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POTASSIUM MOVEMENTS AND ATP IN HUMAN RED CELLS

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It has been known since 1941 that human red cells depend upon glycolysis for the maintenance of concentration gradients of sodium and potassium across the cell membranes (Harris, 1941; Danowski, 1941). Glycolysis is also needed to maintain the concentrations of adenosine triphosphoric acid (ATP) and other phosphate esters, notably 2:3-diphosphoglyceric acid (2:3-PGA; see review by Prankerd, 1955), and ATP is sometimes regarded as the energy transmitter between glycolysis and the active transport of cations. To find if the ATP concentration and the active uptake of potassium were correlated in human red cells, the ATP and potassium concentrations have been measured in cells maintaining, losing and gaining potassium.

The effects of digoxin and ouabain (strophanthin-G), which inhibit the active movements of sodium and potassium without affecting glycolysis (Schatzmann, 1953), were also studied to find if they caused a depletion of intracellular ATP.

The results are consistent with the view that ATP is at least one link in the coupling between glycolysis and the active influx of potassium. The effects of cardiac glycosides, however, show that ATP is not the only link, and are interpreted as indirect evidence for the participation of a membrane carrier in the linked transport of potassium with sodium across the cell membrane. A short account of this work has been published (Whittam, 1957).

METHODS

Preparation of cells. Blood was drawn from the antecubital veins of healthy young men and collected in a flask containing heparin (Pularin, Evans Medical Supplies, 1 mg/10 ml. blood). It was immediately centrifuged and the plasma and buffy coat removed by suction. The cells were then suspended in four volumes of 0·9% (w/v) NaCl solution and allowed to stand for about 1 min before being spun down. After centrifugation the saline and the upper layer of cells were removed and the washing repeated twice more, as this procedure has been shown to eliminate the white cells almost completely (Glynn, 1956). The cell suspension was then free of reducing sugar as tested by Benedict's sugar reagent. The washed cells were finally mixed with saline solution for incubation at

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37° C or for storage at 4° C. When additions of various substances were made to the suspension, concentrated solutions were added to give the concentrations shown in the text.

Incubation of cells. The cell suspensions were shaken in air in stoppered conical flasks in a water-bath at 37° C either fresh, immediately after the cells had been washed, or after storage for five days at 4° C in flasks stoppered with a plug of cotton wool. The temperature of the water-bath at 37° C was controlled by a thermostat and did not vary by more than 0·1° C during the incubations. Samples of the cell suspension were removed for analysis after various times. Precautions to ensure sterile conditions were not taken during cold-storage or incubation at 37° C but the latter never exceeded 5 hr and it is unlikely that bacterial growth would vitiate the results during this period. Samples of the suspension were taken for a haematocrit determination at the beginning and end of each incubation, and in some experiments each time a sample was taken for potassium and phosphate analyses.

Medium. A saline solution buffered with phosphate to pH 7·5 was used and was similar to that of Krebs & Henseleit (1932) save that half of the $MgSO_4$ was replaced by $MgCl_2$. This is recommended by Krebs (1950) in order to make the medium more like human plasma, which contains less sulphate than the plasma of several laboratory animals (Guillaumin, 1940). The ionic composition of the medium was as follows (mM): Na^+, 150; K^+, 5·0; Mg^{2+}, 1·1; Cl^-, 137; SO_4^{2-}, 0·6; $H_2PO_4^-$, 1·4; HPO_4^{2-}, 8·6.

Haematocrit determinations were used as an index of changes in cell volume during an experiment. It was necessary to compare changes in potassium and phosphate esters in the same number of cells (see Maizels, 1943). All cell concentrations are therefore 'corrected' by reference to the initial cell volume, and are therefore expressed in m-mole/l. cells at the start of an incubation at 37° C or of cold-storage at 4° C.

The actual determinations were made by centrifuging the suspension in capillary tubing of uniform bore (13 cm long and 3 mm bore) for 30 min at 1500 *g*. No change in volume occurred when spinning was continued for a further 30 min. Since the volume of trapped fluid between the cells under these conditions is probably not more than 2% (Jackson & Nutt, 1951), the haematocrit value was taken to represent the actual cell volume. Corrections for haemolysed cells were not necessary, since lysis was never more than about 4%.

Analysis procedure

Each sample for analysis was centrifuged for 5 min at 1500 *g* and most of the supernatant fluid removed with a Pasteur pipette and its volume measured by placing it in a 5·0 ml. graduated tube. The volume of cells and saline solution left in the tube was found by difference. The cells (0·5–1·0 ml.) were haemolysed with 0·5 ml. water containing a little saponin; 0·2 ml. of 100% (w/v) trichloroacetic acid (TCA) was added to deproteinize, and the mixture then removed and well shaken and centrifuged. The clear TCA extract was removed and stored at −12° C until the analyses were made. No phosphate esters were found in the medium and a correction for medium in the TCA extract was therefore unnecessary since the esters therein must all have been derived from the cells. When orthophosphate or total acid soluble phosphates were analysed, however, a correction of the TCA extract values for the orthophosphate present in the trapped medium was made. This volume of medium was found by subtraction of the cell volume from the total volume of the suspension to which TCA was added. In the calculation of the volume of water in the TCA extract the cells were assumed to have a water content of 70% (w/v) (Maizels, 1943).

Haemoglobin was estimated as oxyhaemoglobin using a Spekker colorimeter, and an Ilford narrow-band yellow-green filter, to measure the optical density of the solutions. It was estimated in the medium after incubation and in the whole suspension. Lysis was determined by dividing the haemoglobin concentration in the medium by that in the whole suspension and multiplying the dividend by the fraction of the suspension occupied by the medium.

Potassium estimations were made with an EEL flame photometer either on suitable dilutions of the cell TCA extracts or on haemolysates of packed cells. The small amount of TCA (less than 2 mM) did not interfere with the readings, which were made in quadruplicate, alternating the

unknown solutions between standard solutions. The results gave a S.E. of the mean of less than 2%.

