may be presumed to result from degradation of uronic acid residues, but the origin of the absorption of the hydrolysates of the remaining esters is less certain. The structures of these compounds are still not clearly understood and the available methods of preparation usually yield heterogeneous products (see Mori, 1953). However, the nature of the absorbing materials did not fall within the scope of the present investigation and the problem was not pursued further.

SUMMARY

1. A method is described for the analysis of the ester sulphate contents of polysaccharide sulphates of plant and animal origin.

2. The esters are hydrolysed with acid under conditions designed to reduce the rate of formation of ultraviolet-absorbing materials and liberated sulphate is then measured turbidimetrically by a modification of the procedure of Dodgson (1961).

3. Results obtained with the method agreed closely with those obtained by a conventional large-scale gravimetric procedure.

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The Asymmetrical Stimulation of a Membrane Adenosine Triphosphatase in Relation to Active Cation Transport

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The concept that adenosine triphosphate is needed for the active transport of Na^+ and K^+ ions across cell membranes has led to several studies of the effects of these ions on the activity of adenosine triphosphatases in disrupted tissues and cells. A stimulation of enzymic activity has been described when both ions are present in such diverse preparations as suspensions of fragmented erythrocyte membranes (Post, Merritt, Kinsolving & Albright, 1960; Dunham & Glynn, 1961), a microsomal fraction obtained from crab nerve (Skou, 1957, 1960) and brain (Deul & McIlwain, 1961; Järnefelt, 1961), and in a nuclear fraction of a kidney homogenate (Whittam & Wheeler, 1961). The stimulation by Na^+ and K^+ ions is counteracted by digoxin and ouabain and related glycosides, which are known to inhibit the active transport of ions in intact cells. A partial inhibition by digoxin of the hydrolysis of adenosine triphosphate in intact human erythrocytes was shown by Whittam (1958), and this appeared to be the only effect of digoxin on the chemical reactions investigated.

The adenosine triphosphatase of erythrocytes is situated exclusively in the membranes (Clarkson & Maizels, 1952; Herbert, 1956), which, unlike those of other cells and tissues, can be readily obtained in a homogeneous suspension particularly suitable for study. Post *et al.* (1960) found with human erythrocyte membranes that the concentrations of Na^+ and K^+ ions required for half-maximal stimulation of the adenosine triphosphatase were the same as those for the ion-pumping system (Post & Jolly, 1957). Dunham & Glynn (1961)

showed, further, that the partial inhibition of adenosine-triphosphatase activity by low concentrations of ouabain can be overcome by raising the potassium concentration, just as the influx of potassium in the intact cell can be similarly restored in the presence of scillaren (Glynn, 1957). These results, as well as those with intact cells, suggest an intimate connexion between adenosine-triphosphatase activity and active cation transport.

Perhaps the most striking feature of the membrane in the intact cell is its selection of K^+ ions for inward transport from a solution containing less potassium than sodium and its selection of Na^+ ions for outward transport from a solution containing less sodium than potassium. The cell membrane is therefore asymmetric in its selection of Na^+ and K^+ ions for transport, showing preference for K^+ ions on the outside and for Na^+ ions on the inside. In considering the stimulation of adenosine triphosphatase by Na^+ and K^+ ions in cell membranes, questions arise whether the stimulation also occurs in the intact cell, whether it is due to intracellular or extracellular sodium and potassium and whether both ions stimulate from the same side of the membrane. Since fragmented membranes do not permit a test of these points, resort has been made to erythrocyte 'ghosts'. Intact cells were found to be unsuitable: first, because of the difficulty of varying their Na^+ and K^+ ion concentrations and, secondly, because of their low adenosine-triphosphate concentration. Ghosts have therefore been made by the reversal of haemolysis (Hoffman, Tosteson & Whittam, 1960), which allows them to be loaded with adenosine triphosphate and various amounts of sodium and potassium. The results show that the hydrolysis of adenosine triphosphate in ghosts is stimulated by internal, but not by external, Na^+ ions, and also, in contrast, by external K^+ ions. Ouabain inhibits the stimulation by these ions, and the spatial asymmetry of the ion pump is therefore also a feature of the ouabain-sensitive adenosine triphosphatase.

