e}$$

The constant β represents the external K^+ concentration, $[K^+]_e$, at which influx reaches half its maximal value, α . For ouabain-sensitive K^+ influx the appropriate values of α and β were 3.5 $\mu\text{equiv./ml. of cells/hr.}$ and 1.8 mm respectively. This result is similar to the values of β for glucose-dependent K^+ influx (2.2 and 2.5 mm) obtained by Glynn (1956).

Influx in the presence of ouabain could also be fitted to an equation of the type shown above. Fig. 3 shows that the rates of K^+ influx fell close to those associated with values of 1.5 $\mu\text{equiv./ml./hr.}$ and 8 mm for the constants α and β respectively.

Total K^+ influx may be described as the sum of two components of type M , representing active and passive influx. It may also be described, within the experimental error, by a further equation of the type given above, with values for α and β of 4.8 $\mu\text{equiv./ml./hr.}$ and 2.5 mm respectively (Fig. 3). The estimates of β obtained by Glynn (1956) for total K^+ influx in fresh cells (1.7–2.5 mm) were of similar magnitude; the lower value of α (2.1 $\mu\text{equiv./ml./hr.}$) in his experiments would be expected in fresh cells, since their low Na^+ content does not allow a maximal rate of active transport. A linear component of K^+ influx, equal to $0.006[K^+]_e$, was also described by Glynn (1956) for human erythrocytes, but would

Fig. 4. Relationship between active K^+ influx and ouabain-sensitive ATPase activity in media of varied K^+ concentration. The K^+ -dependence of active K^+ influx and of ouabain-sensitive ATPase activity were determined in a single experiment, that illustrated in Figs. 1 and 3. The results have been plotted to show directly the relationship between the ouabain-sensitive components of K^+ influx and of ATPase activity at the various K^+ concentrations (1–20 mm) indicated beside the points on the graph.

only be evident at higher K^+ concentrations than those used here.

Constancy of the K^+ /ATP ratio in media of varied K^+ concentration. The same dependence on external K^+ concentration has been noted for the ATPase activity of erythrocyte 'ghosts' (Whittam & Ager, 1964) and erythrocytes (Fig. 1) and for active K^+ influx. Each process shows saturation kinetics with respect to external K^+ , and is half-maximally activated in medium containing about 2 mm- K^+ . The similar response of active transport and ATPase activity to external K^+ is brought out clearly in Fig. 4, where the rate of active K^+ influx is plotted against the associated ATPase activity (computed as the sum of ouabain-sensitive P_i and lactate production). The points in Fig. 4 fall close to a straight line, indicating that a change in influx was accompanied by a proportional change in ATPase activity. The K^+ /ATP ratio was thus constant over a range of K^+ concentrations from 1 to 20 mm. Its mean value was 2.2 ± 0.1 (6) and the line in Fig. 4 has been drawn with this gradient. With fresh cells incubated in media containing 5–20 mm- K^+ , the mean of five results was 2.4 ± 0.15 (S.E.M.).

Effect of internal Na^+ concentration on K^+ influx. Previous workers have shown that the rate of net active transport of Na^+ and K^+ depends on cell Na^+ content (Flynn & Maizels, 1949; Post & Jolly, 1957; McConaghey & Maizels, 1962). The effect of internal Na^+ concentration on the rate of K^+ influx has now been determined, with erythrocytes loaded with various proportions of sodium chloride and potassium chloride by the 'lactose' procedure.

Table 6. *Values of the K⁺/ATP ratio in erythrocytes of varied Na⁺ content*

Erythrocytes were loaded with different concentrations of Na⁺ by the 'lactose' procedure, and suspended in medium B containing K⁺ (10mm) with or without ouabain (40μM). P_i and lactate production and K⁺ influx were determined after incubation for 1hr. at 37°. Column (a) is the sum of ouabain-sensitive P_i and lactate production.

