J. Physiol. (1961), **156**, *pp.* 274–293
With 11 *text-figures*
Printed in Great Britain

ADENOSINETRIPHOSPHATASE ACTIVITY AND THE ACTIVE MOVEMENTS OF ALKALI METAL IONS

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(*Received* 2 *November* 1960)

It is now generally accepted that the mammalian red cell, like nerve and probably like most cells, maintains its ionic composition by pumping potassium inwards and sodium outwards. In red cells there is good evidence that the energy for this pumping comes from glycolysis (Danowski, 1941; Harris, 1941; Maizels, 1949). It is also well known from the work of Clarkson & Maizels (1952), Caffrey, Tremblay, Gabrio & Huennekens (1956), and Herbert (1956), that the red cell membrane possesses adenosinetriphosphatase (ATP-ase) activity. The experiments to be described in the present paper were designed to see whether this ATP-ase activity is connected with the mechanisms responsible for ion transport. A preliminary account of these experiments has already been published (Dunham & Glynn, 1960).

Four features characteristic of the transport system are (Glynn, 1957*a*):

1. The active movements of sodium and potassium are linked, in the sense that the cell is unable to pump sodium out unless potassium is available outside to be pumped in (Harris & Maizels, 1951; Shaw, 1954; Glynn, 1956).

2. Active transport is prevented by cardiac glycosides at concentrations of about 10^{-5} g/ml. (Schatzmann, 1953; Kahn & Acheson, 1955; Glynn, 1957*b*).

3. Modified cardiac glycosides in which the lactone ring is saturated or attached with the α configuration at C_{17} are much less active, or inactive (Glynn, 1957*b*).

4. At much lower concentrations of glycoside, inhibition can be prevented by raising the potassium concentration in the fluid *outside* the cells (Glynn, 1957*b*).

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Washed human red cell ghosts have been incubated in salt solutions containing ATP, and the rate of splitting has been followed by measurement of the inorganic phosphate formed. The response of the ATP-ase to the factors known to affect cation transport has been observed.

METHODS

Preparation of ghosts

First method. Freshly drawn human blood was used in all experiments. Clotting was prevented with heparin ('Pularin', Evans Medical Supplies) in a concentration of about 5000 u./100 ml. blood. The blood was centrifuged and the plasma and buffy coat removed by suction; the cells were then washed three times with isotonic saline by alternate resuspension and spinning. White cells were almost completely eliminated by removal of the top-most layer of cells with the supernatant after each wash.

The cells were lysed by adding them quickly to 10 volumes of stirred ice-cold glass-distilled water containing 0·1 mM cysteine. The haemolysate was allowed to stand for 10 min, and the cells were then spun down (6 min at 16,000 *g*) and washed with about 6 volumes of ice-cold glass-distilled water of which the pH had been adjusted to between 7·0 and 7·2 and to which a trace of cysteine had been added. The washing was repeated two or three times, until the supernatant contained only a trace of pink colour although the cells remained definitely pink. Some salt was added at this stage (to give a concentration between 1/3 isotonic and isotonic) and the suspension was pipetted into the graduated tubes in which incubation was to be carried out. The quantity of cells added to each tube was generally that which gave a haematocrit of about 10 % during incubation (calculated on the original volume of the cells, not the volume of the ghosts). The addition of salt before pipetting was found to be necessary, since otherwise the ghosts tended to clump, and reproducible results could not be obtained.

Second method. Ghosts prepared by the method just described were usually satisfactory, but occasionally the activity was two or three times as great as was expected and there was very little inhibition by cardiac glycosides. As will be shown later, this is what would be expected if the preparation had been contaminated with calcium ions and, though calcium contamination was never demonstrated, it was found that the properties of a supposedly contaminated preparation returned towards normal if the cells were washed with an isotonic solution of magnesium chloride of a 1 mM solution of ethylenediaminetetraacetate.

The procedure was therefore modified in that isotonic $MgCl_2$ was used to wash the cells before lysis, and after lysis strong $MgCl_2$ solution was added to make the concentration isotonic again. The ghosts were then washed in the usual way with chilled neutral glass-distilled water containing a trace of cysteine.

Third method. Ghosts prepared by the second method always had a normal fraction of glycoside-sensitive activity, but the absolute amount of activity was usually rather low and occasionally very low—about 1/5 of normal. It was thought that the restoration of isotonicity might have made many of the ghosts impermeable to ATP (cf. Székely, Mányai & Straub, 1952), and in one preparation whose activity was very low only 1/5 of the total volume of the ghosts was found to be accessible to ATP. Freezing and thawing increased the activity to normal—presumably by making the membranes leaky again—and in the third method the ghosts were frozen and thawed twice, after washing but before the addition of salt.

Reagents

ATP was obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio, and was purified on Dowex 1 ion-exchange columns by the following procedure: The ATP disodium salt was dissolved in a small quantity of water, and sufficient NaOH added to

bring the pH to between 6 and 8. This solution was poured onto 4 cm (1·5 ml.) columns of Dowex 1 resin, allowing about 0·25 g ATP per column. Each column was then washed with about 50 ml. of a solution containing 20 m-mole HCl and 20 m-mole NH_4Cl per litre. This solution brought down traces of adenosinemonophosphoric acid and adenosinediphosphoric acid. After the ammonium ions had been got rid of by washing each column with about 20 ml. of distilled water, the ATP was eluted with about 10 ml of chilled 250 mM-HCl. The effluent was received in chilled tubes and immediately neutralized with an ice-cold solution of trishydroxymethylaminomethane (Tris) hydroxide. The concentration of ATP was determined by U.V. absorption measurements at 259 mμ. After use the resin columns were washed with about 20 ml of 1 M-HCl and then with water until the pH of the effluent was about 5.

Salts. Except where otherwise stated, the salts used were of 'analytical reagent grade'. The 'spectroscopically pure' salts used in some of the experiments were supplied by Johnson Matthey and Co. Ltd.

Cardiac glycosides. Strophanthin was supplied by British Drug Houses Ltd. Scillaren A and hexahydroscillaren A were a gift from Messrs Sandoz of Basle. Cymarin and 17α-cymarin were a gift from Professor Reichstein of Basle. The glycosides were dissolved in 80 % ethanol (AR grade), and the alcoholic solutions were diluted with water.

Tris was the 'Tris 121 grade' supplied by the Sigma Chemical Company, St Louis, Missouri.

Cysteine was supplied by the Nutritional Biochemical Corporation.

All reagents were made up in glass-distilled water.

