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SYNTHESIS OF ADENOSINE TRIPHOSPHATE AT THE EXPENSE OF DOWNHILL CATION MOVEMENTS IN INTACT HUMAN RED CELLS

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SUMMARY

1. When red cells that have been starved for about 6 hr are loaded with inorganic phosphate and incubated in high-sodium potassium-free media, the ouabain-sensitive efflux of potassium from the cells is accompanied by a ouabain-sensitive incorporation of inorganic phosphate into ATP.

2. The magnitude of the incorporation varies roughly linearly with the concentration of sodium in the medium. The ratio (ouabain-sensitive potassium efflux)/(ouabain-sensitive ATP synthesis) is probably not much less than 2 nor much greater than 3.

3. Potassium in the medium inhibits the ouabain-sensitive incorporation of phosphate. The concentration of potassium necessary for half-maximal inhibition is about the same as the concentration at which, under similar conditions, ouabain-sensitive potassium influx and the stimulation of ouabain-sensitive potassium efflux are both half-maximal.

4. These observations suggest that the ouabain-sensitive efflux of potassium from red cells incubated in high-sodium potassium-free media is associated with a reversal of the entire pump cycle. In media containing sufficient potassium to saturate the pump, the efflux appears to involve the reversal of only part of the cycle.

INTRODUCTION

In the preceding paper (Glynn, Lew & Lüthi, 1970), experiments are described which suggest that the ouabain-sensitive efflux of potassium from human red cells is brought about by a reversal of the mechanism normally responsible for carrying potassium ions into the cell. In media containing potassium in physiological concentrations, the ouabain-sensitive potassium efflux is associated with a larger ouabain-sensitive

potassium influx, and the pump as a whole runs in the forward direction. In media lacking potassium, however, the dependence of ouabain-sensitive potassium efflux on external sodium, and the occurrence of a *net* ouabain-sensitive flux of sodium into the cells, suggest that the ouabain-sensitive potassium efflux is associated with a reversal of the entire pump cycle. Such a reversal should lead to the synthesis of ATP, and the experiments reported here were designed to see whether, and to what extent, ouabain-sensitive incorporation of inorganic phosphate into ATP accompanies the ouabain-sensitive efflux of potassium. Preliminary accounts of these experiments have already been published (Glynn & Lew, 1969*a*, *b*).

METHODS

Preparation of cells. Cells from freshly drawn heparinized blood were washed three times at 37° C with a buffered isotonic salt solution to remove white cells and glucose. The washed cells were incubated at a haematocrit of 20 % for at least 6 hr in a medium which generally contained (mM): choline 150; Mg 1; Tris (pH 7·4 at 37° C) 10; Cl 160. If the cells were to be loaded with ^{42}K, sufficient isotonic KCl labelled with ^{42}K was added to the incubation solution to give a potassium concentration of 10 mM. An hour before the end of the incubation, iodoacetamide was added as a 50 mM solution in more of the incubation medium to give a final concentration of 5 mM. After the incubation the cells were washed twice with an ice-cold solution containing 75 mM trisodium citrate and 45 mM sodium phosphate (pH 7·4), and were then incubated at 37° C for 15 min in about four volumes of a similar solution labelled with [^{32}P]orthophosphate ($^{32}P_i$) and containing iodoacetamide (5 mM). After 15 min the cells generally contained about 30 m-mole orthophosphate per 5 m-mole haemoglobin. They were washed 4–5 times with an ice-cold solution containing (mM): Na 150, Mg 1; Tris (pH 7·4 at 37° C) 5; Cl 156; iodacetamide 5; and were resuspended in about twice their own volume of the same solution.

The method of loading cells with inorganic phosphate by incubating them for a short period in a phosphate/citrate medium is based on the observations of Mollison, Robinson & Hunter (1958), who showed that red cells incubated at 37° C in chloride-free media containing inorganic phosphate took up the phosphate with a half-time of about 5 min.

The procedure described above was varied slightly in some of the experiments. In the experiment of Table 1, the medium used for the 6 hr incubation contained 2 mM-Ca, and before the phosphate-loading step the cells were washed with a medium containing (mM): Na 154; Mg 1; phosphate (pH 7·4) 2·5; Cl 152 instead of the usual phosphate/citrate medium. The cells were incubated in the labelled phosphate/citrate medium for only 10 min. In Expt. 2 of Table 2, 10 mM-K was included in the phosphate solution to minimize the incorporation of inorganic phosphate into ATP during the loading. In the experiment of Fig. 2, the pre-incubation medium contained 150 mM-K instead of choline, and the final wash solution contained choline instead of sodium.