Internal concn. of Na ⁺ (μequiv./ml. of cells)	Ouabain-sensitive ATPase activity (μmoles/ml. of cells/hr.) (a)	Ouabain-sensitive K ⁺ influx (μequiv./ml. of cells/hr.) (b)	K ⁺ /ATP (b):(a)
12	0.8	1.8	2.3
18	1.6	2.8	1.8
31	2.0	4.3	2.1
100	2.0	4.2	2.1

The rate of K⁺ influx rose from 2.6 to 5.2 μequiv./ml. of cells/hr. as the Na⁺ concentration in the cells increased from 10 to 30 μequiv./ml. of cells, and then remained more or less constant as the Na⁺ concentration was increased above this value. In the presence of ouabain, however, the low rate of K⁺ influx (0.6–0.9 μequiv./ml./hr.) was unaffected by changes in cell Na⁺ content. Half-maximal ouabain-sensitive K⁺ influx would be associated with a Na⁺ concentration of roughly 15 μequiv./ml. of cells.

Table 6 shows that the rates of K⁺ influx and ATPase activity increased in constant proportion as the cell Na⁺ concentration was raised from a value (12 μequiv./ml. of cells) giving less than half-maximal activation to the high value of 100 μequiv./ml. of cells. The K⁺/ATP ratio was thus independent of Na⁺ concentration in this range, and had the values 2.3, 1.8, 2.1 and 2.1 with a mean of 2.1 ± 0.1 (4).

Parallel effect of low concentrations of ouabain on K⁺ influx and ATPase activity. ATPase activity and K⁺ influx were determined simultaneously in cells in media containing K⁺ (10mm) and ouabain (0.1μM–0.2mm). Fig. 5 shows the relationship between the inhibition of K⁺ influx caused by a given concentration of ouabain and the fall in ATP hydrolysis under the same conditions. The points fall close to a straight line, indicating that any inhibition of K⁺ influx caused by ouabain was accompanied by a proportional fall in ATPase activity. The mean value of the K⁺/ATP ratio was 2.7 ± 0.1 (6), which is the slope of the line in Fig. 5.

Effect of internal Na⁺ concentration on net movements of Na⁺ and K⁺. The effect of internal Na⁺ concentration on net Na⁺ and K⁺ movements was

Fig. 5. Parallel inhibition of K⁺ influx and of ATPase activity by various concentrations of ouabain. The effects of various concentrations of ouabain on ATPase activity and K⁺ influx were determined in the same experiment. Cells aged 6 weeks were preincubated with adenosine, washed, and incubated for 1hr. at 37° in medium A containing K⁺ (10mm) and ouabain (0.1μM–0.2mm). The Figure shows the relationship between the fall in K⁺ influx and in ATP hydrolysis caused by the concentration of ouabain (μM) indicated beside each point.

also investigated. Fig. 6 shows the changes in cell cation content (in μequiv./ml. of cells) that occurred when cells were incubated for 3.5hr. in media containing K⁺ (10mm) with or without ouabain (40μM). At low internal Na⁺ concentrations, net movements were small; during the incubation, cells with a Na⁺ content of 12–16 μequiv./ml. showed little K⁺ uptake (–2 to +2 μequiv./ml.) and Na⁺ extrusion of only 3.5–6 μequiv./ml. As the Na⁺ content increased to 50 μequiv./ml., net K⁺ uptake rose to 9 and Na⁺ extrusion to 16 μequiv./ml. Further increase in Na⁺ concentration brought little further rise in the net transport. Net K⁺ uptake was always less than net Na⁺ extrusion, but both quantities increased in a similar way with internal Na⁺ concentration, and in each of three experiments reached a plateau with a Na⁺ content of about 50 μequiv./ml. Fig. 6 shows that, in the presence of ouabain, the transport of Na⁺ and K⁺ against their concentration gradients was abolished. The low rates of passive cation transport then observed, rarely exceeding 1.5 μequiv./ml. of cells/hr., indicate how completely the cells recovered a low cation permeability after the treatment with lactose.