Lactic acid estimations on TCA extracts of the whole cell suspension (4·0 ml. suspension and 1·0 ml. 5% (w/v) TCA) were made by the method of Barker & Summerson (1941), and pyruvic acid was estimated likewise by the method of Friedmann & Haugen (1943).

Total acid-soluble phosphate and orthophosphate in the TCA extracts were estimated by the method of Fiske & Subbarow (1925).

Phosphate esters in the TCA extracts were first separated by one-dimensional paper chromatography at room temperature using solvent no. 3 of Gerlach, Weber & Döring (1955). Whatman No. 1 filter paper was first washed with 0·2% (w/v) ethylenediamino-tetraacetic acid solution and water as described by Eggleston & Hems (1952) and a satisfactory separation of ATP, ADP, AMP, 2:3-PGA, orthophosphate, and hexose phosphates was obtained. After trial separations using the same solvent several times, when occasionally all the compounds moved with the orthophosphate, a fresh solvent solution was prepared each time before use. About 100 μl. of TCA extract was accurately applied to the base line of the chromatogram, 7·2 μl. at a time, using a graduated micropipette of a self-filling type similar in principle to that described by Kirk (1950). The paper was dried in a stream of cold air from a hair dryer before the addition of each sample.

After removal from the solvent the chromatograms were dried at room temperature and examined under a low-pressure Hg lamp (Chromatolite, Hanovia, Slough) when compounds containing purines absorbed ultra-violet light and were located as dark areas on a light background. Photographs of the chromatograms under ultra-violet light were also usually made, essentially as described by Markham & Smith (1949).

The chromatograms were sprayed with the molybdate solution of Hanes & Isherwood (1949) and the areas containing phosphates were developed as blue spots by exposing the paper to ultra-violet light (Bandurski & Axelrod, 1951) which was found to be much less troublesome than the use of a jar of H_2S for the reduction of the phospho-molybdate complex. The areas of paper containing the phosphates were ashed with a mixture of H_2SO_4 and $HClO_4$ (Hanes & Isherwood, 1949) and the orthophosphate estimated by the method of Berenblum & Chain (1936).

In order to check the identity of the compound from cell extracts which behaved as ATP on the chromatograms and which absorbed ultra-violet light, the area of paper was cut out before spraying with molybdate and allowed to stand during the night at 4° C in 2 ml. of water. The spectrum of the eluate in the ultra-violet between 240 and 270 mμ was then determined in a Uvispek spectrophotometer (Hilger and Watts Ltd., London). A single peak was found at 260 mμ which is characteristic of adenine compounds, and from the extinction coefficient (Kalckar, 1947) the concentration of purine was calculated. The phosphate in the same eluate was also estimated after acid hydrolysis and the ratio of purine to phosphate was approximately 3:1, which is to be expected for ATP.

Accuracy of phosphate analyses. Duplicate analyses of cells from the same suspension gave values which agreed within 10%.

Influx measurements of potassium were made using ^{42}K and following the procedure of Glynn (1956).

RESULTS

Phosphate compounds in human red cells

Values in the literature for the concentrations of ATP, ADP and 2:3-PGA in fresh, normal human red cells show wide variations which are independent of the analytical methods employed. Thus, values given by different authors for ATP concentrations have a range of four- or fivefold and even values from the same authors show a range of two- or threefold (Table 1). The differences in ATP and ADP concentrations found with the present analytical methods

cannot be ascribed to variable losses of the compounds due to their instability because the same technique of handling different samples of cells was followed, and, moreover, almost identical values were found for cells from the same suspension (see Methods). The observations that red cells from subjects with acidosis contain less 2:3-PGA than normal cells (Rapoport & Guest, 1939) may explain some of the lower values from the wide range for the 2:3-PGA concentration, but there is still a considerable spread for cells from donors

TABLE 1. Concentrations of phosphate esters in human red cells (m-mole/l. cells)

Analytical method	ATP	ADP	2:3-PGA	Reference
Chromatography	0·90±0·08 0·5–1·2 (10)	0·48±0·03 0·4–0·6 (5)	4·62±0·24 3·3–5·4 (8)	This paper. The S.E. of the mean, the range and the number of observations are given
Chromatography	0·3–0·9	0·2–0·4	2·0–2·9	Prankerd & Altman (1954)
Chromatography	1·16	0·25	—	Overgaard-Hansen, Jørgenson & Praetorius (1957)
Chromatography	0·76 0·47–1·11	0·36 0·20–0·43	4·7 3·7–5·9	Fleckenstein & Gerlach (1953); mean values and range
Chromatography	1·0 0·7–1·4	None detected	2·1 1·8–3·3	Rhodewald & Weber (1956); mean values and range
Ion exchange resin	0·45	0·12	1·6	Bartlett, Savage, Hughes & Marlow (1953)
Differential hydrolysis	1·4 (7 min P)	—	4·5	Rapoport & Guest (1941)
Differential hydrolysis	1·1	None detected	3·7	Gourley (1952)
Differential hydrolysis	1·3 (7 min P)	—	5·0	Farmer & Maizels (1939)

apparently healthy. From the values given in Table 1 it seems possible that wide differences may exist in phosphate ester concentrations in red cells from different individuals and from the same individual at different times (e.g. the ATP concentration in red cells obtained on different occasions from the same healthy individual varied from 0·8 to 1·2 m-mole/l. cells).

In spite of the variation in results, a point clearly emerging from Table 1 is that human red cells contain at least two or three times as much ATP as ADP and that the 2:3-PGA concentration is about four times greater than the sum of the ATP and ADP concentrations. About 80% of the acid-soluble phosphate consisted of 2:3-PGA, ATP and ADP.

The variation in potassium concentrations in fresh red cells was small compared with that in phosphate concentrations. The mean potassium concentration from ten experiments was 98·9 ± 1·6 m-mole/l. cells (S.E. of the mean) with a range from 92 to 106. There was no correlation between the potassium and ATP concentrations, a high potassium concentration sometimes being found with a low ATP concentration and vice versa.

