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Structural and Enzymic Aspects of the Hydrolysis of Adenosine Triphosphate by Membranes of Kidney Cortex and Erythrocytes

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The concept of an intimate linkage between active cation movements across cell membranes and adenosine-triphosphatase activity is strongly supported by the parallelism between the effects of cations, notably Na^+ and K^+, and of cardiac glycosides, such as ouabain, on these two processes. The adenosine triphosphatases are activated by suitable concentrations of Na^+ and K^+ ions, this activation being counteracted by low concentrations of glycosides that also inhibit the coupled active transport of Na^+ and K^+ ions. This postulated involvement with cation movements clearly requires the adenosine-triphosphatase activity to be located in cell membranes and to possess the overall spatial asymmetry characteristic of the transport system. The erythrocyte provides excellent material for investigations of these aspects of the problem, and the situation has been more clearly defined in erythrocytes (Glynn, 1962; Whittam, 1962*b*) than in other tissues, such as brain and kidney, where localization of the activity depends on fractional centrifugation of homogenates. It thus seemed important to determine the distribution of this enzymic activity among subcellular fractions of kidney-cortex homogenate and to prepare what appears to be a membrane fraction. A partial fractionation of the erythrocyte membrane was also attempted by preparing elinin, the main lipoprotein component of the membrane (Moskowitz, Dandliker, Calvin & Evans, 1950; Moskowitz & Calvin, 1952).

Previous work has shown that cell-debris fractions from rabbit kidney-cortex homogenate possess adenosine-triphosphatase activity that is inhibited by ouabain (Wheeler & Whittam, 1962; Kinsolving, Post & Beaver, 1963; Taylor, 1963). In an attempt to clarify the nature of the enzyme system and its possible role in other transport processes, the effects of a variety of inhibitors have been studied.

In the interests of clarity and brevity we shall call the component of the adenosine-triphosphatase activity that is observed in the presence of Mg^{2+} ions alone, or Mg^{2+} ions, K^+ ions, Na^+ ions and ouabain, the '(Mg)-adenosine triphosphatase'; and, similarly, the component that requires Mg^{2+}, K^+ and Na^+ ions, and is inhibited by ouabain, the '(NaK)-adenosine triphosphatase'.

METHODS

Materials. ATP (disodium salt), ADP (sodium salt), AMP, ITP (sodium salt), *p*-chloromercuribenzoate and *N*-ethylmaleimide were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. Oligomycin was kindly given by Dr D. E. Griffiths. Imidazole, trichloroacetic acid, phlorrhizin, phloretin, tetrasodium pyrophosphate and ouabain (strophanthin-G) were laboratory-reagent products of British Drug Houses Ltd.; all other compounds used were AnalaR reagents from the same manufacturers. Solutions of oligomycin, phlorrhizin, phloretin and digitonin were made in ethanol; all other solutions were made in glass-distilled water.

Prepration of tissue suspensions

All homogenizations were carried out in media chilled to about 4° and the homogenizer was surrounded by a mixture of ice and water. Centrifugations were performed at 0–4°, and $g_{max.}$ values are quoted.

Kidney-cortex homogenates. Fresh rabbit kidney cortex was obtained as described by Wheeler & Whittam (1962) and homogenized in 0·25 M-sucrose solution containing imidazole–HCl buffer, pH 7·9 (10 mM), by using a glass homogenizer and Teflon pestle (clearance about 0·14 mm.; 700 rev./min.); 5 ml. of medium was used/g. of tissue and after 20 complete strokes of the pestle the homogenate was squeezed through a double layer of muslin.

'800*g* *fraction*'. The cell-debris fraction used for previous investigations (Wheeler & Whittam, 1962) is termed the '800*g* fraction'; it was prepared as follows. The homogenate was centrifuged at 800*g* for 10 min. (MSE Refrigerator centrifuge, 6886 head, or MSE Mistral 6L Refrigerator centrifuge, 69167 head; 2300 rev./min.), the supernatant fluid decanted and the sediment resuspended in the homogenizing medium. (Most of the lower layer of sedimented erythrocytes stuck to the tube and was not resuspended.) The whole procedure was repeated twice and the resulting sediment resuspended as above. In experiments in which the effects of Mg^{2+} and Ca^{2+} ions were investigated, 2–5 mM-EDTA (adjusted to pH 7·9 with N-NaOH) was also present in the media used for homogenizing and for the first wash.

Other fractions. The nuclear fraction was obtained by centrifuging the homogenate at 800*g* for 10 min., as described above, but the sediment was washed only once. The combined supernatant suspensions were centrifuged at 5000*g* (5800 rev./min.) for 15 min., giving a sediment that, after one wash by resuspension and centrifugation, was resuspended to give the mitochondrial fraction. The microsomal fraction was obtained by centrifuging the combined supernatant suspensions from the mitochondrial preparation at 111000*g* for 30 min. (Spinco preparative

ultracentrifuge, model L, type 40 rotor; 35000 rev./min.) and the supernatant fluid was used as the supernatant fraction.