Procedure

Incubation was carried out in 5 or 10 ml. graduated tubes with glass stoppers. The reaction was generally started by the addition of ATP in a volume of fluid equal to one tenth of the total volume, so that the cooling effect was small. At the end of the incubation period the tubes were cooled in iced water for 1 min, and then ice-cold 55 % trichloroacetic acid was added to make the final concentration 5 %. The tubes were kept in iced water until it was convenient to spin down the precipitated protein and estimate the inorganic phosphate in a portion of the supernatant by the method of Fiske & Subbarow (1925). The cooling before the addition of trichloroacetic acid was found to be necessary to get complete deproteinization; if it was omitted the supernatants looked clear after spinning but became cloudy on addition of the strongly acid phosphomolybdate. The need to cool could probably have been avoided by the use of much larger volumes of more dilute trichloroacetic acid, but this would have produced a much larger final volume and so would have decreased the sensitivity of the method. The time during which each tube was kept in iced water before analysis was noted, and a correction was made for the small non-enzymic breakdown of ATP during this period and during the colour development. A small blank correction was also made, to allow for the trace of colour developed in supernatants from control tubes to which no ATP had been added. Preliminary trials showed that the presence of 1 mM cysteine (the concentration used in all experiments) did not affect the phosphate determinations, though concentrations of 5 mM or more caused large errors.

In experiments involving large numbers of tubes, the ATP was added serially at 15 sec intervals at the beginning of the experiment, and the tubes were removed from the water-bath to iced water at 15 sec intervals at the end. Trichloroacetic acid was added to the tubes in the same order exactly 1 min later. The large number of tubes used in some of the experiments and the necessity of washing the syringe at certain stages, to avoid contamination of one tube by the contents of another, meant that sometimes the tubes at the end of a run had been incubated at 37° C for perhaps 20 min longer than those at the beginning. Fortunately, it was found that incubation in the absence of ATP for 1 hr at 37° C led to a decrease in enzyme activity of just under 5 %, so the change in activity between the beginning and end of a run could be neglected.

RESULTS

Preliminary experiments (Figs. 1 and 2) showed that, under the conditions chosen, the amount of phosphate formed in a given time was proportional to the quantity of cells present; for a given quantity of cells the amount increased linearly with time for 1½ hr. In one or two experiments there was a falling off in activity of between 5 and 10% during

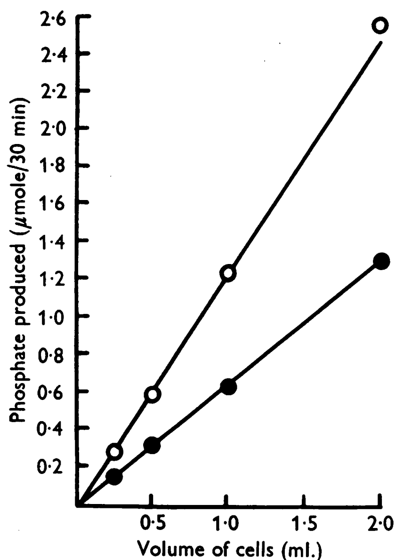


Fig. 1

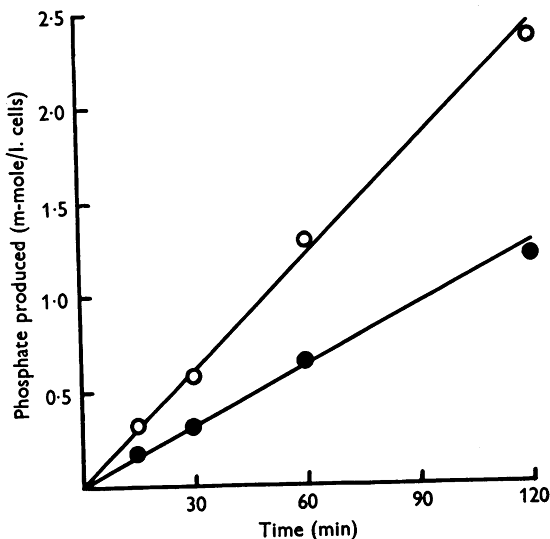


Fig. 2

Fig. 1. The effect of enzyme concentration on the rate of liberation of phosphate from ATP by red cell ghost ATP-ase. Ghosts were prepared by the first method. Temp. 37·1° C; pH 7·1; ATP 2 mM; Mg 5 mM; Na 132 mM; K 10 mM; cysteine 1 mM; ethylenediaminetetraacetate 1 mM; Tris 28 mM. Duration 30 min. ○ glycoside absent; ● strophanthin 10^{-5} g/ml.

Fig. 2. Time curve for the liberation of phosphate from ATP by red cell ghosts in the presence and absence of strophanthin. This experiment was done on the same preparation and under the same conditions as that shown in Fig. 1: symbols as in Fig. 1.

the first hour; the reason for this was never discovered. In subsequent experiments the amount of phosphate formed by a given quantity of cells in a fixed time, usually ½ hr or 1 hr, was used as a measure of activity.

The effects of Na, K and cardiac glycosides

If part of the ATP-ase activity is connected with the pump, and the pump only pumps if both sodium and potassium are present, one would

expect to find this part of the activity dependent on the presence of both sodium and potassium. One might also expect that any activity which appeared only in the presence of both sodium and potassium would disappear on the addition of cardiac glycosides at concentrations known to inhibit the pump. These expectations are realized in the results of two experiments shown in Table 1.

It is clear that a large part of the ATP-ase activity occurs in the absence of alkali metal ions. Addition of sodium alone has no effect; addition of potassium alone has a very slight effect; addition of both together causes a marked increase in activity, and this increase is abolished by the cardiac glycosides strophanthin and scillaren A. Some similar results have recently been reported by Post (1959).

TABLE 1. The effects of Na, K and cardiac glycosides on the ATP-ase activity of red cell ghosts

Expt. no.	[Na] (mM)	[K] (mM)	Activity (m-mole P/l. cells/hr)
IG. 4	0	0	0·75 0·78
	0	16	0·82 0·84
	16	0	0·73 0·83
	16	16	1·34 1·37
In the presence of strophanthin (10^{-4} g/ml.)	16	16	0·78 0·85
RP. 1	0	0	0·57
	0	16	0·64
	64	0	0·55
	64	16	1·33
In the presence of scillaren A ($1·7 \times 10^{-5}$ g/ml.)	64	16	0·58

Conditions of experiments. Ghosts prepared by the third method. Duration 1 hr; temp. 37° C; pH 7·0; ATP 1·5 mM; Mg 0·5 mM in Expt. IG. 4, 0·96 mM in Expt. RP. 1; cysteine 1 mM; Tris to make up 160 mM; 'Specpure' Na and K.

It is convenient, in what follows, to refer to the activity that depends on the presence of both sodium and potassium as 'glycoside-sensitive' and to the remainder as 'glycoside-insensitive'. This is not meant to imply that two separate enzymes are responsible, though they may be.