METHODS

Preparation of 'ghosts'. Human blood (3 weeks old) from a Blood Transfusion Centre was centrifuged, the buffy coat of white cells removed and the erythrocytes were washed twice in a sodium-free choline medium (medium D of Table 1) to avoid contamination of the cells with extracellular sodium. One volume (usually 25 ml.) of washed cells was squirted into either 5 vol. of a solution of 4 mM-disodium ATP and 4 mM-$MgCl_2$ or 5 vol. of water, when haemolysis occurred. After 2–3 min., sufficient 3 M solution of NaCl, KCl or tris–HCl, pH 7·4 (6·4 ml. for 125 ml. of lysing fluid), was quickly added to raise the osmotic pressure of the haemolysate to about 0·3 osmolar. The haemolysate, now iso-osmotic with plasma, was incubated, with occasional shaking, for 30 min. in a water bath at 37° to allow the membranes to regain a low permeability to Na^+ and K^+ ions (Hoffman *et al.* 1960). The ghosts were sedimented by centrifuging for 5 min. at 16 000*g* in a Servall centrifuge (Ivan Servall Inc.) and the clear supernatant was sucked off. The ghosts are in the form of biconcave disks and, as they show some of the properties of cells, they are not referred to as stroma. The ghosts were washed once in the medium in which they were to be incubated later, and finally made into a suspension, such that the ghosts from 25 ml. of packed erythrocytes provided about 11–12 ml. of packed ghosts in 52 ml. of suspension. Packed washed ghosts (0·2 ml.) were analysed for sodium, potassium, orthophosphate and haemoglobin and the concentrations are given as m-moles/l. of packed ghosts. Orthophosphate and haemoglobin were also determined in samples of suspension (1·0 ml.). The volume of ghosts in the suspension was calculated by dividing the amount of haemoglobin in 1 ml. of suspension by the amount of haemoglobin in 1 ml. of packed ghosts sampled just before the suspension was prepared. Usually 1 ml. of suspension contained about 0·2 ml. of ghosts. Orthophosphate found in the suspension was liberated from ATP within the ghosts and its rate of liberation has therefore been expressed as m-moles/l. of packed ghosts present in the suspension.

Chemical estimations. Sodium and potassium were determined by flame photometry (Amoore, Parsons & Werkheiser, 1958). Haemoglobin was estimated colorimetrically (King, 1951) and orthophosphate by the method of Fiske & Subbarow (1925). Acid-labile phosphate was determined by measuring the increase in orthophosphate after heating (for 7 min. at 100°) a sample of a trichloroacetic acid extract to which an equal volume of 2 N-HCl had been added.

Incubation. Samples (2·0 ml.) of the ghost suspension or of the haemolysate were incubated, with constant shaking, in a water bath at 37°, and 0·2 ml. of 50% (w/v) trichloroacetic acid was added, usually after 2 hr.

Chemicals. Sodium ATP was purchased from Sigma Chemical Co., and other chemicals were of AnalaR quality (British Drug Houses Ltd.) wherever possible.

Media. The five media in which the ghosts were incubated (Table 1) were designed so as to include sodium (media A and E) or to exclude sodium (media B, C and D) and to contain potassium concentrations of zero (media C and E), 10 mM (media A and D) and 150 mM (medium B). This combination of media made it possible to incubate ghosts in the presence and absence of either Na^+ or K^+ ions in the medium.

RESULTS

Reversal of haemolysis

The leakage of haemoglobin from the cells into the lysing fluid is due to colloid osmotic haemolysis, in which the osmotic-pressure gradient across the membrane causes water to enter the cells, which swell and lose haemoglobin (Wilbrandt, 1941; Davson, 1943). Although a restoration of impermeability to haemoglobin has been described (Adair, Barcroft & Bock, 1921; Bayliss, 1924), ghosts prepared in this way have been thought to lose irretrievably the low permeability to alkali metals characteristic of the erythrocyte, possibly

Table 1. *Composition of media*

Media in which to wash and incubate ghosts were prepared as shown below, such that sodium and potassium were included or excluded. Sodium was present in media A and E and was replaced with potassium in medium B and with choline in media C and D. No potassium was present in media D and E.

	Composition				
Medium ...	A	B	C	D	E
Main solute ...	NaCl	KCl	Choline chloride	Choline chloride	NaCl
	140 mM-NaCl	—	147 mM-Choline chloride	147 mM-Choline chloride	140 mM-NaCl
	10 mM-KCl 2 mM-$MgCl_2$	150 mM-KCl 2 mM-$MgCl_2$	2 mM-$MgCl_2$ 10 mM-Tris–HCl, pH 7·6	10 mM-KCl 2 mM-$MgCl_2$	2 mM-$MgCl_2$ 10 mM-Tris–HCl, pH 7·6
	10 mM-Tris–HCl, pH 7·6	10 mM-Tris–HCl, pH 7·6	—	10 mM-Tris–HCl, pH 7·6	—

owing to damage to the lipid components of the membrane during lysis (Davson & Ponder, 1938). A partial restoration of low permeability of the ghosts to Na^+ and K^+ ions was described by Teorell (1952), however, and Hoffman *et al.* (1960) obtained a greater restoration by incubation of the haemolysate at 37° after the addition of 3M-salt solution. Use has been made of this method to prepare ghosts with a range of Na^+ and K^+ ion concentrations.