'Cell-membrane' fractions. The modification (Emmelot & Bos, 1962) of Neville's (1960) procedure for preparing cell membranes from liver homogenates was applied to kidney-cortex homogenates. A less pure preparation was also obtained by homogenizing kidney cortex in the sucrose–imidazole–EDTA medium, as described above, and then centrifuging at 1500*g* (3100 rev./min.) for 10 min., as in Neville's (1960) method. The sediment was resuspended in the same medium, centrifuged at 1200*g* (2700 rev./min.) for 10 min., the supernatant fluid carefully pipetted off and the loose buff-coloured upper layer of the sediment resuspended in a few millilitres of medium. The centrifugation (at 1200*g*) was repeated again, the supernatant fluid pipetted off and the upper layer of the sediment freed from the small amount of denser material and resuspended.

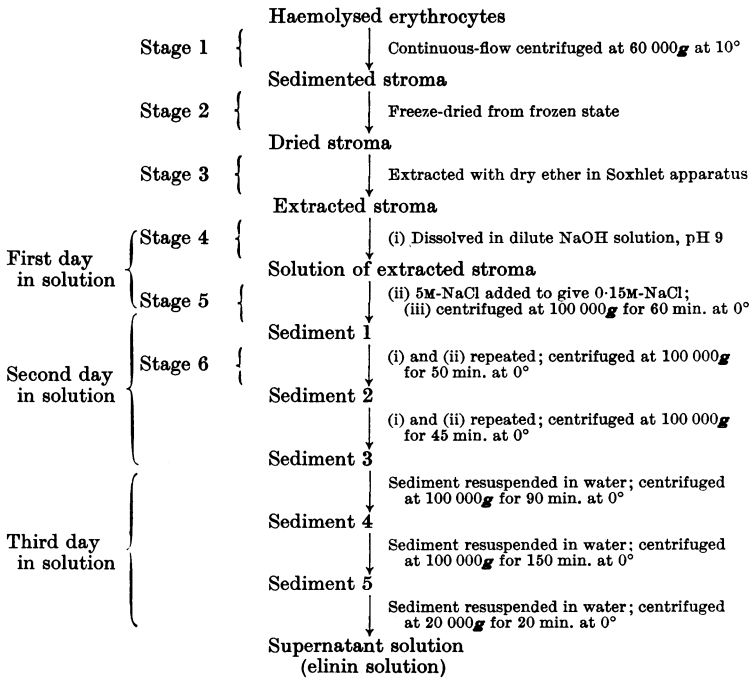
Further fragmentation of the 800g fraction. Samples of this fraction were subjected to further fragmentation by freezing and thawing, and by exposure to ultrasound. The suspensions were frozen by immersion in liquid air or liquid oxygen and then thawed at 37° immediately before their adenosine-triphosphatase activities were measured. A MSE ultrasonic power unit was employed for the ultrasound treatment. The suspensions were placed in 25 ml. beakers and surrounded by a mixture of ice and water during this process; the instrument was tuned by ear (see Hughes & Nyborg, 1962).

Erythrocyte-membrane preparations. Fragmented erythrocyte membranes were prepared as follows. A buffer solution containing EDTA (1 mM; adjusted to pH 7·5 with 0·1 N-NaOH) and imidazole–HCl buffer, pH 7·5 (about 1 mM), was used for both haemolysis and washing. Human blood (3–4 weeks old) was centrifuged at about 18000*g* for 5 min. (Servall SS1 centrifuge; 12000 rev./min.); plasma and white cells were removed with a pipette and the packed erythrocytes washed once by resuspension in 0·15 M-NaCl and centrifuging as above. Then 1 vol. of packed cells was haemolysed in 5 vol. of the buffer solution and the mixture stirred for 10 min. at 0–4° before centrifuging as above. After removal of the supernatant fluid the sedimented 'ghosts' were washed by resuspension in 5 vol. of the buffer solution and repetition of the centrifugation. This washing procedure was repeated twice, centrifuging for 15 min. each time. The final sediment was resuspended in the buffer solution, which was then frozen by immersion in liquid air and thawed at about 4° when required.

The procedure for the isolation of elinin outlined in Scheme 1 is based on that of Moskowitz *et al.* (1950) and Moskowitz & Calvin (1952). In our experiments the washed erythrocytes (from 3–4-week-old blood) were haemolysed in the imidazole–EDTA buffer solution described above, instead of in carbon dioxide-free water, and the haemolysate was centrifuged at about 18000*g* for 10 min. at 4° (compare stage 1 in Scheme 1). Thereafter the procedure outlined in Scheme 1 was followed.