The effect of pH

Figure 3 shows the effect of pH in the presence and absence of cardiac glycosides. The solution was buffered with 35 mM Tris, but over the lower range of the curve the buffering was provided mainly by the ATP itself. The quantity of hydrogen ions liberated during the hydrolysis was calculated to be insufficient to upset the pH in all except the lowest two tubes, where a drop of ½ in pH probably occurred during the run.

The results are not accurate enough to make it worth while to plot the difference curve, which would give the pH sensitivity of the glycoside-sensitive component, but it is obvious that the effects of pH on the two components are not very different.

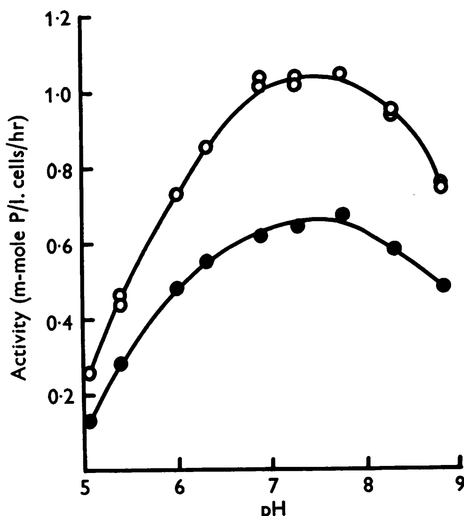


Fig. 3. The effect of pH on the ATP-ase activity of red cell ghosts. Ghosts were prepared by the second method. Temp. 37·1° C; ATP 1·5 mM; Mg 2 mM; K 10 mM; cysteine 0·75 mM; Tris 35·3 mM; Na to make up 160 mM. Duration 1 hr. ○ glycoside absent; ● + strophanthin 10^{-4} g/ml. In this experiment the reaction was started by the addition of the ghost suspension to solutions already containing ATP.

Activation by divalent cations

The ATP-ase studied by Herbert (1956) presumably corresponds to our 'glycoside-insensitive component', as potassium was not present in Herbert's experiments. Herbert's enzyme was strongly activated by magnesium ions; calcium activated weakly when present alone but reversed the activation due to magnesium.

The results given in Table 2 show that even if sodium and potassium are both present there is very little activity in the absence of magnesium. The relation between activity and magnesium concentration, both in the presence and absence of strophanthin, is shown in Fig. 4. The difference, representing the glycoside-sensitive component, is shown as a dotted line.

Since one way of explaining the activating effect of magnesium is to suppose that a complex of Mg and ATP is the true substrate, the concentration of Mg–ATP complex has been calculated for each magnesium concentration. From the titration curves of ATP between pH 8 and pH 2 in the presence and absence of magnesium, Burton & Krebs (1953) conclude that $MgATP^{2-}$ is the principle complex. Burton (1959) has measured the formation constant of this complex, and ΔH for its formation, by an ingenious method in which the

TABLE 2. Dependence of the ATP-ase activity of red cell ghosts on the presence of magnesium

Expt. no.	[Mg] (mM)	Strophanthin (g/ml.)	Activity (m-mole P/l. cells/hr)
DD. 1	0	0	−0·018 0·007 −0·060
	0	10^{-4}	−0·035 0·011 −0·011
	1·5	0	0·425
	1·5	10^{-4}	0·245
JD. 1	0	0	0·036 0·060 0·055
	0	10^{-4}	0·043 0·046 0·031
	2	0	0·490 0·504
	2	10^{-4}	0·271 0·281

Conditions of experiments. Ghosts were prepared by the second method and then washed three times with a buffered isotonic solution of sodium chloride to get rid of magnesium bound to fixed anions. Duration 1 hr; temp. 37° C; pH 7·1; ATP 1·5 mM; Na 120 mM; K 10 mM; cysteine 1 mM; Tris 30 mM. (The negative signs arise when the colour developed is less than the blank.)

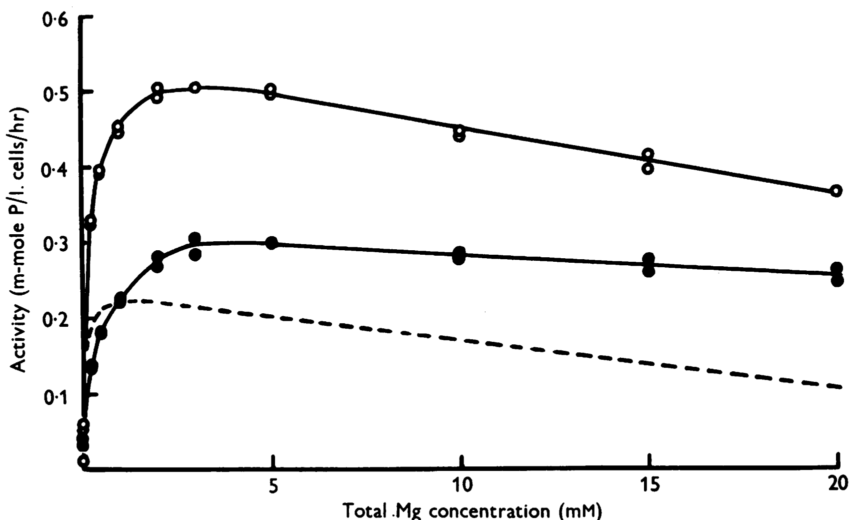


Fig. 4. The effect of Mg on the ATP-ase activity of red cell ghosts. Ghosts were prepared by the second method and then washed three times with a buffered isotonic solution of sodium chloride to get rid of Mg bound to fixed anions. Temp. 37° C; pH 7·1; ATP 1·5 mM; Na 120 mM; K 10 mM; cysteine 1 mM; Tris 30 mM. Duration 1 hr. (○) glycoside absent; (●) strophanthin 10^{-4} g/ml. The difference between the two curves is shown by the dotted line, which therefore represents the 'glycoside-sensitive' component.

concentration of free magnesium is calculated from the absorption spectrum of 8-hydroxyquinoline present in the solution. From Burton's data referring to the formation of complex at 25° C and pH 8 and in the presence of 100 mM-K, it is possible to calculate that the apparent formation constant at 37° C and pH 7 must be about 14000, where the apparent formation constant is defined by the ratio

$$\frac{[\text{MgATP}^{2-}]}{[\text{Mg}^{2+}][\text{ATP}_{\text{total free}}]}.$$

This figure has been used to calculate the concentration of complex at each concentration of magnesium in the present experiments.