Variation of sodium concentration. Additions of mixtures of 3M-NaCl and KCl were made to haemolysates to yield final concentrations of each ion in the range 20–150 mM. The lower values were found when none of the appropriate salt was added and are due to the Na^+ and K^+ ions in the erythrocytes. Fig. 1 shows that the sodium concentration in the ghosts was proportional to the concentration in the haemolysate even after washing the ghosts in a medium A containing 150 mM-Na^+ ions. When only KCl was added to the haemolysate, the sodium concentration (m-moles/l. of packed ghosts) was $49 \pm 4{\cdot}6$ (7) and after the addition of NaCl alone it was $151 \pm 4{\cdot}1$ (7). The lower concentration is about a third of the concentration in the washing medium and illustrates the recovery of a low permeability to sodium. In order to correct the values for the sodium in extracellular fluid in the packed ghosts, a few preparations were washed with sodium-free medium D containing 150 mM-choline chloride. The comparable values were 21 (2) and $120 \pm 8{\cdot}5$ (4), which mean that the amount of sodium readily removed from the packed ghosts is $49 - 21 = 28$ and $151 - 120 = 31$. Although the bulk of this sodium is extracellular, some of it may be due to a small proportion of ghosts being leaky to cations, but this point has not been investigated.

Variation of potassium concentration. The potassium concentration in the washed ghosts was also proportional to the concentration in the haemolysate after the addition of mixtures of 3M-NaCl and KCl (Fig. 1). Thus the minimum potassium

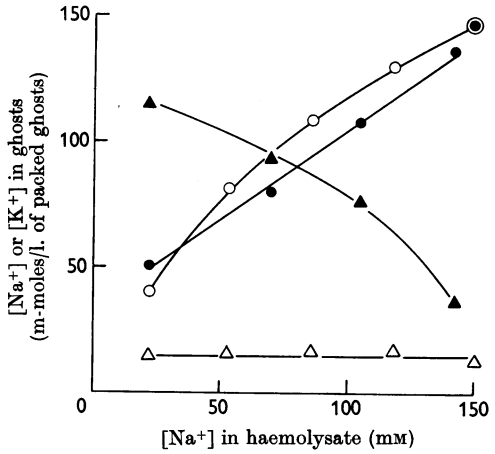

Fig. 1. Dependence of the sodium and potassium concentrations in erythrocyte ghosts on the sodium and potassium concentrations in the haemolysate. Erythrocyte ghosts containing ATP were prepared by adding mixtures of 3M-NaCl and KCl, and of 3M-NaCl and tris–HCl, to haemolysates. The sodium and potassium concentrations were determined in the haemolysates and in the ghosts after washing in medium A. Mixture of NaCl and KCl added: ▲, K^+ ion concentration; ●, Na^+ ion concentration. Mixture of NaCl and tris–HCl added: △, K^+ ion concentration; ○, Na^+ ion concentration.

concentration (m-moles/l. of packed ghosts) when no KCl was added was $13 \pm 1{\cdot}9$ (7), the source being potassium in the erythrocytes, and the maximum concentration after the addition of KCl alone was $115 \pm 4{\cdot}6$ (7). The latter concentration is in marked contrast with the concentration of 10 mM in the washing medium.

Since the haemoglobin remaining in the ghosts was the same whether the ghosts contained low or high concentrations of sodium and potassium, these variations cannot be due to differences in the binding of cations arising from differences in haemoglobin content.

Table 2. *Concentration of adenosine triphosphate and haemoglobin in haemolysate and ghosts*

Samples of haemolysate were taken for the analysis of haemoglobin and of orthophosphate and of acid-labile phosphate just before the sedimentation of the ghosts by centrifuging. The same analyses were also made on the ghosts after they had been washed in medium A. The ATP concentration has been calculated by dividing the concentration of acid-labile phosphate by 2. The shrinkage of the ghosts is calculated by subtracting from 1 the quotient obtained by dividing the concentration in the haemolysate by the concentration in the ghosts.

Salt added to haemolysate	Concn. of ATP (m-moles/l.)		Concn. of haemoglobin (units of E/ml.)		Shrinkage of ghosts	
	Haemolysate	Ghosts	Haemolysate	Ghosts	From ATP values	From haemoglobin values
NaCl	3·45	6·0	29	46	0·43	0·37
NaCl + KCl (3:1)	3·25	5·45	29	47	0·35	0·38
NaCl + KCl (1:1)	3·4	6·20	29	52	0·44	0·44
NaCl + KCl (1:3)	3·5	5·75	29	46	0·40	0·37
KCl	3·15	5·45	29	44	0·36	0·33
				Mean	0·40	0·38