Determinations of tissue concentration and adenosine-triphosphatase activity

Tissue concentration. Dry weights of tissue suspensions were used as measures of tissue concentrations. Trichloro-



Scheme 1. Preparation of elinin from human erythrocyte membranes (see Moskowitz *et al.* 1950; Moskowitz & Calvin, 1952).

acetic acid (15%, w/v) was added to a sample of the tissue suspension, giving a final concentration of 5% (w/v), and the precipitate was centrifuged down. The supernatant solution was drained off and the sediment dried overnight in an oven at about 105° and weighed.

Measurement of adenosine-triphosphate activity. The rate of hydrolysis of ATP in the presence of the various tissue preparations was measured by following the rate of liberation either of orthophosphate or of H^+ ions. The method of following the rate of liberation of orthophosphate was as described by Wheeler & Whittam (1962) and was used in all experiments involving erythrocyte-membrane preparations and in some of those involving fractions of kidney-cortex homogenates. (The incubation times were varied to suit the preparation under examination.)

The hydrolysis of ATP in the presence of equimolar concentrations of Mg^{2+} ions may be represented by the equation:

$$\begin{array}{ccccccc} MgATP^{2-} & & & MgADP^{-} & & HPO_4^{2-} & \\ \Downarrow & & & \Downarrow & & \Downarrow & \\ MgATP^{-} & +xH_2O\rightarrow & & MgADP & + & H_2PO_4^{-} & + yH^+ \end{array}$$

from which it is apparent that the ratio of ATP hydrolysed to H^+ ions released depends on the pH at which the reaction occurs. At any given pH value, however, this ratio will be constant and so the rate of liberation of H^+ will be a measure of the rate of ATP hydrolysis. With apparatus now available it is possible to measure the rate of change of pH very accurately, so that a total change of less than 0·05 pH unit occurs and the reaction rate is unaffected by the pH change. This method has been used successfully by several investigators (Chance & Ito, 1962; Nishimura, Ito & Chance, 1962; Tonomura, Kitagawa & Yoshimura, 1962; Gutfreund & Hammond, 1963), and we thank Dr H. Gutfreund for providing us with details of his apparatus and technique before their publication.

The essential details of the apparatus and circuit that we have used are shown in Fig. 1. The reaction vessel was the end of a 50 ml. plastic centrifuge tube; it was held in a water bath at 37–37·5° and the incubation mixture was stirred with the stirring apparatus from a Radiometer titrator (stirring motor SMP1a; Radiometer, Copenhagen). The standard medium used contained (final concn.): NaCl (100 mM), KCl (20 mM), $MgCl_2$ (2 mM) and imidazole (3–5 mM); it was modified by adding ouabain (0·2 mM), and was adjusted to pH 7·9 by titration with 0·5N-HCl. Other media used for particular experiments are described in the Results section. Before each measurement the glass/calomel electrode was washed with distilled water and the surplus wiped off with tissue paper. Then 10 ml. of the required medium was run into the reaction vessel, and the tissue suspension and any other reagents, in solutions adjusted to pH 7·9 with 0·1N-NaOH or 0·1N-HCl, were added in volumes of about 0·1–0·3 ml. When reagents were in solution in ethanol an equivalent volume of ethanol was added to the control. The final concentration of tissue was always less than 0·6 mg. dry wt./ml. The potential difference between the glass and calomel electrodes was balanced by the potential drawn from the mercury cell and, when temperature equilibration had been attained, as shown by the steadiness of the recording pen, the reaction was started by the addition of 0·2 ml. of 100 mM-ATP (previously buffered to pH 7·9 with N- and 0·1N-NaOH). When the trace was sufficiently long for accurate rate measurement (usually after 40–60 sec.) the system was calibrated by the addition of about 20 μl. of standard HCl or NaOH (0·02–0·04N) from a syringe burette (type SBU1a; Radiometer, Copenhagen) that had been fitted with a glass delivery tube ending just above the surface of the incubation mixture.

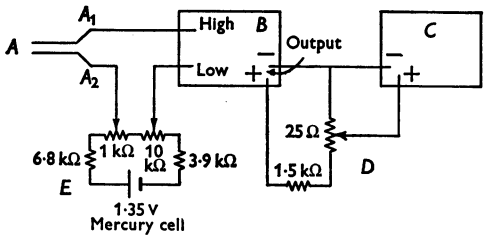


Fig. 1. Apparatus and circuit for measuring small changes in pH. The use of the apparatus for measuring the rate of hydrolysis of ATP is described in the Methods section. The instruments were supplied by Pye and Co. Ltd. (combined glass/calomel electrode, type 450), Electronic Instruments Ltd. (Vibron electrometer, type 33B) and Kipp and Zonen, Delft, Holland (micrograph recorder, type BD_2). *A*, Combined electrode; A_1, glass electrode; A_2, calomel electrode; *B*, electrometer; *C*, recorder; *D*, voltage divider; *E*, backing-off network.