The falling off in glycoside-sensitive activity at high magnesium concentrations suggests that free magnesium might compete with the complex, and, in fact, the observed relationship between glycoside-sensitive activity and the concentrations of complex and of free magnesium fit simple competitive kinetics very well if K_m (the dissociation constant of the enzyme-substrate complex) is 0·077 and K_i (the dissociation constant of the enzyme inhibitor complex) is 0·84. However, when these values for K_m and K_i are used to predict the change in glycoside-sensitive activity in an experiment in which total magnesium is kept constant and total ATP is increased (see, for example, Fig. 5), the results are wildly out. Another possibility that has been considered, suggested by the drop in the last point in Fig. 5, is that Mg-ATP complex is the substrate and that both free magnesium and free ATP act as competitive inhibitors. It should then be possible to find values for three constants, K_m, K_i and K_j (where K_i and K_j are the dissociation constants of the two enzyme-inhibitor complexes) which are compatible with the results of both experiments. In fact this has not been possible and the hypothesis must be rejected.

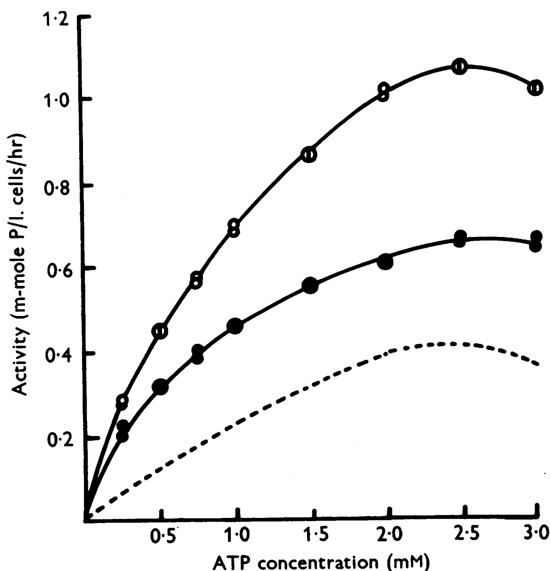


Fig. 5. The effect of ATP concentration on the ATP-ase activity of red cell ghosts. Ghosts were prepared by the second method. Temp. 37° C; pH 7·1; Mg 3 mM; Na 110 mM; K 10 mM; cysteine 1 mM; Tris 30 mM. Duration 1 hr. (○) glycoside absent; (●) strophanthin 10^{-4} g/ml. The difference between the two curves is shown by the dotted line, which therefore represents the 'glycoside-sensitive' component.

Calcium. In the presence of magnesium quite small amounts of calcium increase the ATP-ase activity two- or threefold; higher concentrations of calcium lead to inhibition (Fig. 6). The optimum level of calcium depends on the magnesium concentration (Fig. 7), being roughly three times as high when the magnesium concentration is raised by a factor of three.

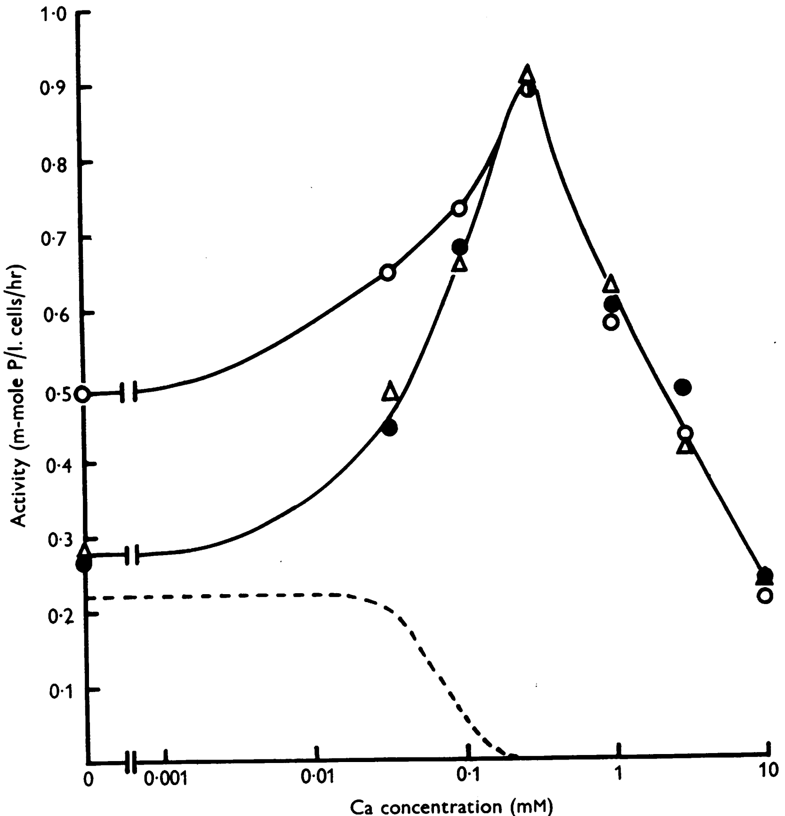


Fig. 6. The effect of calcium in the presence of magnesium on the ATP-ase activity of red cell ghosts. Ghosts were prepared by the second method. Temp. 37° C; pH 7·0; ATP 1·5 mM; Mg 2 mM; Na 110 mM; K 20 mM; cysteine 1 mM; Tris 30 mM. Duration 30 min. (○) glycoside absent; (●) strophanthin 10^{-5} g/ml.; (△) K-free, replaced by extra Tris. The difference between the two curves is shown by the dotted line, which therefore represents the 'glycoside-sensitive' component. Semi-log. scale.

This suggests that the inhibitory effect of calcium depends on a straight Mg–Ca competition. The activation by calcium affects only the glycoside-insensitive component; the glycoside-sensitive component is inhibited even at low calcium concentrations (Fig. 6). Figure 6 also shows that, as might

be expected, the absence of potassium has much the same effect as the presence of strophanthin.