The difference between the rates of transport in the presence and absence of ouabain has been taken as a measure of active Na⁺ extrusion and K⁺ uptake. After making this correction, it was found

that active transport reached half its maximal rate with an internal Na^+ concentration of about $20\mu\text{equiv./ml.}$

Table 7 shows that, in experiments of the type

Fig. 6. Dependence of net Na^+ and K^+ movements on internal Na^+ concentration. Erythrocytes were loaded with different concentrations of Na^+ by the 'lactose' procedure, washed four times with medium *B* containing K^+ (10mm), and suspended at about 15% haematocrit in the same medium, modified to include ouabain ($50\mu\text{M}$) where appropriate. Samples for estimation of cell Na^+ and K^+ were taken initially or after incubation for 3.5hr. at 37° , to allow calculation of the rates of Na^+ extrusion ($\bigcirc$, control; $\bullet$, +ouabain) and K^+ uptake (Δ , control; $\blacktriangle$, +ouabain). Three further experiments gave similar results.

just described, the maximum rate of ATPase activity, in cells containing about $50\text{--}100\mu\text{equiv.}$ of $\text{Na}^+/\text{ml.}$ of cells, was in the range $1.2\text{--}1.5\mu\text{moles/ml.}$ of cells/hr., whereas the associated active movements of Na^+ and K^+ were $3.3\text{--}5.7$ and $2.7\text{--}4.6\mu\text{equiv./ml.}$ of cells/hr. respectively. The Na^+/ATP ratio had values from 2.5 to 3.8 and the K^+/ATP ratio values from 2.1 to 3.3. Table 7 shows, further, that, although the rates of active transport and of ATPase actively fell to less than half the maximal values as cell Na^+ concentration was decreased, the Na^+/ATP ratio remained in a range similar to that in cells rich in Na^+ . Omitting the first three results in Table 3 on account of the error associated with the measurement of low rates of transport, the mean values of the Na^+/ATP and K^+/ATP ratios were 3.2 ± 0.2 (8) and 2.4 ± 0.3 (8) respectively. The mean value for the sum of the Na^+/ATP and K^+/ATP ratios, representing the total number of cations actively transported across the membrane during the hydrolysis of 1mol. of ATP by the ouabain-sensitive ATPase, was 5.6 ± 0.3 (8) (Table 7).

DISCUSSION

Control of metabolism by active transport

The results show that the metabolism of human erythrocytes is partly regulated by the concen-

Table 7. *Stoichiometry between active Na^+ and K^+ movements and ouabain-sensitive ATP hydrolysis by erythrocytes*

Erythrocytes were loaded with various proportions of Na^+ and K^+ by the 'lactose' procedure, and Na^+ and K^+ movements, lactate and P_i production were determined with or without ouabain ($50\mu\text{M}$). Active Na^+ and K^+ movements are taken as the difference between net cation movements in the presence and absence of ouabain, and ouabain-sensitive ATP hydrolysis as the sum of ouabain-sensitive P_i and lactate production

Internal concn. of cations (μ equiv./ml. of cells)		Activity (μ moles/ml. of cells/hr.)			Active cation movements (μ equiv./ml. of cells/hr.)						(Na ⁺ + K ⁺), ATP (VI) + (IV) (I) + (II)
		Ouabain- sensitive P _i production (I)	Ouabain- sensitive lactate production (II)	Ouabain- sensitive ATP hydrolysis (I) + (II)	Na ⁺ extrusion (III)	K ⁺ uptake (IV)	Na ⁺ extrusion + K ⁺ uptake (III) + (IV)	Na ⁺ /ATP	K ⁺ /ATP		
								(III) (I) + (II)	(IV) (I) + (II)		
Na	K										
10	96	0.1	0.4	0.5	0.9	1.4	2.3	1.5	2.6	4.1	
12	108	0.1	0.5	0.6	2.0	0.9	2.9	3.4	1.4	4.8	
16	101	0.2	0.5	0.7	2.6	2.0	4.6	3.5	2.7	6.3	
19	79	0.2	0.7	0.9	2.6	3.1	5.7	2.9	3.6	6.5	
24	89	0.2	0.7	0.9	3.4	1.7	5.1	3.8	1.9	5.6	
31	78	0.3	0.9	1.2	4.3	1.7	6.0	3.6	1.4	5.0	
47	60	0.3	0.9	1.2	4.0	2.9	6.9	3.4	2.4	5.9	
50	65	0.4	1.0	1.4	4.1	4.6	8.7	3.0	3.3	6.4	
78	19	0.3	1.0	1.3	3.3	2.7	6.0	2.5	2.0	4.5	
90	14	0.2	1.2	1.4	4.3	3.4	7.7	3.0	2.4	5.4	
101	12	0.5	1.0	1.5	5.7	3.1	8.8	3.8	2.1	5.9	