RESULTS

Measurement of adenosine-triphosphatase activity

A typical trace of the rate of change of pH during ATP hydrolysis catalysed by the 800*g* fraction of kidney-cortex homogenate is shown in Fig. 2. In this experiment the trace was linear for at least 50 sec., during which time the pH fell from 7·90 to 7·875. The rapid response of the apparatus is shown by the change of about 0·04 pH unit in 2–3 sec. during the calibration with standard acid. The rate of H^+ ion liberation was linear with respect to concentration of tissue in the range 0·1–0·6 mg. dry wt./ml. in the standard medium in the presence and absence of ouabain.

A direct comparison of the two methods of measuring the rate of ATP hydrolysis was made by following the rate of H^+ ion liberation in large volumes of incubation media (20–25 ml.) while samples of the mixtures were withdrawn after known times and their orthophosphate contents measured as described by Wheeler & Whittam (1962). The results show that after the initial 20 sec. the rates of H^+ ion liberation and of orthophosphate liberation were the same at pH 7·9 (Fig. 3). The initial parts of the curves are subject to some uncertainty because (*a*) the zero-time values for orthophosphate concentrations were calculated by extrapolation, and (*b*) rapid changes in pH often occurred during the first few seconds owing to marginal differences in the pH of the ATP

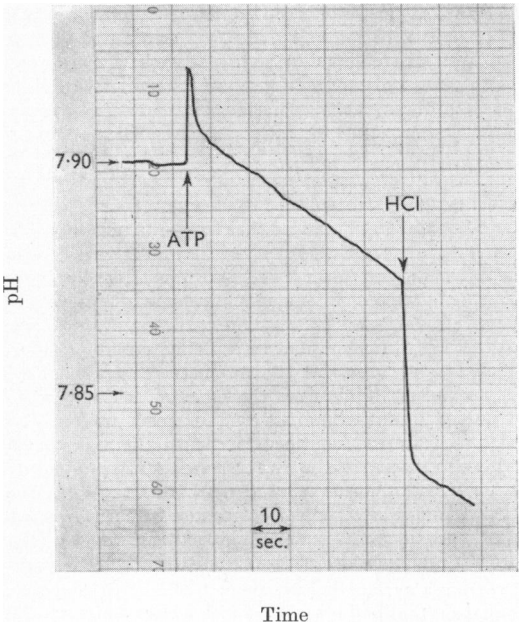


Fig. 2. Typical trace of the pH change recorded during the hydrolysis of ATP by the 800*g* fraction from rabbit kidney-cortex homogenate. About 2 mg. dry wt. of tissue was suspended in the standard medium at 37°, as described in the Methods section. The reaction was initiated by the addition of 0·2 ml. of 100 mM-ATP, as indicated; the downward trace shows the increase in H^+ ion concentration. After about 50 sec., 19·8 μl. of 0·03 N-HCl was added, causing a rapid fall in pH which was used to calibrate the system.

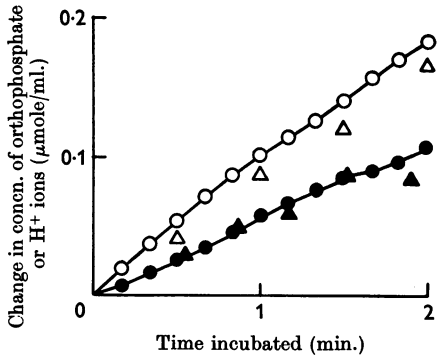


Fig. 3. Comparison of rates of liberation of orthophosphate and H^+ ions during the hydrolysis of ATP at pH 7·9. Samples of the 800*g* fraction from kidney-cortex homogenate were incubated in the standard medium, in the presence (●, ▲) or absence (○, △) of ouabain (0·2 mM), and the rates of liberation of H^+ ions (○, ●) and orthophosphate (△, ▲) measured as described in the Methods section. The zero-time values for orthophosphate concentrations were calculated by extrapolation.

solutions and the media. Nevertheless, the later parts of the lines both show the same linear increase in concentrations of H^+ and orthophosphate ions, indicating that the reaction measured under these conditions was:

$$MgATP^{2-} + H_2O \rightarrow MgADP^- + HPO_4^{2-} + H^+$$

Less than 6 % of the terminal phosphate of ATP was hydrolysed during the complete incubation.

The formation of a phosphorylated intermediate during the enzymic hydrolysis of ATP might manifest itself in the initial rate of H^+ ion liberation being faster than that of orthophosphate (Gutfreund & Hammond, 1963). However, if such a difference does occur, the above results show that it must do so within the first 10–20 sec. of the reaction, when it would be difficult to detect because of the limitation of the orthophosphate measurements.

Reproducibility. Six replicate measurements gave a mean rate ± S.E.M. of ATP hydrolysis (μmole of H^+ ion/tube/min.) of 0·82 ± 0·03, with a range 0·75–0·96.