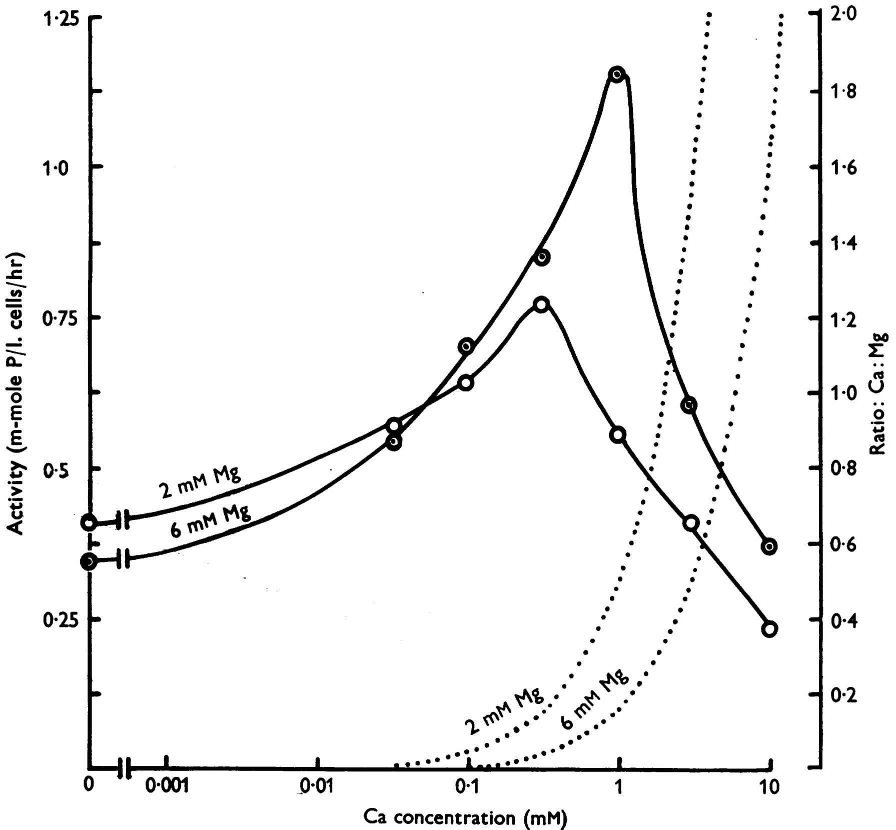


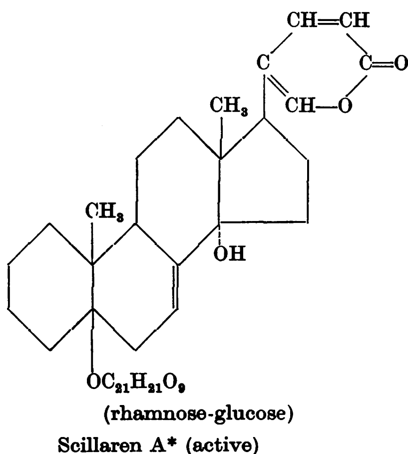
Fig. 7. The effect of calcium at two different levels of magnesium on the ATP-ase activity of red cell ghosts. The dotted lines show the ratio of Ca to Mg. Ghosts were prepared by the second method. (The ghost preparation used in this experiment is different from that used in the experiment shown in Figure 6, and has slightly less activity.) Temp. 37° C; pH 7·0; ATP 1·5 mM; Na 110 mM; K 20 mM; cysteine 1 mM; Tris 30 mM. Duration 1 hr. Semi-log. scale.

Molecular requirements for inhibition

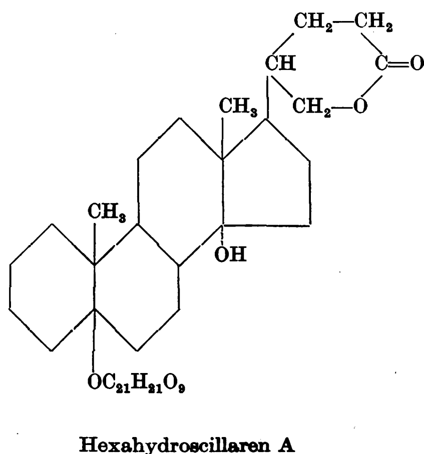
It was mentioned in the introduction that certain modifications to the cardiac glycoside molecule greatly reduce the inhibitory effect on ion pumping. The formulae of two glycosides that are powerful inhibitors of ion pumping, and of two related compounds that are much less active in this respect, are shown on p. 284.

Figure 8 shows a 'dose-effect curve' for the inhibition of the glycoside-sensitive component of the ghost ATP-ase by scillaren A. Marked on the

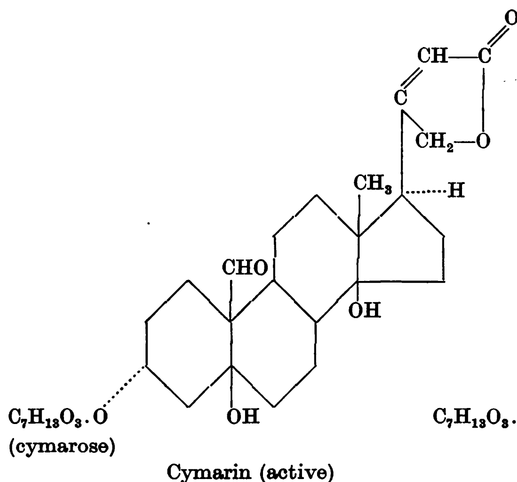
ordinate are the effects of 10^{-6} g/ml. of cymarin, 17 α-cymarin, and hexahydroscillaren A. It is clear that cymarin and scillaren A are about equally effective. Hexahydroscillaren A at a concentration of 10^{-6} g/ml. is less effective than scillaren A at 10^{-7} g/ml.—in other words it has less than one tenth of the activity. 17α-cymarin has no appreciable effect.



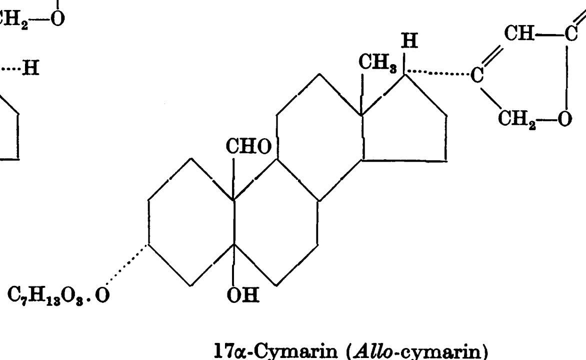
(rhamnose-glucose)
Scillaren A* (active)



Hexahydroscillaren A



(cymarose)
Cymarin (active)



17α-Cymarin (*Allo*-cymarin)

* In the paper by Glynn (1957 *b*) the formulae of scillaren A and of emicymarin are unfortunately shown with the wrong configuration at C_{17}.

So the same molecular features seem to be important for ATP-ase inhibition as are important for inhibiting the ion pump, and also, incidentally, for 'cardiotonic' action (Wollenberger, 1954). In this respect the effect of cardiac glycosides on the membrane ATP-ase differs from their effect on

the viscosity of cardiac actin solutions, for which the molecular requirements are much less stringent (Horvath, Kiraly & Szerb, 1949; Snellman & Gelotte, 1950).