Incubation of fresh cells

Cells incubated in a saline solution containing glucose maintained the concentrations of ATP, ADP and potassium (Table 2), but after 5 hr a 30% fall had occurred in the 2:3-PGA concentration. No other organic phosphate was detected (e.g. 2- or 3-phosphoglyceric acid or phospho-enol pyruvate), but the inorganic phosphate concentration increased, indicating hydrolysis of the 2:3-PGA. The initial glucose concentration was 11 mM, which represents a ninefold excess over that required for a typical consumption of 1·5 m-mole/l. cells/hr (Maizels, 1951; Bernstein, 1953), and it is not clear why the cells should have utilized their store of 2:3-PGA in a buffered suspension when glucose and ATP were still available to facilitate glycolysis.

TABLE 2. Incubation of fresh cells at 37° C. Concentrations of phosphates and of K are shown (m-mole/l. cells) before and after incubation in the presence and absence of glucose

Compound ...	ATP		ADP		AMP	2:3-PGA		Inorganic phosphate		K	
Presence or absence of glucose (11 mM)...	+	−	+	−	−	+	−	+	−	+	−
Hours of incubation											
0	0·95	0·95	0·40	0·40	0	4·8	4·8	4·6	4·6	106	106
1	—	0·75	—	—	0·75	—	3·5	—	—	—	—
2	1·00	0·60	—	0·25	0·65	4·5	2·0	—	—	—	—
3	0·95	0·55	—	—	—	—	1·5	—	—	—	—
4	—	0·45	—	—	0·50	3·5	0·5	—	—	—	—
5	0·85	0·30	0·40	0·10	0·45	3·0	0·5	7·6	13·7	105	96

After 5 hr incubation of cells deprived of glucose the intracellular potassium had fallen by 10 m-mole/l. cells and ATP and ADP by 60%. 2:3-PGA was almost completely hydrolysed with a corresponding rise in inorganic phosphate (Table 2). A similar result was found in cells incubated in the presence of 1 mM-iodoacetate (IAA). About 0·5 m-mole AMP/l. cells was produced but this is 0·45 m-mole/l. cells too little to balance the loss of ATP and the deficit in nucleotide after 5 hr may be due to the formation of small amounts of hypoxanthine or adenine.

Incubation of cold-stored cells

Cells cold-stored with glucose. During incubation of fresh cells at 37° C the potassium concentration either remained constant or decreased, so that in order to compare phosphate changes with a net uptake of potassium, cells first had to be stored at 4° C in order to lower their potassium, and then incubated at 37° C. It was desirable to arrange the time of storage at 4° C so that a measurable net accumulation of potassium could be observed during 5 hr incubation with glucose at 37° C; Ponder (1949) found most potassium uptake after cold storage for 5 days and this period of storage was therefore chosen in the

present experiments. The medium contained glucose during both cold storage and the subsequent incubation at 37° C.

Potassium leaked out of the cells at a very low rate (about 0·1–0·2 m-mole/l. cells/hr) during cold storage, and since ATP was still present this loss was probably due to a direct effect of the low temperature on the mechanism for potassium transport.

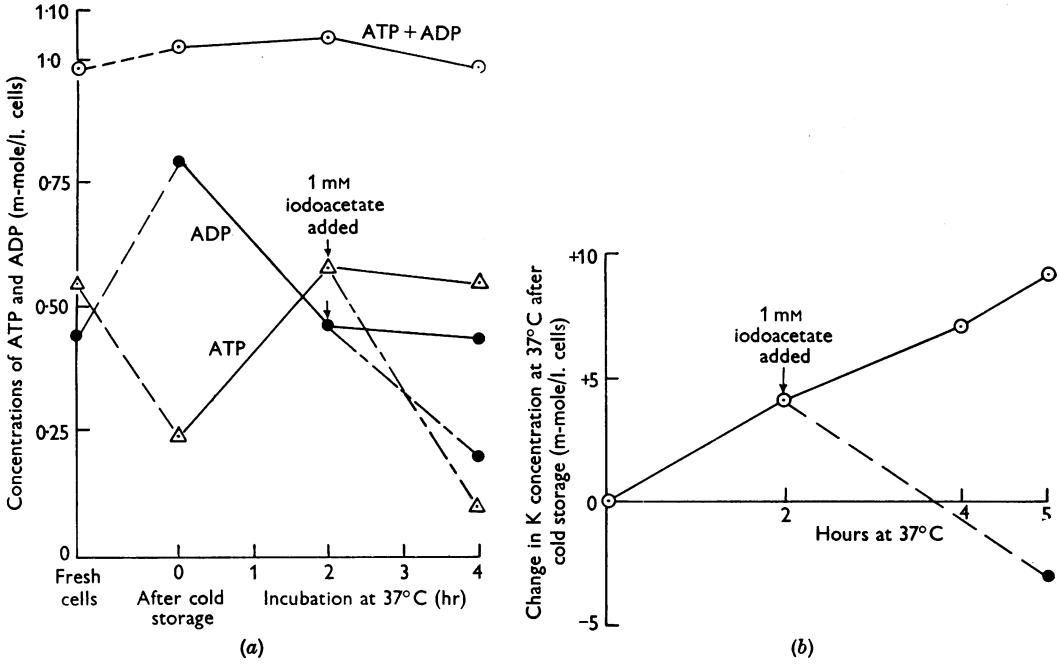

Fig. 1. Changes in ATP and ADP (*a*) and in K (*b*) during storage of human red cells at 4° C and on subsequent incubation at 37° C. The arrows indicate the addition of IAA at 37° C and the broken lines at 37° C show the changes in the IAA-treated cells.

Incubation of cold-stored cells

Potassium was accumulated by the cold-stored cells at 37° C at about 2 m-mole/l. cells/hr (Fig. 1*b*). This uptake was abolished by the addition of IAA (1 mM), which caused a loss of potassium, but, as Taylor, Weller & Hastings (1952) also found, the uptake of potassium was unaffected by physostigmine sulphate (0·5 mM) which was added as an inhibitor of ACh esterase to test if this enzyme was required for potassium uptake.