800*g* *fraction from kidney-cortex homogenate*

Substrate specificity. The specificity of the enzyme system was examined by substituting ITP, ADP, AMP and pyrophosphate for ATP and measuring the initial rates of liberation of orthophosphate under identical conditions at 37° and pH 7·9. The rates of hydrolysis of the various substrates in three experiments have been expressed as percentages of the rate of hydrolysis of ATP measured under optimum conditions with respect to Na^+, K^+ and Mg^{2+} ions (Table 1). AMP was not hydrolysed under these conditions and pyrophosphate was hydrolysed only slowly compared with ATP, and this slow hydrolysis was increased slightly by ouabain, not inhibited. Both ITP and ADP were hydrolysed at appreciable rates, about 39 and 25 % respectively compared with ATP. However, whereas 60 % of the adenosine-triphosphatase activity was inhibited by ouabain, only about 23 % of the 'inosine-triphosphatase' activity and about 40 % of the 'adenosine-diphosphatase' activity was inhibited. Thus the (NaK)-adenosine triphosphatase activity was six times as large as the corresponding activities with ITP or ADP as substrates.

Effects of storage on the two components of the adenosine triphosphatase. The adenosine-triphosphatase activity of the 800*g* fraction was measured in the standard media after being stored at about 4° for 12 days. The (NaK)-adenosine-triphosphatase activity appeared to increase slightly during the first 3–4 days, but the change was too small to be considered significant. After this initial period the activity of this component decreased gradually until after 12 days at 4° it was about 60 % of the

maximum value. The activity of the (Mg)-adenosine triphosphatase remained unchanged throughout the 12 days.

In view of these results, preparations were sometimes kept for up to a week at 4° unless the absolute activity was essential to the experiments, when fresh preparations were used for each experiment.

Table 1. *Substrate specificity of the 800g fraction from kidney-cortex homogenate*

Initial rates of liberation of orthophosphate were measured at 37° in media I and II, with each substrate present in a final concentration of 2 mM and with 0·02–0·06 mg. dry wt. of tissue/ml. Both media were adjusted to pH 7·9 with 5N-HCl and contained (final concn.): I, $MgCl_2$ (2 mM), KCl (20 mM), NaCl (100 mM), imidazole (50 mM); II, the same as I plus ouabain (0·2 mM). The mean values from three experiments are given, each value being expressed as a percentage of the rate of hydrolysis of ATP in medium I.

	Rate of hydrolysis compared with rate of hydrolysis of ATP		
Medium ...	I	II (Ouabain-insensitive)	I–II (Ouabain-sensitive)
Substrate	(Total)		
ATP	100	40	60
ADP	25	15	10
AMP	0	0	0
ITP	39	30	9
Pyrophosphate	8	13	−5

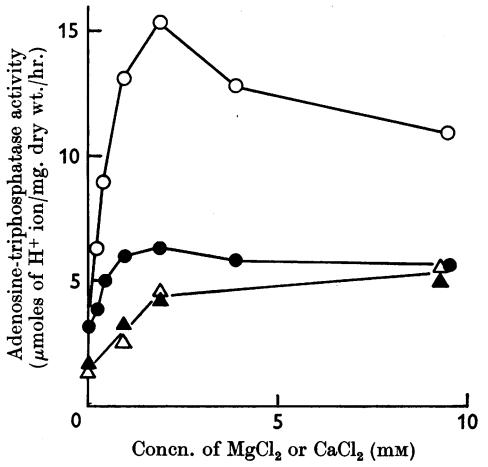


Fig. 4. Activation of adenosine-triphosphatase activity by Mg^{2+} and Ca^{2+} ions. Samples of the 800*g* fraction from kidney-cortex homogenate were incubated in media adjusted to pH 7·9 with 0·5N-HCl and containing (final concn.) NaCl (100 mM), KCl (20 mM), imidazole (3–5 mM) and ATP (2 mM), in the presence (●, ▲) or absence (○, △) of ouabain (0·2 mM). The rates of hydrolysis of ATP were measured in the presence of various concentrations of $MgCl_2$ (○, ●) or $CaCl_2$ (△, ▲), as described in the Methods section. Mean values from four or five experiments are given.