Experimental procedure. Cells loaded with [^{32}P]orthophosphate and, where appropriate, with ^{42}K, were incubated in the test media as described in the preceding paper (Glynn *et al.* 1970). The haematocrit was about 5 % in all experiments. At the end of the incubation period, the cell suspensions were returned to the ice-bath, stirred and centrifuged at 1500 *g* for 3 min. The supernatants were sucked off and kept for the

later determination of ^{42}K, ^{32}P and total inorganic phosphate. The cells were washed once in an ice-cold solution containing (mM): choline 150; Mg 1; Tris (pH 7·4 at 37° C) 8; Cl 158; and were lysed in 2–3 ml. distilled water. The volume of haemolysate in each tube was made up to 5 ml. with a solution containing (mM): ATP 0·75; ADP 0·75; inorganic phosphate 0·4; and 0·5 ml. of 55 % (w/v) trichloracetic acid was added to each. The tubes were allowed to remain in the ice-bath for at least 5 min and were then centrifuged for 3 min at 1500 *g*. After extraction of the trichloracetic acid with ether, the supernatants were chromatographed on Dowex 1 resin as described by Garrahan & Glynn (1967*b*). To determine the specific activity of intracellular inorganic phosphate, separate lots of haemolysate were deproteinized with trichloracetic acid and analysed for total inorganic phosphate by the method of Weil-Malherbe & Green (1951), and for ^{32}P. During 30 min incubation about half of the intracellular inorganic phosphate was lost to the suspending medium, and in some experiments it was more convenient to determine specific activity in the medium at the end of the incubation. In experiments in which specific activity was estimated both in the cells and in the medium the two estimates were not significantly different.

Measurement of radioactivity. ^{42}K was measured with a Panax sodium iodide crystal scintillation counter, ^{32}P with a Nuclear Chicago liquid scintillation counter using Bray's (1960) solution. Where it was necessary to measure ^{42}K in solutions which also contained [^{32}P]orthophosphate, the phosphate was converted into phosphomolybdate and extracted into isobutanol as described by Weil-Malherbe & Green (1951).

Sources of materials. [^{32}P]orthophosphate and ^{42}K were obtained from the Radiochemical Centre, Amersham. ATP, ADP, choline, Tris ('Trizma' grade) and ouabain were from Sigma London Ltd.; the choline was recrystallized from hot absolute ethanol. Sodium and potassium chlorides were supplied 'specpure' by Johnson Matthey Ltd., London.

RESULTS

Ouabain-sensitive incorporation of inorganic phosphate into ATP

Table 1 summarizes the results of an experiment in which cells that had been starved for 6 hr, loaded with labelled inorganic phosphate and poisoned with iodoacetamide, were incubated for 30 min in high-sodium media with and without ouabain. In this Table, the incorporation of radioactivity into ATP and ADP is shown separately, but, since labelled ADP is thought to be formed from labelled ATP through the action of adenylate kinase, it is convenient to consider merely the total incorporation of inorganic phosphate into 'energy-rich' phosphate ($\sim$P). The results show that, for a litre of cells, in 30 min about 50 μ-mole inorganic phosphate were incorporated into $\sim$P by a ouabain-sensitive mechanism. The addition of 8·5 mM-K to the incubation medium reduced phosphate incorporation to the level found with ouabain.

Figure 1 shows the results of a similar experiment in which phosphate incorporation in the presence and absence of ouabain was measured in media containing different concentrations of potassium. Increasing the potassium concentration had no effect on the incorporation of phosphate

when the media contained ouabain, but reduced the ouabain-sensitive incorporation to zero. The inhibitory effect of potassium was half-maximal at a concentration of 1·3 mM, which is about the concentration at which stimulation of ouabain-sensitive potassium efflux by external potassium was found to be half-maximal under similar conditions (Glynn *et al.* 1970).