During 5 days cold storage about 60 % of the initial ATP was hydrolysed to ADP, but on incubation at 37° C the ADP thus formed was re-phosphorylated to ATP, which then remained constant (Fig. 1*a*). The concentration of 2:3-PGA remained constant during cold storage with glucose but fell about 40 % during incubation at 37° C.

Fig. 1 shows that potassium was accumulated at about the same rate during the whole of the 4 hr incubation and that the ATP concentration increased during the first 2 hr and then remained constant. (Intermediate values during the first 2 hr were not obtained because the small changes in the potassium concentration would have been within experimental error.) The result obtained might suggest that once ATP was available, both during and after its resynthesis, the rate of uptake of potassium was not correlated with the ATP concentration. This conclusion would be premature, however, because if the ATP concentration increased during the first few minutes to its 2 hr level the rate of uptake would still correlate with the ATP concentration. Since the measurement of net changes in potassium concentration is too insensitive to distinguish between these possibilities, the simultaneous measurement of potassium influx (with tracer potassium) and ATP concentration at frequent intervals during the first 2 hr of incubation is required.

Fate of 2:3-*PGA*

The loss of 2:3-PGA during the incubation of fresh cells at 37° C showed that it was utilized much more when glucose was absent than when this was present and the question arises as to the end product of its metabolism. Estimations of lactic and pyruvic acids were therefore made on cell suspensions to test if these acids were the products. No lactic acid was detected in a glucose-free suspension whilst cells containing glucose produced about 3·9 m-mole lactic acid/l. cells/hr. Fig. 2 shows the loss of 2:3-PGA and production of pyruvic acid

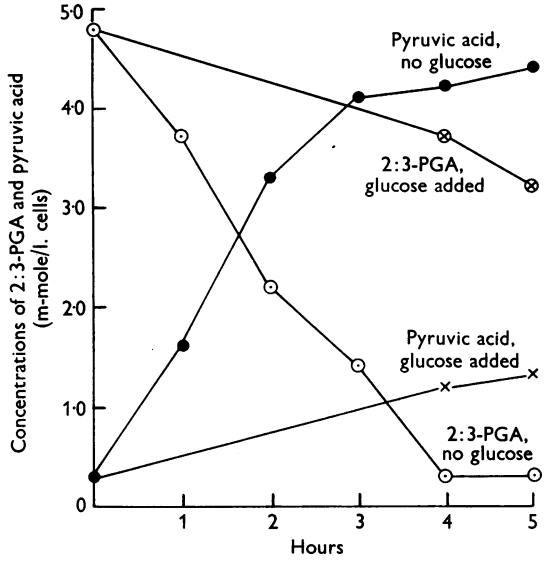

Fig. 2. Conversion of 2:3-PGA to pyruvic acid during the incubation at 37° C of fresh red cells in the presence and absence of glucose.

during 5 hr incubation of cells in the presence and in the absence of glucose. In each condition 2:3-PGA was quantitatively converted to pyruvic acid, the rate in the glucose-free suspension during the first 2 hr being 1·5 m-mole/l. cells/hr.

Pyruvic acid is to be expected because in the absence of glucose no co-enzyme 1 will be reduced by triosephosphate dehydrogenase and therefore lactic acid dehydrogenase, lacking its co-enzyme, will be unable to convert pyruvic acid to lactic acid. In accord with this result, Pappius & Denstedt (1954) have observed an accumulation of pyruvic acid and a decrease in 'difficultly-hydrolysable' phosphate (probably 2:3-PGA) in blood cold-stored without glucose.

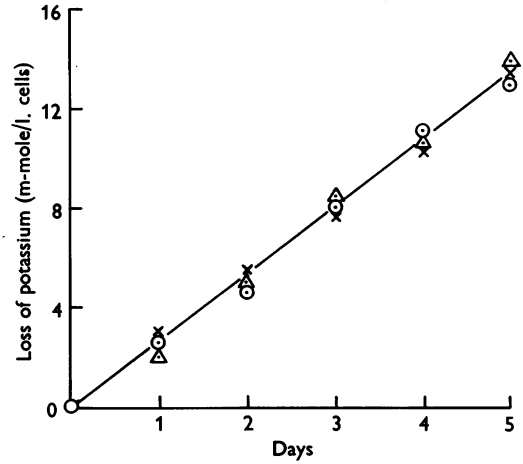

Fig. 3. Loss of potassium from red cells at 4° C shown to be unaffected by glucose deprivation or removal of calcium and magnesium from the saline. Cf. Table 5, which shows that digoxin also did not increase the loss of potassium during cold storage. ⊙, glucose added; ×, no glucose; △, no glucose, no Ca, no Mg.

Glucose and calcium removal, and potassium loss at 4° C

To test whether the removal of glucose and Ca would increase the loss of potassium during cold storage at 4° C, fresh cells were washed three times with a Ca- and Mg-free saline containing 0·2% (w/v) ethylenediamine tetraacetic acid (versene). Glucose was completely washed out of the cells and the versene presumably combined with ionic Ca on the outside of the cells. The cell suspension was divided into three lots and to one was added glucose and Ca and Mg, to another was added Ca and Mg, whilst the third one was left without additions. The cells were cold-stored, and Fig. 3 shows that the loss of potassium was identical whether or not glucose or Ca and Mg were present. The factors limiting the loss of potassium during cold storage were therefore independent of glucose and Ca and Mg. On incubation at 37° C the cold-stored cells also accumulated potassium to the same extent whether Ca and Mg were both present or both absent in the saline.