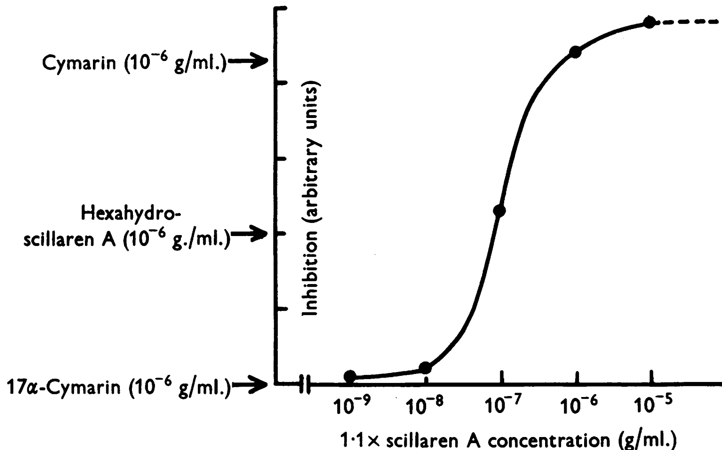


Fig. 8. Features of the cardiac glycoside molecule required for inhibition of red cell ghost ATP-ase. The continuous line is a 'dose–effect' curve for scillaren A. The effects of certain related compounds are shown on the ordinate. Ghosts were prepared by the third method. Temp. 37·1° C; pH 7·1; ATP 1·5 mM; Mg 1 mM; Na 64 mM; K 16 mM; cysteine 1 mM; 'Tris' 80 mM. Duration 1 hr. The tubes were placed in the water-bath 45 min before the addition of ATP. Semi-log. scale.

The effect of potassium on inhibition by cardiac glycosides

The fourth feature of the sodium–potassium pump was that inhibition by very low concentrations of cardiac glycosides could be prevented by raising the potassium concentration outside the cells (Glynn, 1957*b*). The effect resembled competitive inhibition, though competition between potassium ions and cardiac glycoside molecules does not seem very likely and the data did not accurately fit simple competitive kinetics. Table 3 shows the effect of potassium concentration on the inhibition of the glycoside-sensitive component of the ATP-ase by strophanthin at a concentration of 5×10^{-8} g/ml. The decrease in inhibition as the potassium concentration is raised is striking. Two experiments with slightly higher concentrations of strophanthin gave similar results, except that the reversal was less complete, and remained incomplete even when the potassium concentration was increased further. Thus, with strophanthin at a concentration of 10^{-7} g/ml. inhibition of the 'sensitive' component decreased from about 90 % with 0·25 mM-K^+ to 18 % with 8 mM-K^+ and then remained at about this level from 15 to 64 mM-K^+. With strophanthin at 2×10^{-7} g/ml. inhibition was 35 % at 8 mM-K^+, 39 % at 16 mM-K^+, and

35% at 32 mM-K$^+$, the differences between these figures not being significant. The interpretation of this levelling off is not clear but it cannot be explained on simple competitive kinetics. It could conceivably be due to a separate non-competitive inhibition, either by strophanthin itself or an impurity in the strophanthin preparation.

TABLE 3. The effect of potassium concentration on inhibition by strophanthin of the 'glycoside-sensitive' ATP-ase activity of red cell ghosts

K concn. (mM)	Total glycoside-sensitive activity (m-mole P/l. cells/hr)	Activity in the presence of strophanthin (5×10^{-8} g/ml.) (m-mole P/l. cells/hr)	Inhibition (%)
0·25	0·188	0·027	86
0·5	0·364	0·134	63
1	0·619	0·290	53
2	0·778	0·498	36
4	0·855	0·686	20
8	0·926	0·782	15
16	1·02	0·823	19
32	1·04	0·964	7

Conditions of experiment. Ghosts prepared by the third method. Duration 1 hr; temp. 37° C; pH 7·2; ATP 1·5 mM; Mg 0·5 mM; Na 60 mM; cysteine 1 mM; Tris to make up 160 mM. The tubes were placed in the water-bath an hour before the addition of ATP. The total glycoside-sensitive activity was determined with 10^{-4} g strophanthin/ml. and at its highest accounted for 56% of the total ATP-ase activity (see Fig. 9).

Sodium and potassium interaction

So far nothing has been said about the concentrations of sodium and potassium necessary to activate the glycoside-sensitive component of the ATP-ase. Figure 9 shows that with 60 mM-Na$^+$ present the activity increases with increasing potassium concentration up to about 4 mM-K$^+$ and then levels off. The concentration of potassium for half-maximum activity is about 1 mM. A similar result is shown by the top curve of Fig. 10. When activity is plotted as a function of sodium at a fixed potassium concentration of 16 mM (Fig. 11, top curve), activity increases up to about 32 mM-Na$^+$ and then levels off. The sodium concentration for half-maximum activity is about 4 mM. However, these statements do not adequately summarize the behaviour of the ATP-ase, because the effect of sodium concentration depends on the concentration of potassium and vice versa. Thus, in Fig. 11, although with 16 mM-K$^+$ activity increases with increasing sodium concentration till 32 mM-Na$^+$ and then levels off, with only 0·5 mM-K$^+$ there is an optimum at 8 mM-Na$^+$, and higher levels of sodium give less activity. Similarly, looking at the results in Fig. 11 the other way round, with 64 mM-Na$^+$ activity increases as potassium is raised from 0·5 to 2 to 16 mM, but with 2 mM-Na$^+$ there is slightly less activity at

2 mM-K^+ than at 0·5 mM-K^+, and less again at 16 mM-K^+. Again, in Fig. 10, with 64 mM-Na^+ activity is more than doubled as potassium is raised from 0·5 to 8 mM, while with only 2 mM-Na there is a slight decrease in activity over this range.

All these results may be summarized by saying that both sodium and potassium are necessary, but that for optimum activity the ratio between them must be neither too great nor too small.