Activation and inhibition by Mg^{2+} and Ca^{2+} ions. The preparations exhibited only a low and variable adenosine-triphosphatase activity unless either Mg^{2+} or Ca^{2+} ions were added to the incubation media (Fig. 4). (The rates of hydrolysis measured in the presence of Ca^{2+} ions alone varied considerably from experiment to experiment and the results were not as consistent as those obtained in the presence of Mg^{2+} ions.) The addition of ouabain had no effect on the activity measured in the presence of Ca^{2+}, Na^+ and K^+ ions, but the much higher activity due to Mg^{2+}, Na^+ and K^+ ions was partly inhibited by ouabain. The extent of the stimulation due to Mg^{2+} or Ca^{2+} ions, measured in the presence of ouabain to prevent activation by Na^+ and K^+ ions, was about the same, but for half-maximal stimulation only about 0·4 mM-magnesium chloride was required, compared with about 1·3 mM-calcium chloride. Moreover, in the presence of Ca^{2+} ions the rate of hydrolysis of ATP continued to rise slightly as the concentration of Ca^{2+} ions was increased above that of ATP, whereas the enzymic activity due to Mg^{2+} ions reached a maximum when the concentration of Mg^{2+} ions equalled that of ATP and fell at higher concentrations. The fall in activity at high concentrations of Mg^{2+} ions was considerable in the presence of Na^+ and K^+ ions, but was only slight when ouabain was also added.

When Ca^{2+} ions were added to media containing Na^+ ions, K^+ ions and 2 mM-ATP (magnesium salt), the (NaK)-adenosine-triphosphatase activity was progressively inhibited as the concentration of Ca^{2+} ions was increased, and was completely abolished by 2–3 mM-calcium chloride (Table 2). Thus it appears that Ca^{2+} ions compete with Mg^{2+} ions at the site where the latter is required for the (NaK)-adenosine-triphosphatase activity, and are more readily bound there than are Mg^{2+} ions, resulting in a selective inhibition of the (NaK)-adenosine-triphosphatase activity similar to that caused by ouabain in lower concentrations.

Inhibition by phlorrhizin and phloretin. In view of the well-established inhibition of renal and intestinal absorption of glucose by phlorrhizin, and of sugar entry into erythrocytes by phloretin (see Lotspeich, 1961; Crane, 1960), it was decided to see if these compounds had any effect on the adenosine-triphosphatase activity of the kidney-cortex preparation. As Table 2 shows, both phlorrhizin and phloretin inhibited the (NaK)-adenosine-triphosphatase activity but had no effect on the ouabain-insensitive component. Under the experimental conditions given in Table 2, 0·1 mM concentrations of both the glycoside and the aglycone inhibited about 50 % of the (NaK)-adenosine-triphosphatase activity, and 0·3–0·4 mM-phlorrhizin caused complete inhibition. These compounds are therefore

similar to ouabain and Ca^{2+} ions in selectively inhibiting the component of the adenosine-triphosphatase activity that requires Na^+ and K^+ ions.

Inhibition by oligomycin. The inhibition by oligomycin of the active transport of Na^+ and K^+ ions in liver slices has been reported and correlated with the associated inhibition of respiration (van Rossum, 1962). There is an interdependence between active cation transport and respiration in brain and kidney cortex (Whittam, 1961, 1962*a*; Whittam & Willis, 1963) that appears to be mediated by (NaK)-adenosine-triphosphatase activity (Blond & Whittam, 1964; Whittam & Blond, 1964), so that the action of oligomycin on this enzymic activity is of some importance with regard to the nature of energy transformation. We have found that oligomycin had no significant effect on the ouabain-insensitive adenosine-triphosphatase activity of the kidney-cortex preparation. On the other hand, up to 80 % of the (NaK)-adenosine-triphosphatase activity was inhibited by this antibiotic, a concentration of about 10 μg. of oligomycin/ml. being required for maximal inhibition and about 1 μg./ml. for half-maximal inhibition (Table 2).

Inhibition by p-chloromercuribenzoate and by N-ethylmaleimide. Ungar & Romano (1963) have shown that there is a correlation between the number of ionizable thiol groups in surviving rat diaphragm and the movements of Na^+ and K^+ ions across it. Hence it seems possible that such thiol groups might be involved in (NaK)-adenosine-triphosphatase activity, and so we have examined the effects of *p*-chloromercuribenzoate and *N*-ethylmaleimide on this activity.

Table 2 shows that as little as 10 μM-*p*-chloromercuribenzoate inhibited both components of the adenosine-triphosphatase activity, but the (NaK)-adenosine-triphosphatase activity was preferentially inhibited. Thus the (NaK)-adenosine-triphosphatase activity fell by about 65 % (from 9·5 to 3·3) whereas the remaining activity decreased by only 35 % (from 8·1 to 5·3) under the same conditions. *N*-Ethylmaleimide (1·0 mM) caused much smaller

Table 2. *Preferential inhibition of the ouabain-sensitive adenosine-triphosphatase activity*

Initial rates of H^+ ion liberation from ATP were measured at 37° in media A and B, with tissue concentrations of 0·27–0·55 mg. dry wt./ml. and the various inhibitors as indicated below. Both media contained (final concn.): NaCl (100 mM), KCl (20 mM), $MgCl_2$ (2 mM), ATP (2 mM) and imidazole (3 mM); medium B contained in addition ouabain (0·2 mM), and both media were adjusted to pH 7·9 with 0·5N-HCl. Mean values for at least two experiments are given.