ATP breakdown at different external potassium concentrations

Table 3 shows the results of an experiment to test whether the hydrolysis of ATP in glucose-free cells was affected by changes in the external sodium and potassium concentrations, which are known to change critically the fluxes of

these ions (Glynn, 1956). A cell suspension was prepared with the saline potassium concentration equal to 0, 5 and 100 mM, the balance for isotonicity being made up with sodium chloride. The suspension had a low haematocrit of 5% so that changes in medium potassium with an initial zero concentration, due to leakages from the cells, would be negligible. It might be expected that at zero and 100 mM external potassium concentrations the ATP hydrolysis would be retarded, since the linked active potassium uptake and sodium

TABLE 3. Break-down of ATP and 2:3-PGA in glucose-free red cells in saline with different Na and K concentrations (m-mole/l. cells)

Compound ...	ATP			2:3-PGA		
Na concn. (mM) ...	150	145	50	150	145	50
K concn. (mM) ...	0	5	100	0	5	100
Hours of incubation						
0	0·80	0·80	0·80	5·00	5·00	5·00
1	0·75	0·70	0·75	4·65	4·75	4·40
2	0·70	0·65	0·60	3·45	3·60	3·30
3	0·55	0·45	0·50	2·35	2·25	2·00

extrusion is abolished with zero external potassium (Glynn, 1956), and with 100 mM-K the concentration gradients of sodium and potassium between cells and saline are much reduced. However, the results show that the break-down of ATP and 2:3-PGA proceeded at the same rate at the three different potassium concentrations (Table 3). Clearly no sparing effect on ATP break-down was elicited by the changes in sodium and potassium composition of the saline solution.

Potassium influx and ATP concentration

The results already described have been of net movements of potassium either into or out of cells, and it has been shown that a potassium uptake was accompanied by a maintenance or increase in ATP concentration and a potassium loss by a fall in ATP. It also emerged that 2:3-PGA almost disappeared from a glucose-free suspension, owing to conversion to pyruvic acid, which suggests that 2:3-PGA, as a reserve source of energy, may be capable of maintaining potassium influx in the absence of glucose. To test whether potassium influx was maintained in the absence of glucose, and whether its decline was correlated with a fall in ATP, the following experiment was performed.

A washed glucose-free suspension of cells was divided into five flasks and ^{42}KCl was added to them at intervals of 1 hr. To measure the potassium influx as a function of time, samples were removed from the flasks at 30 and 60 min after the addition of ^{42}K, and after centrifugation the cells were washed and analysed for ^{42}K and haemoglobin. A graph of activity against time was drawn for each flask and from the slopes of the straight lines obtained the influx at each hourly interval was calculated. Measurements of ATP and 2:3-PGA were also made on cells taken at hourly intervals. The same measurements were also made on cells incubated in the presence of glucose. The results

(Fig. 4) show that, as expected, the potassium influx and ATP concentration in cells containing glucose were the same after 4 hr incubation as initially. The initial influx was 2·0 m-mole/l. cells/hr and the initial ATP concentration was 1·2 m-mole/l. cells. The potassium influx into glucose-deprived cells, on the other hand, was reduced by 15% even after 1 hr incubation and the fall continued, until after 4 hr incubation the influx was but 57% of its initial value. When expressed as a percentage of the initial value, the ATP concentration fell in almost the same way as the potassium influx. 2:3-PGA was utilized in a way similar to that shown in Fig. 3. The rate of decline in potassium influx and ATP concentration was thus very close, and Fig. 4 shows that in spite of its conversion to pyruvate, 2:3-PGA was unable to maintain the potassium influx and ATP concentration at their initial levels.

Ratio of potassium influx to ATP hydrolysis

From the work of Rapoport & Luebering (1952), which demonstrated the conversion of 2:3-PGA to 3-PGA during its metabolism to pyruvic acid, it is reasonable to assume that only one molecule of ATP is formed per molecule of

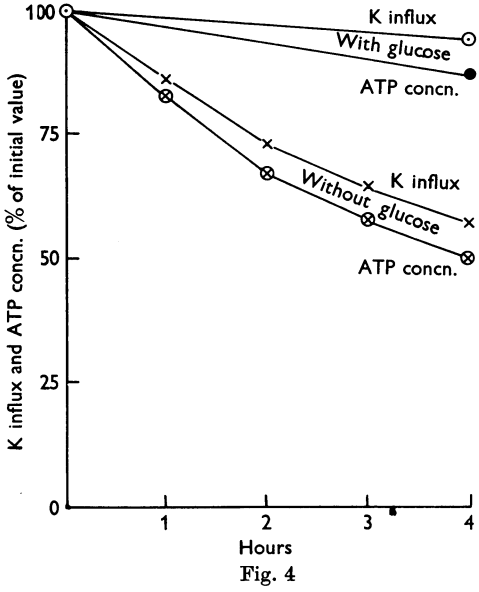

Fig. 4

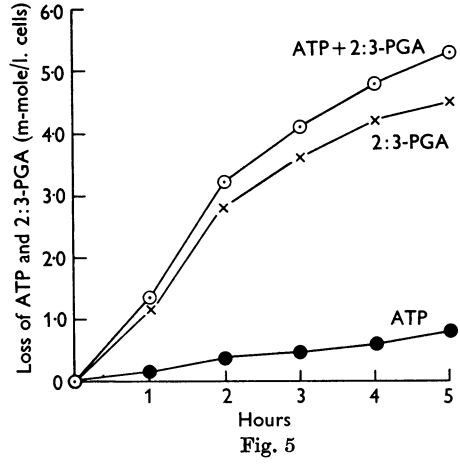

Fig. 5

Fig. 4. Change in potassium influx and ATP concentration in human red cells in the presence and absence of glucose. Results are plotted as percentages of the initial values. Potassium influx values were obtained by measuring the rate of uptake of ^{42}K, which was added to separate suspensions of cells at hourly intervals.

Fig. 5. The total loss of ATP and 2:3-PGA from cells deprived of glucose during incubation at 37° C. The total loss of the two compounds (⊙) is given as a measure of the total hydrolysis of ATP, assuming that one molecule of ATP was synthesized per molecule of 2:3-PGA metabolized.