Mean + s.e.m. for last eight values in columns ... 3.2±0.2 (8) 2.4±0.3 (8) 5.6±0.3 (8)

trations of internal Na^+ and external K^+ . It is these concentrations that determine the activity of the transport system that utilizes energy from ATP. The rate of lactate production is an indication of the rate of ATP synthesis, and, like respiring cells, erythrocytes appear to adjust their production of ATP according to the demand for ATP for active transport. These effects of Na^+ and K^+ do not arise from interference with glycolytic enzymes because they were abolished by ouabain, which does not cause large changes in cellular cation content during an incubation of 4 hr. (Whittam, 1958). Stimulation of pyruvate-kinase activity by K^+ and inhibition by Na^+ have been observed in extracts of muscle and nerve (Boyer, Lardy & Phillips, 1943; Utter, 1950), but these effects are the opposite of those in our experiments, which must therefore arise from a different cause. Again, ouabain had no effect in the absence of external K^+ and its effect was least when the internal Na^+ concentration was low. Thus the conditions under which lactate production was altered by Na^+ and K^+ are those under which the transport of these ions occurs at different rates. This finding is the converse of the well-established dependence of active transport on metabolism. It means that there is interdependence between the processes of ATP synthesis and hydrolysis. This conclusion is further supported by work with oligomycin, which is like ouabain in inhibiting active transport, the membrane ATPase and lactate production (Whittam, Wheeler & Blake, 1964). In the electric organ of *Electrophorus electricus*, Aubert, Chance & Keynes (1964) have demonstrated that glycolysis may be stimulated as a result of increased active cation transport.

The above conclusion does not agree with earlier work of Schatzmann (1953), Solomon, Gill & Gold (1956), Kunz & Sulser (1957), Nagano & Nakao (1962), and Jacob & Jandl (1964), who found that glucose utilization in fresh erythrocytes was unaffected by cardiac glycosides. The experimental discrepancy has not been resolved, but it may arise from the marked sensitivity to pH of the ouabain-sensitive component of lactate production and the low sensitivity of their analytical methods. On the other hand, the present results are in line with other recent reports that ouabain retards glycolysis. Murphy (1963) found that the rate of glucose utilization of fresh erythrocytes, incubated in a medium containing Ca^{2+} , was decreased by 15% when active transport was inhibited by ouabain at pH 7.5. Sen & Post (1964) and Minakami, Kakinuma & Yoshikawa (1964) also found that ouabain caused a fall in lactate production. The effects are the same whether or not Ca^{2+} is present in the medium. Of particular interest is the presence of an ouabain-sensitive component of lactate produc-

tion in spherocytic cells in which rates of transport are raised above normal (Jacob & Jandl, 1964). The present results extend these findings in directly showing how vectorial effects of Na^+ and K^+ on lactate production can be ascribed to an action on active transport, and the associated membrane ATPase. Active transport thus appears to act as a control of glycolysis as well as of respiration.