		Adenosine-triphosphatase activity (μmoles of H^+ ion liberated/mg. dry wt./hr.)			
	Medium	A	B	A – B	
Addition	Final concn. (mM)	Total	Ouabain-insensitive	Ouabain-sensitive	Percentage fall
None	—	16·3	8·2	8·1	0
$CaCl_2$	0·10	13·1	6·8	6·3	22
	0·50	10·6	7·1	3·5	52
	0·90	9·1	7·0	2·1	74
	1·9	7·8	6·6	1·2	85
	9·3	5·6	6·6	−1·0	111
None	—	15·8	7·5	8·3	0
Phlorrhizin	0·01	14·8	8·3	6·5	22
	0·10	11·4	5·8	5·6	33
	0·37	7·1	—	—	—
	1·9	8·0	8·2	−0·2	102
None	—	16·6	7·5	9·1	0
Phloretin	0·01	15·7	8·5	7·2	21
	0·10	11·5	7·2	4·3	53
	0·19	10·5	5·7	4·8	47
None	—	17·6	8·1	9·5	0
p-Chloromercuribenzoate	0·01	8·6	5·3	3·3	65
N-Ethylmaleimide	1·0	14·5	6·2	8·3	13
	(μg./ml.)				
None	—	16·3	5·5	10·8	0
Oligomycin	0·1	15·5	—	—	—
	0·5	12·7	5·3	7·4	31
	1·0	11·9	6·7	5·2	52
	2·0	11·4	5·2	6·2	43
	10	8·1	5·8	2·3	79
	20	8·3	6·4	1·9	82

Table 3. *Distribution of adenosine-triphosphatase activity among subcellular fractions of rabbit kidney-cortex homogenate*

Rabbit kidney-cortex homogenates were fractionated by centrifugation into nuclear, mitochondrial, microsomal and supernatant fractions, as described in the text. Samples of each fraction (final concn. 0·07–0·92 mg. dry wt./ml.) were incubated for 5 min. at 37° in media I and II, and the rates of liberation of orthophosphate from ATP measured. The media were adjusted to pH 7·9 with 5N-HCl and contained (final concn.): I, ATP (2 mM), $MgCl_2$ (2 mM), NaCl (100 mM), KCl (20 mM) and imidazole (50 mM); II, ATP (2 mM), $MgCl_2$ (2 mM) and imidazole (50 mM). Mean values of the results from four experiments are given and the S.E.M. is indicated where possible.

		Adenosine-triphosphatase activity						
		(μmoles of orthophosphate liberated/mg. dry wt./5 min.)			(m-moles of orthophosphate liberated/kg. of cortex/5 min.)			
	Medium ...	I	II	I − II				
Fraction	No. of observations	(Total)	(Mg^{2+} ion-dependent)	(Na^+ ion-plus-K^+ ion-dependent)	(Total)	(Mg^{2+} ion-dependent)	(Na^+ ion-plus-K^+ ion-dependent)	$\frac{I-II}{II}$ ratio
Homogenate	4	1·00±0·01	0·70±0·02	0·30±0·03	145±8	102±4	43±7	0·4
Nuclear	4	1·05±0·12	0·60±0·09	0·44±0·06	48±4	27±2	21±4	0·8
Mitochondrial	4	0·97±0·09	0·77±0·05	0·18±0·06	24±4	20±3	5±1	0·2
Microsomal	3	1·57	1·17	0·39	59	44	15	0·3
Supernatant	2	0·55	0·65	−0·10	5	6	−1	−0·2
Total					136	97	40	0·4

inhibitions, and the (Mg)-adenosine-triphosphatase activity was decreased more (23 %) than the (NaK)-adenosine-triphosphatase activity (13 %). (The reagents were present in the reaction mixture for up to about 5 min. before the rates of ATP hydrolysis were measured; the effect of such preincubation times was not investigated.)

Effect of digitonin. The inhibition in human erythrocytes of K^+ ion influx and Na^+ ion efflux by digitonin (10 μM) has been reported by Pfleger, Rummel, Seifen & Baldauf (1961). Digitonin, however, in concentrations from 1 μM to 0·1 mM had no effect on the (NaK)-adenosine-triphosphatase activity of the kidney-cortex preparation, although the (Mg)-adenosine-triphosphatase activity was stimulated slightly in the presence of 0·1 mM-digitonin.