2:3-PGA utilized. The rate of disappearance of 2:3-PGA from the cells was measured (see Fig. 3) and has accordingly been taken to represent the rate of ATP synthesis due to 2:3-PGA metabolism. Since the intracellular ATP concentration showed a progressive fall (Fig. 4) the total rate of hydrolysis of ATP must have been equal to the rate of synthesis from 2:3-PGA plus the rate of loss of endogenous ATP. The total loss of ATP computed in this way is shown in Fig. 5. The amounts of ATP hydrolysed and the mean values for potassium influx during each of the first 4 hr of incubation are given in Table 4. The ratio

TABLE 4. Comparison of K influx and ATP hydrolysis in glucose-deprived cells

	ATP hydrolysed (m-mole/l. cells)	Mean K influx (m-mole/l. cells)	Mean K influx / ATP hydrolysed
Experiment 1			
1st hour	1·37	1·93	1·4
2nd hour	1·41	1·64	1·2
3rd hour	1·03	1·42	1·4
4th hour	0·83	1·26	1·5
Experiment 2			
1st hour	1·35	2·00	1·5
2nd hour	1·45	1·73	1·2
3rd hour	1·15	1·51	1·3
4th hour	1·00	1·29	1·3

of potassium transported inwards to ATP hydrolysed was about 1·4. It is worth emphasizing that this ratio is derived solely from measurements of potassium influx and the loss of 2:3-PGA and ATP in cells deprived of glucose, and the only assumptions made are that one molecule of ATP was synthesized during the conversion of 2:3-PGA to pyruvic acid and that all the intracellular ATP was available for the transport mechanism. The similar results of a duplicate experiment (number 2) are also shown in Table 4.

If ATP is indeed the source of the energy for the linked active transport of sodium and potassium, the fraction of the free energy of hydrolysis of ATP utilized in this way can be calculated. For a linked transport of sodium and potassium the free energy required for the movement of one equivalent of cation is

$$RT \ln \frac{[K]_i [Na]_o}{[K]_o [Na]_i},$$

where the subscripts refer to the internal (i) and external (o) concentrations—concentrations here correspond respectively to m-mole/l. water in cells or external medium—and other terms have their usual significance. Substitution of values for the internal and external cation concentrations and the influx value for the first hour, gives the energy requirement as 3030 cal. The standard free energy of hydrolysis of the terminal phosphate group of ATP is about 5600 cal (Vladimirov, Vlassova, Kolotilova, Lyzlova & Panteleyeva, 1957) and thus the percentage of the observed hydrolysis energy required for the cation

transport is 75% $\left(=\frac{1{\cdot}4 \times 3030}{5600}\right)$. The available ATP was thus sufficient to satisfy the energy requirement of the cation transport, even on the disadvantageous assumption that the whole of the potassium influx and sodium efflux were active.

Effect of digoxin

Schatzmann showed in 1953 that digoxin and other cardiac glycosides prevented the uptake of potassium at 37° C by cold-stored human red cells which otherwise occurred in the presence of glucose, and moreover, that this effect was not due to an inhibition of glycolysis. More recent work on the kinetics of sodium and potassium movements in the presence of digoxin has shown that the active fluxes of these ions are reduced at least to the values found in cells deprived of glucose (Kahn & Acheson, 1955; Joyce & Weatherall, 1955; Glynn, 1955, 1957). It therefore seemed worth testing whether digoxin might act by interfering with the supply of ATP from glycolysis. To test this possibility incubations were made at 37° C with both fresh and cold-stored cells both in the presence and absence of glucose.

Freshly washed cells were suspended in two lots of medium, identical save that glucose was added to one and not to the other. Each sample was further divided into two lots, to one of which digoxin was added to give a final concentration of 0·01 mM. This arrangement was designed to show whether digoxin caused a greater loss of potassium than glucose deprivation alone. Within the limits of experimental error, the results of incubation at 37° C (Table 5) show that digoxin in the presence of glucose caused only the same net loss of potassium (2 m-mole/l. cells/hr) as that from cells deprived of glucose, and also that the addition of digoxin did not increase the loss of potassium from glucose-deprived cells. The potassium concentration was maintained in the control cells without digoxin. In contrast to the loss of potassium with digoxin in the presence of glucose, the concentrations of ATP, ADP, 2:3-PGA and inorganic phosphate remained the same in cells incubated both with and without digoxin.

In a glucose-free suspension, the addition of digoxin (or of ouabain) markedly retarded the hydrolysis of ATP during the first 2 hr of incubation (Fig. 6*a*). In two other experiments of 1 hr duration the inhibition of dephosphorylation of ATP was 79 and 94%. This sparing effect was observed only during the first 2 hr and the rate of hydrolysis after then was about the same as that in cells without the glycoside. Fig. 6*a* shows that the utilization of 2:3-PGA in glucose-free cells was unaffected by digoxin or ouabain. Fig. 6*b* shows the total loss of ATP and of 2:3-PGA.

Table 5 shows the results of cold storage of cells with and without digoxin in the presence and absence of glucose. During cold storage, the same amount of potassium was lost under each of the four conditions and this shows, first, that

TABLE 5. Incubation of fresh and cold-stored red cells in the presence and absence of digoxin (10^{-5} M) and glucose (11 mM). Cells were incubated for 5 hr at 37° C either fresh or after cold storage at 4° C for 5 days. Concentrations are given in m-mole/l. cells

Compound	ATP	ADP	2:3-PGA	Inorganic phosphate: Cells	Inorganic phosphate: External saline medium	Total acid-soluble phosphate	K
Fresh cells							
Before incubation	0·95	0·40	5·5	4·0	10·0	19·0	104
After incubation with glucose	0·85	0·40	3·0	7·5	7·5	17·0	103
with glucose and digoxin	0·90	0·50	3·5	7·0	7·5	17·5	94
without glucose	0·60	0·40	1·5	13·5	10·0	20·0	95
without glucose, with digoxin	0·60	0·40	1·5	14·0	10·0	20·0	94
Cold-stored cells							
After storage with glucose	0·55	0·50	5·5	5·5	8·5	19·5	84
After incubation at 37° C	0·70	0·50	1·5	11·0	10·5	17·5	88
After storage with glucose and digoxin	0·50	0·45	5·5	6·5	8·5	19·5	83
After incubation at 37° C	0·65	—	2·0	12·5	8·5	19·0	76
After storage without glucose	0·70	0·70	0·5	15·5	9·0	21·0	84
After incubation at 37° C	0	0·40	0	14·5	9·5	18·5	78
After storage without glucose, with digoxin	0·60	0·70	1·0	16·0	9·0	20·5	86
After incubation at 37° C	0	0·35	0	13·0	10·5	16·0	79