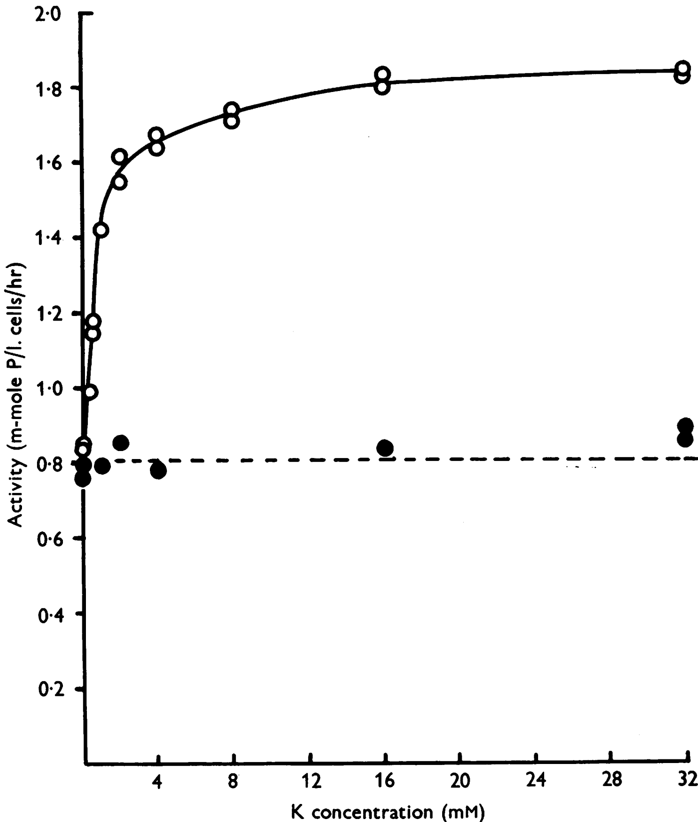


Fig. 9. The effect of potassium concentration on the ATP-ase activity of red cell ghosts in the presence and absence of strophanthin. 60 mM-Na; ○ glycoside absent; ● strophanthin 10^{-4} g/ml. The results plotted here were obtained on the same preparation, under the same conditions and at the same time as those given in Table 3.

DISCUSSION

The dependence of ion transport on glycolysis in some cells, on respiration in others, and on either in yet others, suggests that energy may be supplied to the pump in the form of ATP rather than directly from any of the individual steps in glycolysis or respiration. Support for this idea

comes from experiments with dinitrophenol. Dinitrophenol uncouples respiration from phosphorylation and is found to inhibit ion pumping in nerve and nucleated red cells, which depend on respiration, but not in mammalian red cells, which depend on glycolysis (Maizels, 1954; Hodgkin & Keynes, 1955). Further support for the role of ATP comes from the experiments of Caldwell (1956) on squid axons, and of Dunham (1957*a*, *b*)

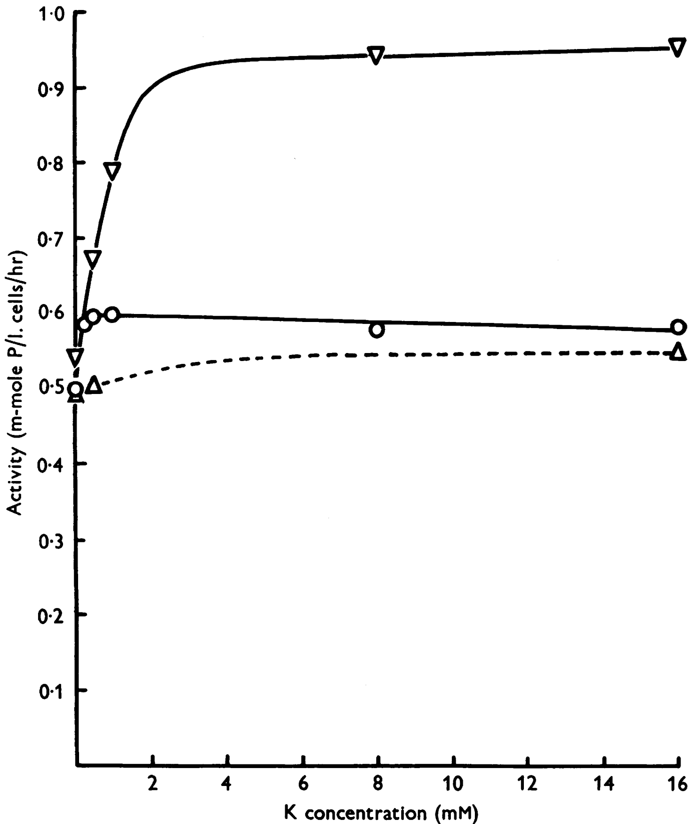


Fig. 10. The effect of potassium concentration on the ATP-ase activity of red cell ghosts at two different sodium concentrations. Ghosts were prepared by the third method. Temp. 37·1° C; pH 7·1; ATP 1·5 mM; Mg 1 mM; cysteine 1 mM; Tris to make up 160 mM. Na and K were 'Specpure' reagents. Duration 1 hr. ▽ 64 mM-Na; ○ 2 mM-Na; △ 0 mM-Na.

and Whittam (1958) on mammalian red cells, showing a correlation between the rate of ion transport and the concentration or rate of splitting of 'energy-rich' phosphate. More direct evidence is provided by experiments in which ATP was injected intracellularly into cyanide-poisoned squid axons (Caldwell & Keynes, 1957), or incorporated into 'reconstituted'

ghosts (Gardos, 1954), or removed from intact red cells by poisoning glucose-free cells with iodoacetate and then allowing them to phosphorylate added glucose (Dunham, 1957*b*).

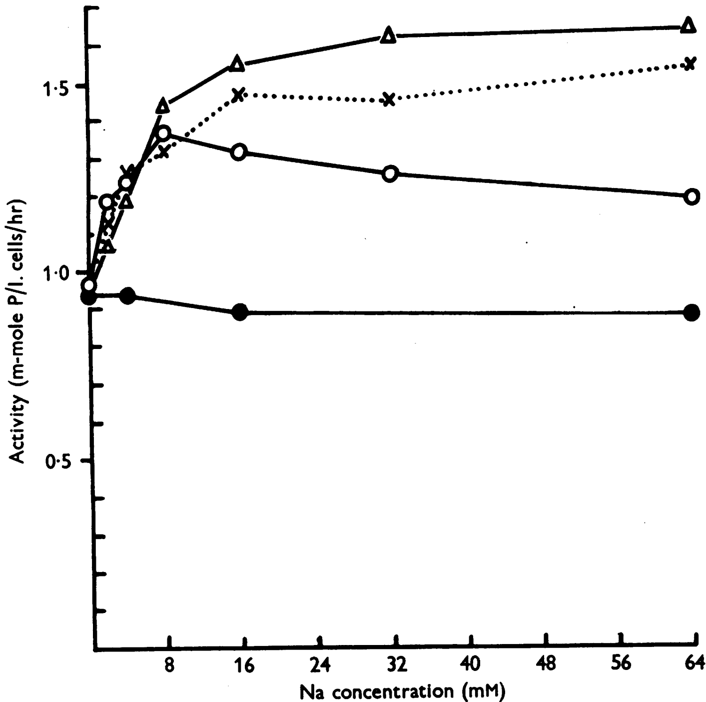


Fig. 11. The effect of sodium concentration on the ATP-ase activity of red cell ghosts at three different potassium concentrations. Ghosts were prepared by the third method. Temp. 37·2° C; pH 7·2; ATP 1·5 mM; Mg 0·5 mM; cysteine 1 mM; Tris to make up 160 mM. Na and K were 'Specpure' reagents. Duration 1 hr. △ 16 mM-K, × 2 mM-K, ○ 0·5 mM-K, ● 16 mM-K+strophanthin 10^{-4} g/ml.