Magnitude of the regulation. What is the extent of the control of erythrocyte metabolism by this enzymic activity? The activity can be expressed as the sum of lactate and P_i production that is sensitive to ouabain, and Fig. 2 shows that this sum can vary from about 0.5 to 1.5 $\mu\text{moles/ml.}$ of cells/hr., the increase being due to an increase in internal Na^+ concentration. The activity of 0.5 $\mu\text{mole/ml.}/\text{hr.}$ corresponds to that in fresh cells. Similar effects of internal Na^+ and external K^+ on P_i production were described by Laris & Letchworth (1962). The ouabain-insensitive lactate and P_i production is independent of Na^+ concentration and is represented by a value of about 2.0 $\mu\text{moles/ml.}/\text{hr.}$ It thus appears that there is a basal energy production, unaffected by ouabain, and a transport-controlled part that can vary from 25% (i.e. 0.5/2.0) to 75% (i.e. 1.5/2.0) of the basal. The former value refers to fresh cells, and suggests that about 20% [i.e. 0.5/(0.5+2)] of the normal energy production is used and controlled by the active-transport system for Na^+ and K^+ . Murphy (1963) found a value of 15% for fresh cells, in close agreement with our value. The comparable value for cells rich in Na^+ , in which rates of transport are higher, is about 43% [i.e. 1.5/(1.5+2)]. The energy devoted to active transport in human erythrocytes is therefore not insignificant.

Possible mechanisms of the regulation. The effects of Na^+ and K^+ thus depend on the activity of an enzyme system not in the glycolytic pathway. The mechanism of the regulation has not been established, but there are two points where control appears to be likely. One is at the stage of 3-phosphoglycerate kinase, which would be stimulated by an increase in ADP concentration arising from ATPase activity. Jones, Norris & Landon (1963) found that glycolysis was stimulated by more than 50% in a fraction of rat kidney due to an interaction between membrane ATPase activity and 3-phosphoglycerate kinase. Another point of control that is likely is at the level of sugar phosphates at the reaction catalysed by phosphofructokinase. This enzyme is affected by AMP, ADP and ATP concentrations in a way that would explain the observed changes in lactate production (see Passonneau & Lowry, 1962; Mansour, 1963; Newsholme & Randle, 1964).

Constant values of the K⁺/ATP and Na⁺/ATP ratios

A further aspect of the results is that when the rates of active Na⁺ and K⁺ transport were varied there was a parallel change in the activity of the membrane ATPase. The changes in enzymic activity were comparable whether the rates of transport were varied by altering the concentration of internal Na⁺, external K⁺ or ouabain, suggesting that the transport rate and enzymic activity are different ways of measuring the same membrane property. The mean K⁺/ATP ratio in fresh cells was 2.4 ± 0.15 (5), and the mean value from all the different kinds of experiments was 2.5 ± 0.1 (35). The mean Na⁺/ATP ratio was 3.2 ± 0.2 (8). These values, derived from measurements over a fivefold range of transport rate, are in good agreement with previous values found under a single condition by Sen & Post (1964). Their values were 3.1 ± 0.1 (6) for the Na⁺/ATP ratio and 2.4 ± 0.1 (6) for the K⁺/ATP ratio. Glynn (1962) also reported a value of about 3 for the Na⁺/ATP ratio from work with erythrocyte 'ghosts', and Gardos (1964) a value of 2.05 ± 0.24 (range 1.75–2.42) for the K⁺/ATP ratio.

Transformation of energy involved in active transport

The values of the K⁺/ATP and Na⁺/ATP ratios are of interest in relation to the question of energy transformation, in which the free energy of the hydrolysis of ATP is converted into osmotic work. On the assumption that the activity coefficients for Na⁺ and K⁺ are similar in the intracellular and extracellular fluids, the free-energy change, ΔG , associated with the extrusion of $n\text{Na}^+$ ions into, and the uptake of $m\text{K}^+$ ions from, a medium containing 10mm-K⁺ and 140mm-Na⁺ is given by:

$$\Delta G = 2.3 RT \{n \log (140/[\text{Na}^+]_i) + m \log ([\text{K}^+]_i/10) + (n-m)EF\}$$

where R is the gas constant, T the absolute temperature, F the faraday, E the membrane potential (approx. 0.01 v), and $[\text{Na}^+]_i$ and $[\text{K}^+]_i$ the intracellular Na⁺ and K⁺ concentrations in $\mu\text{equiv./ml.}$ of cell water. ΔG now represents the free energy utilized in steady-state conditions for active Na⁺ and K⁺ movements during the hydrolysis of 1mole of ATP in a manner sensitive to ouabain. This value was clearly highest when the cells contained a low concentration of Na⁺ and a high concentration of K⁺, since the external medium contained 140mm-Na⁺ and 10mm-K⁺: it may be calculated that ΔG did not exceed 9kcal. in the present experiments.