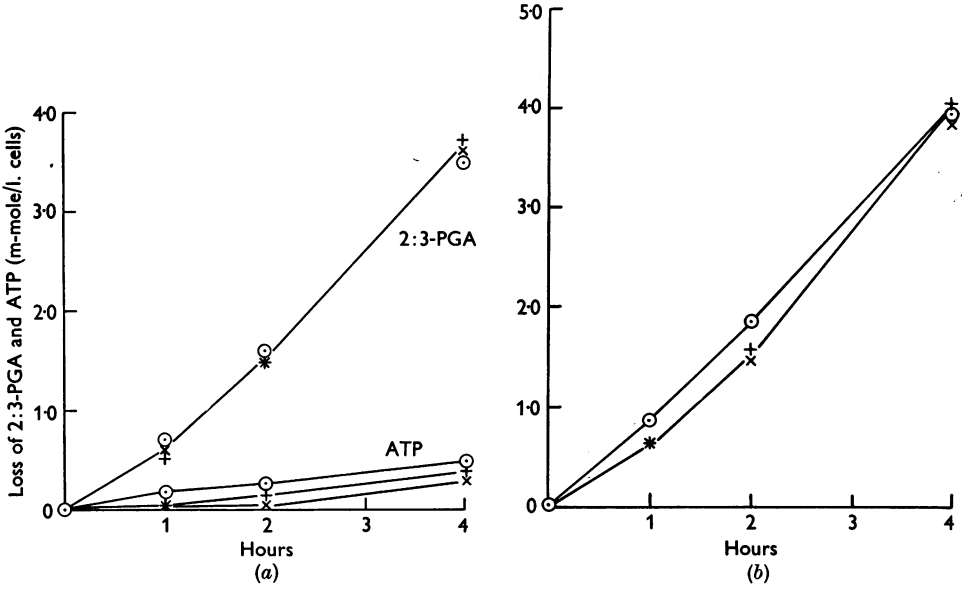

Fig. 6. The effect of ouabain (10^{-6} M) and digoxin (10^{-5} M) on ATP hydrolysis in red cells deprived of glucose during incubation at 37° C. The loss of 2:3-PGA was unaffected by the glycosides but the values are plotted (*a*) separately and (*b*) after addition to the ATP values in order to show the total hydrolysis of ATP and the initial retardation effect of ouabain and digoxin. ⊙, cells without glycoside added; ×, 10^{-6} M ouabain added; +, 10^{-5} M digoxin added.

glucose deprivation did not increase the loss of potassium after 5 days storage (see also Fig. 3, and Maizels, 1949), and secondly, that the addition of digoxin did not increase the loss of potassium at 4° C either in the presence or absence of glucose.

After cold storage with glucose the same concentrations of the different phosphate fractions were found in cells stored with and without digoxin. Thus, the ATP fell during cold storage whilst the other phosphates did not change markedly. In agreement with Schatzmann (1953), it was found that digoxin prevented the recovery of potassium on 5 hr incubation at 37° C which occurred with glucose (4 m-mole K/l. cells), and, instead, a loss of 7 m-mole K/l. cells was observed. The phosphates of the various fractions were again unaffected by digoxin in the presence of glucose (Table 5) and a small increase in ATP plus ADP, a large fall in other organic phosphates and a rise in orthophosphate are shown by the values.

In the absence of glucose the cells lost the same amount of potassium (6–7 m-mole/l. cells) at 37° C in suspensions both with and without digoxin, and this loss was the same as that found in the cells with glucose and digoxin. After cold storage without glucose most of the 2:3-PGA had disappeared, with a corresponding rise in inorganic phosphate, and after 5 hr at 37° C the cells had lost about 80% of ATP plus ADP. The endogenous store of 2:3-PGA was thus utilized during cold storage when, evidently, the cells were not completely metabolically inert.

Digoxin thus inhibited the active influx of potassium in both fresh and cold-stored cells and the net losses were the same with digoxin as with glucose deprivation. It is unlikely to cause these effects by a disturbance of phosphate transfers involving ATP since the ATP concentration was not reduced by digoxin. A similar result was found when digoxin was added to give a final concentration of 0·001 mM.

DISCUSSION

Glycolysis, ATP and the transport of potassium

It is known that synthesis of ATP requires energy, supplied either by respiration or glycolysis, and that inhibition of metabolism consequently stops the synthesis of ATP. The fact that it also eventually eliminates the active fluxes of cations has led to the view that the hydrolysis of ATP provides the energy for the migration of sodium and potassium against concentration gradients, but it is not direct evidence. The only unequivocal evidence that ATP is linked to movements of cations against electrochemical gradients is that of Caldwell & Keynes (1957) who demonstrated an efflux of sodium from cyanide-poisoned nerve axons when ATP was injected. Support for a similar linkage in red cells is given by Prankerd (1956) and in this paper.

Human erythrocytes in which the potassium concentrations were rising,

falling and being maintained constant have been analysed for phosphate esters and potassium, and the main finding is that ATP was always present when the intracellular potassium was rising or being maintained, whilst a fall in ATP occurred when potassium was leaking into the medium. Thus, during the incubation of fresh cells with glucose at 37° C both potassium and ATP were maintained constant, and during similar incubation after cold storage with glucose a rise in the intracellular concentrations of both ATP and potassium were observed. Conversely, when the potassium concentration was falling, as during incubation with IAA or in the absence of glucose, a fall of ATP and of potassium occurred. Gerlach (1956) and Prankerd & Altman (1954) have made similar observations on the phosphate compounds. These results are only compatible with, and are not direct evidence for, the ATP hypothesis, since the same results would be obtained if the active transport of potassium and the synthesis of ATP depended separately upon glycolysis without the transport of potassium being directly dependent upon ATP.