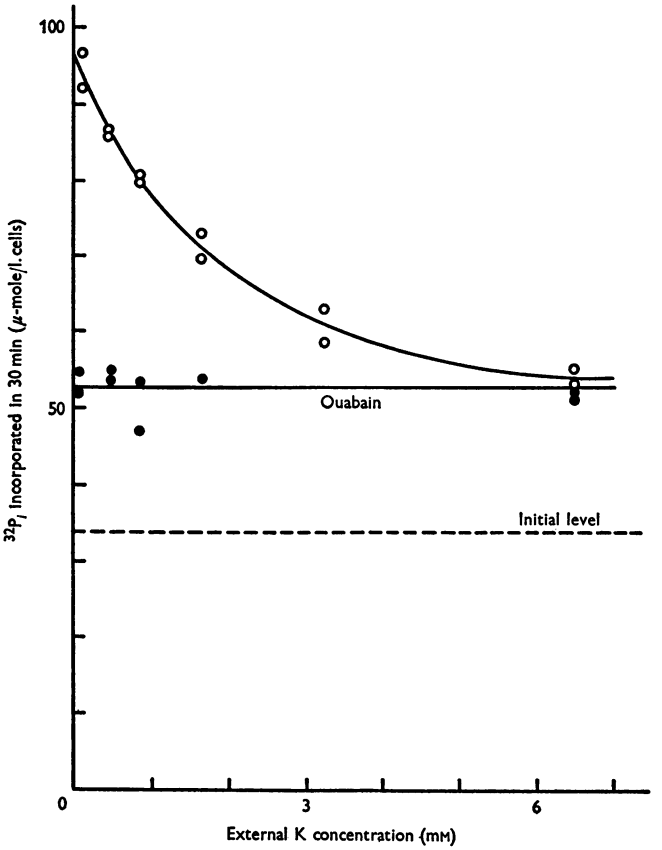

Fig. 1. ATP synthesis as a function of external potassium concentration. Cells starved for 6 hr, treated with iodoacetamide and loaded with $^{32}P_i$ as described in the Methods section, were incubated at 37° C for 30 min in media containing different concentrations of potassium, with and without ouabain. The basic incubation medium contained (mM): Na 150; Mg 1; Tris (pH 7·4 at 37° C) 5; Cl 156; iodoacetamide 5. K, when present, replaced an equivalent amount of Na. The ouabain concentration was 5×10^{-5} g/ml. At the end of the pre-incubation the cells contained only 3·28 m-mole Na/l. cells.

When starved red cells are incubated in K-free media, the ouabain-sensitive efflux of potassium is negligible when the external sodium concentration is 5 mM, and increases roughly linearly as the sodium

concentration is increased (Glynn *et al.* 1970). Phosphate incorporation by cells incubated in K-free media might, therefore, also be expected to vary more or less linearly with the external sodium concentration, and Fig. 2 shows that it does.

Stoicheiometry

In the experiments in which Garrahan & Glynn (1967*b*) demonstrated a ouabain-sensitive incorporation of inorganic phosphate into ATP in resealed ghosts, it was not possible to get any idea of the stoicheiometric relations between ATP synthesis and ion movements because the amount of synthesis was small and the irrelevant movements of ions were large. In intact cells the synthesis is greater and the membranes are much less

TABLE 1. Incorporation of inorganic phosphate into ATP by phosphate-loaded cells poisoned with iodoacetamide and incubated in high-sodium media

Medium			Fraction of $^{32}P_i$ incorporated into ATP (%)	Fraction of $^{32}P_i$ incorporated into ADP (%)	Estimated synthesis of ATP (μ-mole/l. orig. cells)
Na (mM)	K (mM)	Ouabain (g./ml.)			
154	0	0	1·09	0·401	143
			1·12	0·382	
154	0	5×10^{-5}	0·657	0·243	86·2
			0·669	0·237	
145	8·5	0	0·671	0·235	86·0
			0·653	0·241	

Cells starved for 6 hr and loaded with $^{32}P_i$, as described in the Methods section, were incubated at 37° C for 30 min. The incubation media contained 1 mM-$MgCl_2$, 2·5 mM phosphate buffer (pH 7·4) and 5 mM iodoacetamide in addition to the constituents shown in the Table. In this experiment the specific activity of the orthophosphate (P_i) in the loaded cells was not measured, and ATP synthesis has been calculated on the assumption that the intracellular specific activity was equal to the specific activity in the loading solution. The true specific activity in the cells must have been a little lower, because of P_i originally present and P_i that entered from the incubation media.

leaky, so it is worthwhile to compare ouabain-sensitive phosphate incorporation with ouabain-sensitive potassium efflux. Table 2 summarizes the results of two experiments in which both incorporation and efflux were measured in cells incubated in high-sodium potassium-free media. A difficulty in experiments of this kind is that some of the newly formed ATP is hydrolysed during the incubation, so that the observed phosphate incorporation leads to an underestimate of ATP synthesis. In the second experiment of Table 2 an attempt was made to measure ATP hydrolysis

by taking some of the cells at the end of the 30 min incubation and measuring the loss of labelled ~P when they were incubated for a further 30 min in an unlabelled phosphate/citrate medium. During the second 30 min incubation the specific activity of intracellular phosphate dropped sharply, because of the entry of unlabelled phosphate and the loss of radioactive phosphate, and by the end of the incubation period the specific activity was only 0·13 of its initial value. Knowing the amounts of labelled ~P at the end of the loading period, at the end of the first 30 min incubation and at the end of the second 30 min incubation, we may calculate the rates of formation of ATP in the following way.