Independent variation of sodium and potassium concentrations. Fig. 1 shows that a low sodium concentration was accompanied by a high potassium concentration, and the sum of these concentrations, whatever the value of the individual concentrations, was approximately constant. In later experiments it became necessary to vary the sodium and potassium concentrations independently, namely to vary the sodium concentration with the potassium concentration kept constant and vice versa. This was achieved by substituting tris–HCl for either NaCl or KCl in the 3M solution added to the haemolysate. When tris–HCl was added in place of KCl, the sodium concentration in the ghosts showed the same proportionality to the concentration in the haemolysate and the potassium concentration was constant at about 18 m-moles/l. of packed ghosts (Fig. 1). The sodium concentration was also varied in ghosts that contained more potassium by a partial replacement of 3M-KCl with tris–HCl. After washing in medium D, the concentrations (m-moles/l. of packed ghosts) were: K^+, 81 and 82; Na^+, 14 and 84. After washing in medium A the respective values were: K^+, 79 and 75; Na^+, 41 and 100 (Table 4). A variation in the potassium concentration with a constant sodium was similarly obtained by adding mixtures of 3M-KCl and tris–HCl to the haemolysate. In one experiment, the sodium and potassium concentrations (m-moles/l. of packed ghosts) after washing in medium D were 84 and 82, and after replacement of KCl with tris the sodium concentration was unchanged (80) whereas the potassium concentration was decreased to 16. The sodium and potassium concentrations in the ghosts could thus be independently varied by replacing one or other of these cations with tris.

Concentrations of adenosine triphosphate and haemoglobin in the ghosts. Analyses were made of ATP and haemoglobin in the haemolysate and in the packed ghosts after the latter had been washed. The ATP concentrations in the extracts have been calculated on the assumption that 2 moles of acid-labile phosphate were produced from 1 mole of ATP, as this was found with the stock ATP solution. The ATP concentration in the ghosts (5·45–6·0 m-moles/l. of packed ghosts) was independent of the kind of salt added to the haemolysate, as was also the haemoglobin concentration (44–52 extinction units/ml.) (Table 2). Both concentrations, however, were about 60% above the concentrations in the haemolysate from which the ghosts were separated. This observation must mean that the ghosts had shrunk between the time of haemolysis and sedimentation, as Bayliss (1924) showed, and the higher concentrations of haemoglobin in the ghosts are not due to adsorption of haemoglobin on the corpuscles (Brinkman & Szent-Gyorgi, 1923). The shrinkage of the ghosts was the same when calculated from the ATP (40%) or from the haemoglobin (38%) values.

Hydrolysis of adenosine triphosphate in erythrocyte ghosts

Preliminary experiments with ghosts prepared from erythrocytes that had been lysed in water showed that orthophosphate was not liberated from ATP added to the medium in which the ghosts were incubated. Nor was ATP broken down in the particle-free supernatant obtained when the ghosts were sedimented by centrifuging. The orthophosphate produced during the incubation of ghosts prepared from a haemolysate containing ATP must therefore have been derived from the fission of ATP within the ghosts.

Effect of time of incubation. In order to decide on a convenient period of incubation, the orthophosphate was determined on samples of a suspension of ATP-containing ghosts after various periods of incubation (30–120 min.). Fig. 2 shows that the amount of orthophosphate produced was linear with time up to 2 hr., which was a convenient period

for routine incubation. The addition of 0·1 mM-ouabain caused a fall in production of orthophosphate of about 50%.

Omission of sodium from the medium. One aim of this work was to find whether the stimulation of adenosine triphosphatase in fragmented membranes by sodium is also found in the intact membranes of ghosts, and whether the stimulation depends on internal or external sodium. Ghosts were incubated in sodium-free medium and Table 3 shows that replacement of medium sodium with choline had no effect on the adenosine-triphosphatase activity. In media in which 10 mM-K^+ ions were present in the high-sodium and sodium-free media (A and D respectively), the rates of production of orthophosphate (m-moles/l. of packed ghosts/hr.) were 2·4–2·9 and were lowered by 52–65% to 1·4–1·8 when 0·1 mM-ouabain was added. The sensitivity of the adenosine triphosphatase to ouabain was therefore also unaltered in the sodium-free medium.

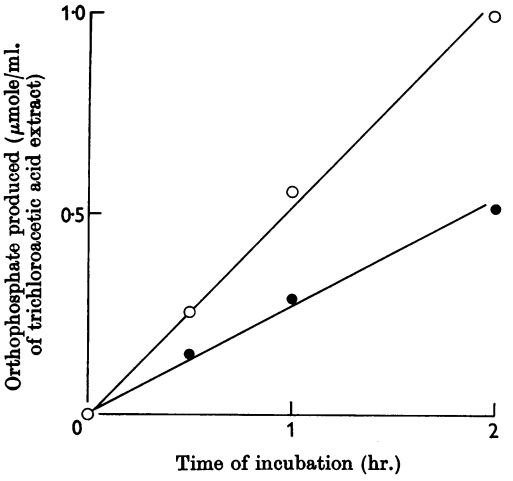

Fig. 2. Increase in liberation of orthophosphate from ATP with increase in incubation time. Erythrocyte ghosts containing ATP and about 140 m-moles of Na^+ ion/l. and 15 m-moles of K^+ ion/l. were prepared by the addition of 3M-NaCl to a haemolysate. They were incubated for various periods in medium A (0·19 ml. of packed ghosts/ml. of suspension) in the presence of 0·1 mM-ouabain (●) and without ouabain (○) at 37°. Orthophosphate was determined in 0·4 ml. of trichloroacetic acid extract obtained by adding 0·2 ml. of 50% (w/v) trichloroacetic acid to 2·0 ml. of suspension.