The most suggestive indication of a causal linkage between active potassium influx and ATP, and evidence against a dependence of influx upon the whole of the reactions of glycolysis, are provided by the observation that in cells producing not lactic acid but pyruvic acid the rates of fall in influx and ATP concentration were very similar (Table 4). This correlation suggests that in the absence of glucose ATP prevented an abrupt fall in potassium influx to its final low level. If the influx had been coupled to a glycolytic enzyme at a point before phosphoglyceromutase, such a fall would be expected, but since these enzymes were inactive in glucose-free conditions they may be excluded as being directly concerned in active potassium transport in human red cells. A dependence upon the oxidation and reduction of co-enzyme 1 can also be excluded. The possible enzymes with which potassium influx could be linked in the absence of glucose are enolase, pyruvate kinase and phosphoglyceromutase, because these are involved in the conversion of 2:3-PGA to pyruvic acid. Although these enzymes cannot be excluded it seems simpler to regard potassium influx and sodium efflux as being due to the operation of an energy-requiring carrier mechanism that utilizes ATP.

Suggestions as to the precise role of ATP such as facilitation of the formation or break-down of carrier-cation complexes, or their movement across the membrane, remain speculative at the present time. Energy calculations involving ATP hydrolysis and flux measurements can easily be misleading (Gillespie, Maw & Vernon, 1953; Whittam & Davies, 1954), but the present results show that the cation fluxes would require only about 75% of the energy of hydrolysis of ATP.

The role of 2:3-*PGA in red cells*

The role of 2:3-PGA in mammalian red cells is still obscure, for its conversion to pyruvic acid—ineffective as regards the complete maintenance of

potassium influx—can hardly represent its physiological function. The present results agree with earlier ones in indicating the dynamic nature of 2:3-PGA and its close connexion with glycolysis (see Rapoport & Guest, 1939; also review by Guest & Rapoport, 1941). Farmer & Maizels (1939) suggested that the base-binding power of 2:3-PGA, other phosphorylated compounds and glutathione in the red cell may be equal to that of haemoglobin. Moreover, with the suitable pK value of 6·78 (Rubin, 1938), 2:3-PGA could participate in the acid-base buffering action of the blood in a way that intimately links this important physiological function with red cell metabolism.

ATP and 2:3-PGA

Rapoport & Luebering (1951) showed that human red cells contain an enzyme, glycerate-2:3-diphosphatase, which catalyses the hydrolysis of 2:3-PGA to 3-PGA. 3-PGA can be converted to phospho-enolpyruvate, which reacts with ADP to form ATP, and hence one molecule of ATP is formed per molecule of 2:3-PGA. Prankerd & Altman (1954), however, suggested that 2:3-PGA could give rise to two molecules of ATP by first undergoing a transformation to 1:3-PGA. This mechanism assumes the unlikely conversion of a secondary alcohol phosphate (2:3-PGA) into an acyl phosphate (1:3-PGA) and, furthermore, Rapoport & Luebering (1952) have shown that the diphosphoglycerate mutase reaction is practically irreversible and proceeds only in the direction of 1:3-PGA to 2:3-PGA. It is likely therefore that the catabolism of 2:3-PGA provides the cell with only one molecule of ATP at the reaction catalysed by pyruvate kinase (Solvonuk & Collier, 1955).

Red cells and digoxin

Schatzmann (1953) showed that the accumulation of potassium by red cells was prevented by digoxin, although the glycolysis was unaffected, and further work on the kinetics of the sodium and potassium movements has shown that the active fluxes were abolished (Joyce & Weatherall, 1955; Kahn & Acheson, 1955; Solomon, Gill & Gold, 1956; Glynn, 1957). The results of the phosphate analyses show clearly that the effect of digoxin on the transport of potassium in the presence of glucose was not due to a depletion of intracellular ATP, and the effect may be due to the inactivation of a membrane carrier directly.

The sparing effect on ATP hydrolysis found in glucose-free cells (as also found by Dunham, 1957), in the presence of ouabain and digoxin, is added support for the carrier-ATP hypothesis for sodium and potassium transport, for the retardation in ATP hydrolysis by an agent known to eliminate active fluxes of sodium and potassium strongly suggests that the active fluxes normally utilize ATP.

SUMMARY

1. Measurements of ATP, ADP and 2:3-diphosphoglyceric acid (2:3-PGA) have been made in human red cells maintaining, increasing and losing their intracellular potassium.

2. Concentrations in human red cells (in m-mole/l. cells with S.E. of the mean) were: ATP, $0{\cdot}90 \pm 0{\cdot}08$; ADP, $0{\cdot}48 \pm 0{\cdot}03$; 2:3-PGA, $4{\cdot}62 \pm 0{\cdot}24$ and K, $98{\cdot}9 \pm 1{\cdot}6$. Fresh cells incubated in the presence of glucose maintained their initial phosphate and potassium concentrations, whilst in the absence of glucose potassium leaked out, ATP and 2:3-PGA were hydrolysed and adenylic acid accumulated. The 2:3-PGA hydrolysed was quantitatively converted to pyruvic acid. The hydrolysis of ATP and 2:3-PGA in glucose-free cells was unaffected when the external potassium concentrations were altered so as to reduce the concentration gradients.

3. During cold storage at 4° C ATP was partly hydrolysed to ADP and the potassium concentration fell; these changes were reversed on subsequent incubation with glucose at 37° C. Potassium leakage at 4° C was unaffected by removal of glucose and of Ca and Mg from the saline solution.

4. The rates of fall in potassium influx and ATP concentrations in glucose-free cells were similar, suggesting that potassium influx was facilitated by the presence of ATP.

5. Digoxin and ouabain caused a net loss of potassium from fresh red cells containing glucose but did not reduce the ATP concentration, whilst after cold storage the uptake of potassium on incubation at 37° C was prevented but not the resynthesis of ATP. In glucose-free cells digoxin and ouabain caused an initial delay in the hydrolysis of ATP.

6. The conclusion has been reached that ATP is at least one of the links in the coupling between glycolysis and the active transport of potassium in human red cells and that a membrane carrier, inactivated by digoxin and ouabain, utilizes ATP in the transport of potassium across the membrane.

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