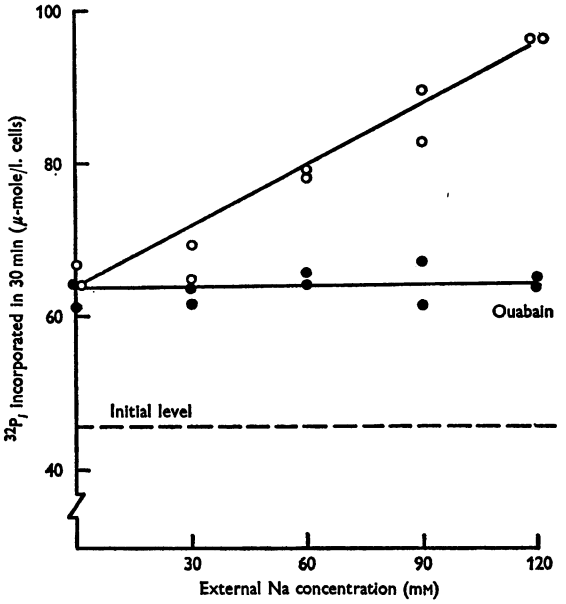

Fig. 2. ATP synthesis as a function of external sodium concentration. Cells starved for 9 hr, treated with iodoacetamide and loaded with $^{32}P_i$ as described in the Methods section were incubated at 37° C for 30 min in media containing different concentrations of Na, with and without ouabain. The basic incubation medium contained (mM): choline 150; Mg 1; Tris (pH 7·4 at 37° C) 8; Cl 158; iodoacetamide 5. Sodium replaced an equivalent amount of choline. The ouabain concentration was 5×10^{-5} g/ml.

Let:

α be the rate of formation of ATP in the control tubes,

α' be the rate of formation of ATP in the ouabain tubes,

β be the rate constant for the hydrolysis of ATP,

x_t be the quantity of labelled ~P at time t in the control tubes,

x'_t be the quantity of labelled ~P at time t in the ouabain tubes.

TABLE 2. The relation betwen ouabain-sensitive potassium efflux and ouabain-sensitive ATP synthesis in cells incubated in high-sodium potassium-free media

Expt.	Medium: Na (mM)	Medium: K (mM)	Medium: Ouabain (g/ml.)	K efflux in 30 min (μ-mole/l. orig. cells)	$^{32}P_i$ incorporated into ~P after 30 min (μ-mole/l. orig. cells)	$^{32}P_i$ incorporated into ~P after a further 30 min in unlabelled phosphate/citrate solution (μ-mole/l. orig. cells)
1	150	0	0	995 ± 5	176 173	—
	150	0	5×10^{-5}	777 ± 9	116 111	—
	141	8·5	0	—	114 122	—
2	150	0	0	706 ± 14	143·3 ± 2·2	118·7 ± 1·6
	150	0	5×10^{-5}	506 ± 17	88·7 ± 1·7	—

	Expt. 1	Expt. 2
Ouabain-sensitive K efflux (μ-mole/l. orig. cells)	218 ± 10	200 ± 22
Apparent ouabain-sensitive synthesis of ATP (μ-mole/l. orig. cells)	61·2	54·6 ± 2·8
$\frac{\text{Ouabain-sensitive K efflux}}{\text{Apparent ouabain-sensitive ATP synthesis}}$	3·56	3·66 ± 0·44
Ouabain-sensitive synthesis of ATP corrected for ATP break-down (μ-mole/l. orig. cells)	—	75·4
$\frac{\text{Ouabain-sensitive K efflux}}{\text{Corrected ouabain-sensitive ATP synthesis}}$	—	2·65

Cells loaded with ^{42}K and $^{32}P_i$ as described in the Methods section were incubated at 37° C for 30 min at a haematocrit of about 5 %. The basic incubation medium contained (mM): Na 150; Mg 1; Tris (pH 7·4 at 37° C) 5; Cl 156; iodoacetamide 5: potassium, when present, replaced an equivalent quantity of sodium. In the second experiment, cells that had been incubated without ouabain were transferred to an unlabelled phosphate/citrate solution and incubated for a further period of 30 min, during which the specific activity of the intracellular P_i fell to 0·13 of its initial value. Each figure for potassium efflux represents the mean of five tubes (± 1 S.E.) in the first experiment and the mean of seven tubes in the second. In both experiments, the specific activity of effluent potassium was used to calculate the efflux. $^{32}P_i$ incorporation under each set of conditions was measured in two tubes in the first experiment and in three tubes in the second.