Omission of potassium from the medium. The active efflux of sodium from erythrocytes is prevented by depriving the medium of potassium (Harris & Maizels, 1951; Glynn, 1956), and if the adenosine triphosphatase is closely linked with the ion pump, a decrease in adenosine-triphosphatase activity is to be expected when ghosts are incubated in potassium-free media. The absense of potassium in the media, in spite of its presence in the ghosts (about 18 m-moles/l. of packed ghosts), caused falls in the rate of production of orthophosphate that were the same in the high-sodium medium A (46%) and in the sodium-free medium D (48%). The rates (m-moles/l. of packed ghosts/hr.) fell from 2·4 to 1·3 (1·1) and from 2·7 to 1·4 (1·3) respectively (Table 3). The part of the adenosine-triphosphatase activity sensitive to ouabain was decreased from 1·4–1·5 to 0·4–0·5 m-moles/l. of packed ghosts/hr. by omission of K^+ ions from the medium (Table 3).

Table 3. *Decrease in adenosine-triphosphatase activity with omission of potassium from the medium*

Ghosts with high sodium and low potassium concentrations, prepared by the addition of 3M-NaCl to a haemolysate, were washed and incubated at 37° for 2 hr. in media A, C, D and E, in which 10 mM-KCl was either added or omitted. One lot of ghosts was incubated in medium B containing no NaCl and 150 mM-KCl. An addition of 0·1 mM-ouabain was made before the incubation of a part of each suspension. Estimations of orthophosphate were made on the suspensions both before and after incubation, after deproteinization with trichloroacetic acid (0·2 ml. of 50%, w/v, to 2·0 ml. of suspension) and sodium and potassium were estimated on ghosts isolated before incubation. The volume of ghosts in each suspension (0·18–0·20 ml. of packed ghosts/ml. of suspension) was determined by comparing the extinction of haemoglobin in the suspension with that in unit volume of packed washed ghosts.

Main solute in medium	Concn. of cation in ghosts (m-moles/l. of packed ghosts) Na^+	K^+	$Na^+ + K^+$	Concn. of K^+ in medium (mM)	Concn. of ouabain in medium (mM)	Increase in orthophosphate during incubation (m-moles/l. of packed ghosts/hr.)	Decrease in liberation due to ouabain of orthophosphate (m-moles/l. of packed ghosts/hr.)
NaCl	149	18	167	0	0	1·3	0·4
					0·1	0·9	
				10	0	2·4	1·5
					0·1	0·9	
Choline chloride	115	20	125	0	0	1·4	0·5
					0·1	0·9	
				10	0	2·7	1·4
					0·1	1·3	
KCl	—	—	—	150	0	2·9	1·9
					0·1	1·1	

The membrane adenosine triphosphatase is therefore stimulated by 10 mM-K^+ ions both in the sodium and choline media, and it was investigated whether the stimulation was further increased by raising the K^+ ion concentration to 150 mM (medium B). The orthophosphate produced in two experiments was approximately the same (2·4–2·9 m-moles/l. of packed ghosts/hr.), however, when the ghosts were incubated in the presence of 10 and 150 mM-K^+ ions, and ouabain caused a similar decrease in rate of 1·4–1·9 (Table 3). The adenosine triphosphatase was therefore maximally stimulated by 10 mM-K^+ ions in the medium. This result shows that Na^+ ions in the medium may also be replaced with K^+ ions as well as with choline.

Stimulation of adenosine triphosphatase by sodium in the ghosts

Simultaneous variation of sodium and potassium concentrations. The method of preparation of ghosts allows a test to be made of the effects of sodium and potassium concentrations in the ghosts over a tenfold range on the adenosine-triphosphatase activity. The liberation of orthophosphate was therefore measured after the incubation of ghosts in medium A (150 mM-Na^+ and 10 mM-K^+ ions) in the presence and absence of 0·1 mM-ouabain. The medium was the same for all ghosts; the concentrations of Na^+ and K^+ ions in the ghosts were the only variables.

The rate of production of orthophosphate was proportional to the sodium concentration and inversely proportional to the potassium concentration until there was a two- to three-fold difference (Fig. 3). The rate reached a maximum when the sodium concentration was about 100 m-moles/l. of packed ghosts. The variations in sodium and potassium concentrations had no effect on the orthophosphate produced in the presence of 0·1 mM-ouabain, which decreased the maximum rate by about 50 % in the results of two experiments shown (Fig. 3).