The results reported in the present paper show that there is a close parallelism between the properties of an ATP-ase in the red cell membrane, and the properties of the pump responsible for shifting ions across that membrane. It is difficult to avoid the conclusion that the action of this ATP-ase somehow provides energy for the transport of sodium and potassium.

If, as seems likely, the sodium and potassium ions that activate the ATP-ase are also the ions that are pumped across the membrane, then it follows that *external* potassium and *internal* sodium are the activating ions. As the ghosts used in these experiments were highly permeable to sodium and potassium, there is no direct evidence on this point, but there is some

indirect evidence relating to potassium ions. The prevention by potassium of glycoside inhibition in intact cells is due to *external* potassium, because, in the relevant experiments, the internal potassium was high and remained unchanged. Unless the resemblance between this effect and the potassium reversal of ATP-ase inhibition is coincidental, it follows that the prevention of ATP-ase inhibition must also be due to *external* potassium. It is not certain that the effects of potassium in activating the ATP-ase and in preventing the inhibition of the ATP-ase occur at the same site, but it seems likely.

The interpretation of the rather complicated relationship between sodium and potassium concentration and activity is not clear. If there are sites with different affinities for Na^+ and K^+ and if the rate of ATP splitting depends on how these sites are filled, then a possible explanation would be that too high a ratio of K^+ to Na^+ displaces Na^+ from the sodium sites and too low a ratio displaces K^+ from the potassium sites. However, it is not necessary to postulate the existence of sites with different affinities. If Tris were unable to reach the sites at which the alkali metal ions exert their effects, the local concentration of each of these ions would depend on the concentration of the other and mutual interaction would occur. There are other possibilities. A serious limitation of the present experiments is that because the ghosts used were highly permeable to cations it was not possible to vary the concentration on the two sides of the membrane independently. Experiments with relatively impermeable ghosts would be helpful.

It has not been possible to decide whether the 'glycoside-sensitive' and 'glycoside-insensitive' components of the ATP-ase are two separate enzymes. The ratio between the size of the two components depends on the magnesium concentration at which activity is measured (Fig. 4). Different preparations have slightly different ratios, and in a given preparation the ratio of 'sensitive' to 'insensitive' activity tends to decrease slowly if the enzyme is stored for a week at 5° C. In one experiment the fraction of activity that was sensitive to cardiac glycosides was considerably increased by hypertonicity, but this effect has not been investigated further. All attempts to extract the ATP-ase into solution have failed. Acetone, Morton's (1955) butanol procedure, various other alcohols and digitonin have been tried. Since it has not been possible to detach the glycoside-sensitive ATP-ase from the membrane, and, under the experimental conditions used, it is likely that individual transport units in the membrane remained intact and continued to pump sodium outwards and potassium inwards, it may be that any or all of the observed alterations in ATP-ase activity resulted from a primary action on some other component of the transport system. This does not affect the argument that

a close resemblance between the properties of the pump and of the ATP-ase points to a role for the ATP-ase in ion transport.

The effect of calcium in very low concentrations is remarkable, though whether it has any physiological significance is not clear. It may be significant that red cells normally contain no appreciable calcium (Ponder, 1948).

There is evidence that active movements of sodium and potassium across nerve and muscle membranes resemble those across the red cell membrane in being linked (Keynes, 1954; Hodgkin & Keynes, 1955), in being inhibited by cardiac glycosides (Matchett & Johnson, 1954; Edwards & Harris, 1957; Caldwell & Keynes, 1959), and, in nerve anyway, in depending on energy from ATP (Caldwell, 1956; Caldwell & Keynes, 1957). The question therefore arises whether an ATP-ase similar to that in red cells might exist in nerve and muscle membranes. In muscle, where it is not possible to get the membrane free from the contents of the cell, and where the contents have very considerable ATP-ase activity, this is not going to be an easy question to answer. In nerve, however, there is some evidence that such an ATP-ase may exist. Libet, in 1948, described ATP-ase activity in squid axons, and showed that the activity remained behind when the axoplasm was extruded. However, nothing is known of the effects of alkali metal ions on this enzyme, nor is it known whether the activity is in the mitochondria, or attached to the axon membrane or even in the neurilemma. Of more interest is the ATP-ase described by Skou (1957) in submicroscopic particles obtained from finely minced crab nerves. In the presence of magnesium, this ATP-ase is activated to some extent by sodium and much more by sodium plus potassium, and these properties led Skou to suggest that the enzyme may be responsible for sodium and potassium transport. Unfortunately it is not known how the particles carrying the ATP-ase activity are related to the original axon membrane.

The enzyme in the red cell membrane responsible for the splitting of ATP has been referred to throughout this paper as an ATP-ase because what is observed is the formation of inorganic phosphate. But it is quite possible that phosphate transfer occurs first, and is followed more or less immediately by hydrolysis. If phosphate transfer does occur, the phosphate must for a time be attached to some constituent of the membrane, and it might be possible to identify the phosphorylated compound if the ATP split were labelled in the terminal group with ^{32}P. However, as the intermediate might be highly unstable, or present only in trace amounts, or both, the method might not work.

SUMMARY

1. The ATP-ase activity of human red cell ghosts has been shown to consist of two components.

2. The first component requires the presence of magnesium ions but occurs in the absence of alkali metal ions and is not inhibited by cardiac glycosides. In the presence of magnesium, activity is greatly increased by small amounts of calcium but inhibited by larger amounts.

3. The second component requires the presence of magnesium and also of both sodium and potassium ions. It is completely inhibited by cardiac glycosides in concentrations sufficient to inhibit ion transport in intact red cells. Calcium ions inhibit at both low and high concentrations.

4. Experiments with certain modified cardiac glycosides suggest that the same molecular features are important for inhibition of the ATP-ase as for inhibition of the ion pump.

5. ATP-ase inhibition by very low concentrations of cardiac glycosides is prevented by raising the potassium concentration, just as, in intact cells, inhibition of the active potassium influx by low concentrations of cardiac glycosides may be prevented by raising the potassium concentration.

6. The effect of potassium concentration on the glycoside-sensitive component of the ATP-ase depends on the concentration of sodium and vice versa.

7. The close resemblance between the properties of the glycoside-sensitive ATP-ase and the ion pump suggests that they are intimately connected.

We wish to thank Dr J. B. Chappell for several helpful discussions, Professor Reichstein and Messrs Sandoz of Basle for gifts of cardiac glycosides, and the U.S. Public Health Service for a grant for apparatus.

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