The difficulties in estimating the free-energy change ($\Delta G'$) associated with ATP hydrolysis are

formidable, since a knowledge is required of the activities of ATP, ADP and P_i (or their Mg²⁺ complexes) and of H⁺ ions (George & Rutman, 1960). Writing concentrations in place of activities, $\Delta G'$ is given by:

$$\Delta G' = \Delta G^0 - 2.3RT \left\{ \text{pH} - 7.5 - \log \left(\frac{[\text{ADP}][\text{P}_i]}{[\text{ATP}]} \right) \right\}$$

Taking the standard free energy of ATP hydrolysis, ΔG^0 , as -8.5kcal./mole (see Burton, 1958), this calculation gives a value for $\Delta G'$ in 'lactose-treated' cells of -13kcal./mole . This value is considerably in excess of the energy (9kcal.) utilized for active transport during the ouabain-sensitive hydrolysis of 1mole of ATP in these cells.

The results confirm that the rates of active transport of Na⁺ and K⁺ are determined by $[\text{Na}^+]_i$ and $[\text{K}^+]_i$, in keeping with the observation that ATP hydrolysis in erythrocyte 'ghosts' is dependent on $[\text{Na}^+]_i$ and $[\text{K}^+]_i$ (Whittam, 1962b; Glynn, 1962). It is particularly striking that the values for the Na⁺/ATP and K⁺/ATP ratios are constant even though the electrochemical gradient was varied. The Na⁺/O₂ ratio for frog skin also is constant for different values of the electrochemical gradient, and Zerahn (1956) has discussed the implications of this finding. His considerations can now be applied to the present results with erythrocytes.

Vectorial nature of the membrane adenosine triphosphatase

How is it possible for the energy released during the hydrolysis of ATP to be utilized for the unidirectional transport of ions against an electrochemical gradient? It is evident intuitively, and may be demonstrated by means of irreversible thermodynamics, that coupling between a scalar chemical reaction and a vectorial flow of matter cannot occur in an isotropic system (Jardetsky & Snell, 1960; Katchalsky & Kedem, 1962). However, Moszynski, Hoshiko & Lindley (1963) show that a one-dimensional flux can be coupled to a reaction which possesses asymmetry in occurring only at one side of a membrane. The reaction catalysed by the (Na⁺ + K⁺)-activated ATPase fulfils this criterion, since the enzyme acts only on internal ATP and appears to liberate the products of hydrolysis inside the cell (Schatzmann, 1964; Sen & Post, 1964; Whittam & Ager, 1964). Other authors have pointed out that a chemical reaction occurring in the cell membrane may itself possess marked asymmetry and thus be a vector quantity (see Katchalsky & Kedem, 1962). The present results appear to provide a direct illustration of coupling between a chemical reaction which has vectorial characteristics and the unidirectional transport of ions. Energy made available by ATP

hydrolysis is used for the movement of ions against electrochemical gradients. There is no information at present on the possibility of the opposite form of energy transformation, an increase in chemical energy at the expense of ionic gradients.

The present studies have not been concerned with the chemical nature of the mechanism that causes the hydrolysis of ATP. Although the sequence of events that lead to ATP hydrolysis is still somewhat obscure, it is attractive to speculate that changes in the configuration of a single enzyme might constitute the means by which Na^+ and K^+ are carried across the membrane. There is some evidence that the configuration of a soluble enzyme changes during the formation of the enzyme-substrate complex, as, e.g., during the interaction between glucose 6-phosphate and phosphoglucose mutase (see Koshland, Yankeelov & Thoma, 1962). The apparent flexibility of 'active centres', and steric effects in general, may assume particular importance for those enzymes that form part of a membrane. Thus it might be supposed that internal Na^+ is involved in inducing the fit of ATP with the active centre of the $(\text{Na}^+ + \text{K}^+)\text{-activated ATPase}$. Overall ATP hydrolysis requires external K^+ as well, however, and active ion movements may be connected with a rearrangement of the enzyme to its initial state, triggered by K^+ . Na^+ and K^+ may be regarded as enzymic cofactors, but differ from ones in a homogeneous milieu in being raised to a higher energy level as ATP is hydrolysed.