Independent variation of sodium and potassium in the ghosts. Although the result in Fig. 3 is suggestive, it does not prove that the increase in production of orthophosphate was due to stimulation by internal Na^+ ions, for the same result would be obtained if a high potassium concentration was inhibitory. This possibility may seem unlikely, but it was nevertheless tested with ghosts with the same sodium concentration but different potassium concentrations, and vice versa, prepared as described above. Suspensions of ghosts made in both media A and D (with 150 mM-Na^+ ions and without Na^+ ions, and 10 mM-K^+ ions) were incubated with and without 0·1 mM-ouabain. After incubation in the choline medium (D), the production of orthophosphate (m-moles/l. of packed ghosts/hr.) of 2·5–2·6 and its decrease by ouabain of 1·6 were identical in ghosts containing 80 and 84 m-moles of Na^+ ions/l. of packed ghosts in spite of a fivefold difference in potassium concentration from 16 to 82 m-moles/l. of packed ghosts (Table 4). Secondly, ghosts with the same potassium concentration (81 and 82 m-moles/l. of packed ghosts) but different sodium concentrations (14 and 84 m-moles/l. of packed ghosts) showed a difference in adenosine-triphosphatase activity sensitive to ouabain from 1·0 to 1·6 m-moles/l. of packed ghosts/hr.

Similar results were obtained after the same batch of ghosts was incubated in medium A (150 mM-Na^+ and 10 mM-K^+ ions). With approximately the same sodium concentration (83 and 100 m-moles/l. of packed ghosts), a fivefold increase in potassium concentration from 14 to 75 m-moles/l. of packed ghosts had no effect on the adenosine-triphosphatase activity, sensitive to ouabain, of 1·7–1·9 m-moles of orthophosphate/l. of packed ghosts/hr. On the other hand, with a constant potassium concentration of 75–79 m-moles/l. of packed ghosts, an increase in sodium concentration from 41 to 100 m-moles/l. of packed ghosts caused a 58 % increase in the ouabain-sensitive production of orthophosphate, from 1·2 to 1·9 m-moles/l. of packed ghosts/hr. In another experiment, in which the sodium concentration in the ghosts was constant (34–39 m-moles/l. of packed ghosts), an increase in potassium concentra-

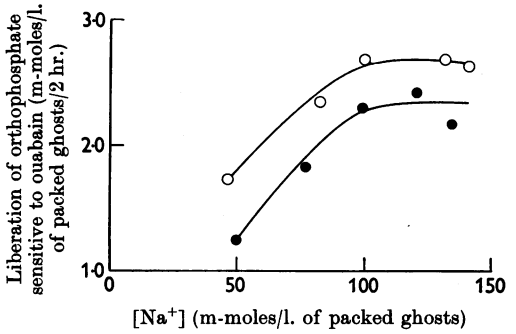

Fig. 3. Stimulation by Na^+ ions in ghosts of the membrane adenosine triphosphatase. Erythrocyte ghosts containing ATP and a range of sodium and potassium concentrations were prepared by making haemolysates iso-osmotic by the addition of mixtures of 3 M-KCl and NaCl. The liberation of orthophosphate from ATP was measured in two experiments after incubation for 2 hr. at 37° in the presence and absence of 0·1 mM-ouabain in medium A (Table 1) and the production of orthophosphate sensitive to ouabain is plotted against the sodium concentration in the ghosts. The volume of ghosts in each suspension (0·18–0·2 ml. of packed ghosts/ml. of suspension) was determined by comparing the extinction of haemoglobin in the suspension with that in unit volume of packed washed ghosts.

Table 4. *Increase in adenosine-triphosphatase activity with increase in sodium concentration in erythrocyte ghosts*

Additions of mixtures of 3 M-solutions of NaCl + tris, of NaCl + KCl and of KCl + tris were made to samples of a haemolysate and the ghosts were suspended in media A (140 mM-Na^+ and 10 mM-K^+ ions) and D (147 mM-choline chloride and 10 mM-K^+ ions). Parts of the ghost suspensions were incubated with and without 0·1 mM-ouabain and the orthophosphate was determined before and after incubation. The sodium and potassium concentrations in the ghosts isolated before incubation were measured. The volume of ghosts in each suspension (0·18–0·20 ml. of packed ghosts/ml. of suspension) was determined by comparing the extinction of haemoglobin in the suspension with that in unit volume of packed washed ghosts.