Define unit specific activity as the specific activity of inorganic phosphate during the first 30 min incubation.

Let t_0 denote the end of the loading period, t_1 the end of the first 30 min incubation and t_2 the end of the second 30 min incubation.

For the control tubes during the first 30 min incubation,

$$\frac{dx}{dt} = \alpha - \beta x. \tag{1}$$

Integrating, and choosing 30 min as the unit of time,

$$\frac{\alpha - \beta x_1}{\alpha - \beta x_0} = e^{-\beta}. \tag{2}$$

Similarly, for the ouabain tubes,

$$\frac{\alpha' - \beta x_1'}{\alpha' - \beta x_0} = e^{-\beta}. \tag{3}$$

During the second 30 min incubation,

$$\frac{dx}{dt} = \alpha e^{-kt} - \beta x, \tag{4}$$

where k defines the rate of fall of specific activity of inorganic phosphate (assumed to be exponential).

Integrating,

$$x_2 = \frac{\alpha}{k - \beta}(e^{-\beta} - e^{-k}) + x_1 e^{-\beta}. \tag{5}$$

k is readily calculated from the known drop in specific activity during the second 30 min incubation, and eqns. (2), (3) and (5) may then be solved by substitution of known values for x_0, x_1, x_1' and x_2. The solutions are

$$\alpha = 179{\cdot}7 \ \mu\text{-mole/l. cells in 30 min},$$
$$\alpha' = 104{\cdot}3 \ \mu\text{-mole/l. cells in 30 min},$$
$$\beta = 0{\cdot}72 \ (30 \text{ min})^{-1}.$$

The ouabain-sensitive formation of ATP in 30 min was therefore $179{\cdot}7 - 104{\cdot}3 = 75{\cdot}4$ μ-mole/l. cells.

Hence the ratio

$$\frac{\text{ouabain-sensitive potassium loss}}{\text{ouabain-sensitive ATP synthesis}} = \frac{200}{75{\cdot}4}$$
$$= 2{\cdot}65.$$

It is not easy to give a precise estimate of the error of the corrected figure for ATP synthesis, but from the estimated error of the uncorrected figure it is clear that the true ratio is unlikely to have been much greater than 3 or much less than 2.

The above argument assumes that the rate constant for the hydrolysis of ATP was the same during the two 30 min incubation periods. Although this is likely to have been roughly true, transfer of cells from a chloride medium to a phosphate/citrate medium is likely to have led to some increase in intracellular pH and, as intracellular chloride exchanged for phosphate, to a decrease in cell volume. The expected effects of these changes on the rate constant for the hydrolysis of ATP are in opposite directions, and it is unlikely that the net effect could have been large enough to make much difference to the already rough estimate of ATP synthesis.

DISCUSSION

The experiments reported here show that the ouabain-sensitive efflux of potassium into high-sodium potassium-free media is associated with an incorporation of inorganic phosphate into ATP. Very roughly, a molecule of ATP is formed for every two to three potassium ions leaving the cell. The parallelism between the effects of increasing the external sodium concentration on ouabain-sensitive potassium efflux and ouabain-sensitive phosphate incorporation (cf. Fig. 2 of Glynn *et al.* 1970, and Fig. 2 of this paper) suggests that the stoicheiometry does not change greatly when the driving force is increased by an increase in the external sodium concentration, but the measurements are not accurate enough to exclude small changes.

In media containing potassium ions at concentrations sufficient to saturate the potassium entry mechanism, the ouabain-sensitive efflux of potassium is greater than it is in potassium-free media. It also differs from the efflux into potassium-free media (i) in being unaffected by the external concentration of sodium (Glynn *et al.* 1970), (ii) in being unaccompanied by a ouabain-sensitive entry of sodium (Garrahan & Glynn, 1967*a*), and (iii) in not being associated with a synthesis of ATP (this paper). Under these conditions, reversal of the potassium entry mechanism therefore appears to occur without reversal of the parts of the system concerned with sodium translocation or with phosphorylation by ATP.

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