The notion that a conformational change is involved in active transport has also been put forward by Hokin & Hokin (1963) and Judah & Ahmed (1964). These authors suggest respectively that lipoprotein or phosphoprotein carriers undergo changes of configuration according to their degree of phosphorylation. A problem that remains unresolved is whether separate cation-binding sites are responsible for Na^+ and K^+ carriage or whether there is rotation of a single binding site such that its specificity is high for Na^+ at the inner and for K^+ at the outer surface of the membrane (see Whittam, 1962b).

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REFERENCES

- Adam, H. (1963). In *Methods of Enzymatic Analysis*, p. 573. Ed. by Bergmeyer, H. U. New York and London: Academic Press Inc.
- Aubert, X., Chance, B. & Keynes, R. D. (1964). *Proc. Roy. Soc. B*, **160**, 211.
- Bartlett, G. R. (1959). *J. biol. Chem.* **234**, 466.
- Bartlett, G. R. & Schafer, A. W. (1961). *J. clin. Invest.* **40**, 1185.
- Bilodeau, F. & Elliott, K. A. C. (1963). *Canad. J. Biochem. Physiol.* **41**, 779.
- Bolingbroke, V. & Maizels, M. (1959). *J. Physiol.* **149**, 563.
- Boyer, P. D., Lardy, H. A. & Phillips, P. H. (1943). *J. biol. Chem.* **149**, 529.
- Burton, K. (1958). *Nature, Lond.*, **181**, 1594.
- Clarkson, E. M. & Maizels, M. (1955). *J. Physiol.* **129**, 476.
- Davson, H. (1939). *Biochem. J.* **33**, 389.
- Elshove, A. & Van Rossum, G. D. V. (1963). *J. Physiol.* **166**, 531.
- Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
- Flynn, F. & Maizels, M. (1949). *J. Physiol.* **110**, 301.
- Gabrio, B. W., Hennessey, M., Thomasson, J. & Finch, C. A. (1955). *J. biol. Chem.* **215**, 357.
- Gardos, G. (1964). *Experientia*, **20**, 387.
- George, P. & Rutman, R. J. (1960). *Progr. Biophys.* **10**, 2.
- Glynn, I. M. (1956). *J. Physiol.* **134**, 278.
- Glynn, I. M. (1957). *J. Physiol.* **136**, 148.
- Glynn, I. M. (1962). *J. Physiol.* **160**, 18r.
- Glynn, I. M. (1964). *Pharm. Rev.* **16**, 381.
- Hoffman, J. F. (1964). *Fed. Proc.* **23**, 210.
- Hohorst, H. J. (1963). In *Methods of Enzymatic Analysis*, p. 266. Ed. by Bergmeyer, H. U. New York and London: Academic Press Inc.
- Hohorst, H. J., Kreutz, F. H. & Bücher, T. (1959). *Biochem. Z.* **332**, 18.
- Hokin, L. E. & Hokin, M. R. (1963). *Fed. Proc.* **22**, 8.
- Jacob, H. S. & Jandl, J. H. (1964). *J. clin. Invest.* **43**, 1704.
- Jardetsky, D. & Snell, F. M. (1960). *Proc. nat. Acad. Sci., Wash.*, **46**, 616.
- Jones, V. D., Norris, J. L. & Landon, E. J. (1963). *Biochim. biophys. Acta*, **71**, 277.
- Judah, J. D. & Ahmed, K. (1964). *Biol. Rev.* **39**, 160.
- Kahn, J. B. (1962). *J. Pharmacol.* **136**, 197.
- Katchalsky, A. & Kedem, O. (1962). *Biophys. J.* **2**, 53.
- King, E. J. (1951). *Microanalysis in Medical Biochemistry*, 2nd ed., p. 33. London: J. and A. Churchill Ltd.
- Koshland, D. E., Yankeelov, J. A. & Thoma, J. A. (1962). *Fed. Proc.* **21**, 1031.
- Krebs, H. A. & Hems, R. (1953). *Biochim. biophys. Acta*, **12**, 172.
- Kühler, W. & Bretschneider, H. J. (1963). *Pflüg. Arch. ges. Physiol.* **277**, 141.
- Kunz, H. A. & Sulzer, F. (1957). *Experientia*, **13**, 365.
- Laris, P. C. & Letchworth, P. E. (1962). *J. cell. comp. Physiol.* **60**, 229.
- McConaghey, P. D. & Maizels, M. (1962). *J. Physiol.* **162**, 485.
- Maizels, M. (1935). *Biochem. J.* **29**, 1970.
- Mansour, T. E. (1963). *J. biol. Chem.* **238**, 2285.
- Minakami, S., Kakinuma, K. & Yoshikawa, H. (1964). *Biochim. biophys. Acta*, **90**, 434.
- Moszynski, J. R., Hoshiko, T. & Lindley, B. D. (1963). *Biochim. biophys. Acta*, **75**, 447.
- Murphy, J. M. (1963). *J. Lab. clin. Med.* **61**, 567.
- Nagano, K. & Nakao, M. (1962). *J. Biochem., Tokyo*, **52**, 99.
- Newsholme, E. A. & Randle, P. J. (1964). *Biochem. J.* **93**, 641.
- Passonneau, J. V. & Lowry, O. H. (1962). *Biochem. biophys. Res. Commun.* **7**, 10.
- Post, R. L. & Jolly, P. C. (1957). *Biochim. biophys. Acta*, **25**, 118.
- Post, R. L., Merritt, C. R., Kinsolving, C. R. & Albright, C. D. (1960). *J. biol. Chem.* **235**, 1796.