Expt. no.	Main solute in medium	Concn. of cation in ghosts (m-moles/l. of packed ghosts) Na^+	K^+	$Na^+ + K^+$	Concn. of ouabain (mM)	Increase in orthophosphate during incubation (m-moles/l. of packed ghosts/hr.)	Decrease in production of orthophosphate due to ouabain (m-moles/l. of packed ghosts/hr.)
39	Choline chloride	80	16	96	0	2·6	1·6
					0·1	1·0	
		84	82	166	0	2·5	1·6
					0·1	0·9	
		14	81	95	0	2·2	1·0
					0·1	1·2	
	NaCl	83	14	97	0	2·8	1·7
					0·1	1·1	
		100	75	175	0	3·2	1·9
					0·1	1·3	
		41	79	120	0	2·5	1·2
					0·1	1·3	
40	NaCl	39	24	63	0	1·8	0·7
					0·1	1·1	
		34	136	170	0	1·9	0·9
					0·1	1·0	
		144	17	161	0	2·5	1·8
					0·1	0·7	

tion from 24 to 136 m-moles/l. of packed ghosts had no effect on the rate of production of orthophosphate. An increase in sodium concentration to 144 m-moles/l. of packed ghosts, however, caused a doubling of the production of orthophosphate sensitive to ouabain (from 0·7–0·9 to 1·8 m-moles/l. of packed ghosts/hr.) (Table 4).

The results in Table 4 therefore prove that the stimulation of adenosine triphosphatase when the sodium and potassium concentrations in the ghosts were varied (Fig. 3) is due to an increase in sodium concentration and that a fivefold change in the potassium concentration had no effect. The potassium concentration was never less than 12 m-moles/l. of packed ghosts and the results do not indicate whether the stimulating effect of internal Na^+ ions depends upon the presence of K^+ ions at this or a lower concentration. It is clear, however, that internal K^+ ions are not inhibitory and that the main control of adenosine-triphosphatase activity is by internal Na^+ ions and external K^+ ions.

Sensitivity of adenosine triphosphatase to ouabain

The effect of the ouabain concentration on the adenosine triphosphatase was measured in two experiments with ghosts containing a high sodium concentration (about 140 m-moles/l. of packed ghosts) incubated in medium A containing 150 mM-Na^+ and 10 mM-K^+ ions. The amount of orthophosphate liberated from ATP decreased rapidly within the range of ouabain concentration 0–0·5 μM and maximum inhibition was produced with 1·0–2·5 μM. The concentration of 100 μM used in the experiments was therefore more than enough to give maximum inhibition of the part of the adenosine triphosphatase sensitive to ouabain.

DISCUSSION

Asymmetrical features of the membrane adenosine triphosphatase

Attention has already been drawn to the role of ATP in facilitating active ion movements (Whittam, 1961) and to the many common features of the adenosine triphosphatase in fragmented-erythrocyte membranes and the ion pump in intact cells (Post *et al.* 1960; Dunham & Glynn, 1961). A point about the enzyme that required clarification, however, was the site of activation by Na^+ and K^+ ions which could not be investigated with fragmented membranes. This paper describes how erythrocyte ghosts containing ATP added at the time of haemolysis and a range of sodium and potassium concentrations are suitable materials with which to investigate the effects on the adenosine triphos-

phatase of variations of both internal and external concentrations of Na^+ and K^+ ions. The separation of ghosts with a low permeability to Na^+ and K^+ ions (Hoffman *et al.* 1960) itself raises problems that cannot be adequately discussed at present, as so little is known about the reversal of haemolysis. It may suffice to say that, besides regaining a low permeability, ghosts are able actively to pump both Na^+ and K^+ ions (Gardos, 1954; Hoffman & Tosteson, 1956; Hoffman, 1960).

The results have shown that the hydrolysis of ATP within the ghosts is stimulated by K^+ ions in the medium and by Na^+ ions inside the ghosts. Glynn (1962) has described experiments that show similar effects. Replacement of medium Na^+ ions with either choline or K^+ ions did not cause a fall in activity of adenosine triphosphatase, whereas deprivation of K^+ ions caused a fall approaching that caused by ouabain (Table 3). On the other hand, increases in internal sodium concentration, accompanied by equivalent falls in potassium concentration, caused increases in activity of adenosine triphosphatase. The sodium concentration giving half-maximal effects could not be determined because ghosts with less than about 20 m-moles of sodium/l. of packed ghosts were not prepared. Ghosts were also prepared in which the concentrations of the two ions were varied independently and the results showed that the stimulating effect of increasing the internal sodium concentration was independent of the potassium concentration.

The relevance of these findings to active movements of Na^+ and K^+ ions lies in two main facts about the active efflux of Na^+ ions: first, it is dependent on the presence outside the cells of K^+ ions, which are pumped inwards (Harris & Maizels, 1951; Glynn, 1956), and secondly, together with the coupled uptake of K^+ ions, it is directly proportional to the sodium concentration in the cells (Post & Jolly, 1957; M. E. Ager & R. Whittam, unpublished work). The higher rates of adenosinetriphosphatase activity found when ghosts containing high sodium concentrations are incubated in a medium containing 10 mM-K^+ ions are thus correlated with higher rates of ion pumping than those when the internal sodium concentration is low or when no K^+ ions are in the medium.