- Pranker, T. A. J. (1956). *Biochem. J.* **64**, 209.
- Rubinstein, D., Kashket, S. & Denstedt, O. F. (1956). *Canad. J. Biochem. Physiol.* **34**, 61.
- Schatzmann, H. J. (1953). *Helv. physiol. acta*, **11**, 346.
- Schatzmann, H. J. (1964). *Experientia*, **20**, 551.
- Sen, A. K. & Post, R. L. (1964). *J. biol. Chem.* **239**, 345.
- Shaw, T. I. (1955). *J. Physiol.* **129**, 464.
- Solomon, A. K. (1952). *J. gen. Physiol.* **36**, 57.
- Solomon, A. K., Gill, T. J. & Gold, G. L. (1956). *J. gen. Physiol.* **40**, 327.
- Szentkiralyi, E. M. (1957). *Arch. Biochem. Biophys.* **67**, 298.
- Utter, M. F. (1950). *J. biol. Chem.* **185**, 499.
- Whittam, R. (1958). *J. Physiol.* **140**, 479.
- Whittam, R. (1960). *J. Physiol.* **154**, 614.
- Whittam, R. (1961). *Nature, Lond.*, **191**, 603.
- Whittam, R. (1962a). *Biochem. J.* **82**, 205.
- Whittam, R. (1962b). *Biochem. J.* **84**, 110.
- Whittam, R. & Ager, M. E. (1964). *Biochem. J.* **93**, 337.
- Whittam, R., Wheeler, K. P. & Blake, A. (1964). *Nature, Lond.*, **203**, 720.
- Whittam, R. & Willis, J. S. (1963). *J. Physiol.* **168**, 158.
- Wyatt, G. R. (1955). In *The Nucleic Acids*, vol. 1, p. 245. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
- Zerahn, K. (1956). *Acta physiol. scand.* **36**, 300.