It therefore seems that erythrocyte membranes contain an adenosine triphosphatase, acting on internal ATP, that is stimulated synergically by Na^+ and K^+ ions and, as shown before, the activity of this enzyme due to such stimulation is opposed by ouabain. The fact that the ions stimulate the enzyme from different sides of the membrane shows that the enzyme also shares spatial asymmetry with the ion-transport system. Thus the synergy of Na^+ and K^+ ions requires Na^+ ions on the inside and K^+ ions on the outside of the membrane. These are, of course, the locations of the ions from which they are actively transported and it may be supposed that the operation of the ion pump in moving Na^+ ions outwards and K^+ ions inwards is perhaps linked to the rotation in the membrane of the enzyme or of a part of it, such that the active centre of the enzyme is alternately exposed to the outside and the inside. Hydrolysis of ATP would occur when Na^+ ions were attached to the active centre facing the inside. If the affinity of the enzyme were high for Na^+ ions when the active centre faced inwards and low when it faced outwards, and vice versa for K^+ ions, rotation of the enzyme in the membrane might provide the chemical unit responsible for the ion movements.

SUMMARY

1. A study has been made of the stimulation by Na^+ and K^+ ions of the adenosine triphosphatase in human erythrocyte membranes.

2. Erythrocyte ghosts containing adenosine triphosphate and a range of sodium and potassium concentrations were prepared by the reversal of haemolysis. They had a low permeability to Na^+ and K^+ ions, the concentrations of which were varied independently both in the ghosts and in the incubation media.

3. During incubation at 37°, the rate of liberation of orthophosphate from adenosine triphosphate within the ghosts was measured. Replacement of Na^+ ions of the medium with either choline or K^+ ions did not affect the rate but omission of K^+ ions from the medium caused a fall of about 50 %. Maximum stimulation was shown by 10 mM-K^+ ions in the medium. The membrane adenosine triphosphatase was therefore stimulated by K^+ ions, but not by Na^+ ions, in the medium.

4. The sodium and potassium concentrations in the ghosts were varied and it was shown that the adenosine triphosphatase is stimulated twofold by an increase in sodium concentration. Fivefold variations in potassium concentration were without effect. The membrane adenosine triphosphatase was therefore stimulated by Na^+ ions inside the ghosts.

5. The synergic stimulation of the adenosine triphosphatase by internal Na^+ and external K^+ ions was counteracted by ouabain.

6. The membrane adenosine triphosphatase is stimulated asymmetrically by Na^+ and K^+ ions and spatial asymmetry is another property shared with the membrane ion pump for Na^+ and K^+ ions.

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Substrate Attachment in Enzymes

THE INTERACTION OF PYRIDOXAL PHOSPHATE WITH AMINO ACIDS

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A number of reactions in which amino acids take part are catalysed by enzymes which require pyridoxal phosphate. The aldehyde group of the latter compound is believed to form an imine (Schiff base) with the amino group of the substrate; the apoenzymes then bring about reactions (decarboxylation, transamination etc.) by inducing electronic rearrangements within the Schiff base. The Schiff bases can also chelate with certain metals. Many of the reactions catalysed by 'B_6 enzymes' can be simulated in chemical systems in the presence of pyridoxal and a suitable metal. The mechanisms of both the reactions *in vitro* and their enzymic counterparts have been discussed in detail by Snell (1958).

The chemical models can be related to enzyme action only if it is known: (*a*) how readily pyridoxal phosphate can combine with amino acids; (*b*) whether chelating metals influence this reaction; (*c*) what is the relation, if any, between the affinity of pyridoxal phosphate and an amino acid under physiological conditions, and the affinity of pyridoxal phosphate enzymes for the amino acid.

The union of both pyridoxal and pyridoxal phosphate with amino acids has been studied by Metzler (1957) and Matsuo (1957), but information is not available in all cases over the pH ranges within which the enzymes act.

Pyridoxal phosphate enzymes may operate under widely varying conditions. The pH optimum of bacterial glutamate decarboxylase is 4·5 (Gale, 1946), but alanine racemase (Wood & Gunsalus, 1951) and serine deaminase (Selim & Greenberg, 1959) show optima at pH 8·1 and 8·3 respectively. The structure of pyridoxal phosphate changes considerably over this range. The phenolic —OH and the pyridine —N= groups have pK values of 4·14 and 8·7 respectively. The zwitterion thus predominates at the pH optima of most pyridoxal phosphate enzymes, unless these pK values are drastically altered by attachment to the apoenzyme. At the acid and alkaline ends of the range, however, a substantial portion of anion or cation will be present. The secondary phosphate group dissociates with a pK of 6·2 (Williams & Neilands, 1954). The pK for dissociation of the Schiff bases