Localization of Na^+ ion-plus-K^+ ion-dependent adenosine triphosphatase in kidney cortex and in erythrocyte membranes

Distribution of adenosine-triphosphatase activity among subcellular fractions of kidney-cortex homogenate. The adenosine-triphosphatase activities of various fractions of rabbit kidney-cortex homogenates were measured to determine the location of the (NaK)-adenosine triphosphatase. The results, expressed as absolute rates in terms of dry weight of tissue and as total activity/kg. of cortex, are given in Table 3. The agreement between the adenosine-triphosphatase activity of the whole homogenate and the sum of the activities in the four fractions shows that practically complete recovery of the activity was obtained. In terms of ATP hydrolysed/unit dry wt. of tissue, the (NaK)-adenosine-triphosphatase activity was highest in the nuclear fraction, the microsomal fraction being slightly less active. On the other hand, the (Mg)-adenosine-triphosphatase activity expressed in these units was lower in the nuclear fraction than in any of the other fractions, and was little more than half the value of the microsomal activity. Consideration of the total activities in each fraction reveals that on this basis, also, the nuclear and microsomal fractions account for most of the (NaK)-adenosine triphosphatase, with about 50 and 37 % respectively of the total. Moreover, the proportion of (NaK)-adenosine-triphosphatase activity to (Mg)-adenosine-triphosphatase activity was highest in the nuclear fraction, being about 0·8, which was more than twice that in the microsomal fraction. Another important feature of the distribution is that, in addition to almost 90 % of the (NaK)-adenosine-triphosphatase activity being concentrated in the nuclear and microsomal fractions, about 80 % of the total (Mg)-adenosine-triphosphatase activity was extramitochondrial.

Further fractionation of kidney-cortex homogenates. The membrane preparation obtained by application of Neville's (1960) procedure to kidney cortex showed very high adenosine-triphosphatase activity in the presence of Mg^{2+}, Na^+ and K^+ ions (Table 4). Thus the activity of this membrane preparation described in Expt. III (Table 4) was three-and-a-half times that of the 800*g* fraction prepared from the same kidney. However, when the activities of the two preparations were measured in the presence of ouabain, it was found that the ouabain-insensi-

tive adenosine-triphosphatase activity in the membrane preparation was six times as high as that in the 800*g* fraction, whereas the ouabain-sensitive activity was slightly lower in the membrane preparation. The procedure therefore provided no means of enhancing the (NaK)-adenosine-triphosphatase activity, because this was about the same in the membrane preparations as in the usual 800*g* fractions, and constituted a smaller proportion of the total activity than in the 800*g* fractions.

The relatively crude membrane preparation obtained by substituting the sucrose–imidazole–EDTA medium for Neville's (1960) bicarbonate medium and omitting the density-gradient centrifugation exhibited a low (Mg)-adenosine-triphosphatase activity, but the ouabain-sensitive activity was about the same or slightly higher than that normally found in the 800*g* fraction (Expts. IV and V in Table 4). Because of the low (Mg)-adenosine-triphosphatase activities, the (NaK)-adenosine-triphosphatase activities contributed about 70 % of the total in these preparations, which was a higher proportion than that found in any other preparation.

Fragmentation of the 800g fraction by freezing and thawing or by exposure to ultrasound. Freezing and thawing the 800*g* fraction doubled the activity of the (Mg)-adenosine triphosphatase but had no effect on the (NaK)-adenosine triphosphatase (Expt. I in Table 5). Hence, although freezing and thawing gave preparations with high adenosine-triphosphatase activity, the proportion of the activity due to the (NaK)-adenosine triphosphatase was always lower in such preparations than in the normal 800*g* fraction.

The effect of subjecting the preparation to ultrasound for 1–2 min. was similar to that of freezing and thawing. Thus in typical experiments (Expt. II in Table 5) the (Mg)-adenosine-triphosphatase activity was increased up to 300 %, whereas the (NaK)-adenosine-triphosphatase activity rose by only 30 % under the same conditions; hence the percentage inhibition by ouabain fell from about 60 to 30–40 %. Exposure to ultrasound for longer periods resulted in a fall in the activities of both components of the adenosine-triphosphatase activity, their relative proportions remaining more or less constant.

Localization of adenosine-triphosphatase activity in erythrocyte membranes: conditions for measuring the activity. Preliminary experiments with fragmented erythrocyte membranes showed that the amount of orthophosphate liberated from ATP in a medium containing (final concn.) sodium chloride (100 mM), potassium chloride (20 mM), imidazole–hydrochloric acid buffer, pH 7·5 (25 mM), magnesium chloride (3 mM) and ATP (3 mM), with or without ouabain (0·1 mM), was a linear function of the incubation time for up to 60 min. The enzymic activity also varied linearly with the dry weight of tissue within the range 0·4–2·0 mg./ml.

During these preliminary investigations a preferential inhibition of the (Mg)-adenosine-triphosphatase activity by EDTA was noted. Variation of the Mg^{2+} ion concentration in the absence of EDTA and in the presence of 3 mM-ATP gave the results shown in Fig. 5. It is clear that the two components of the adenosine-triphosphatase activity responded differently to the Mg^{2+} ion concentration when the ATP concentration was constant. Thus in the presence of 3 mM-ATP the (NaK)-adenosine triphosphatase showed a marked peak of activity in the presence of 3 mM-magnesium chloride, whereas the (Mg)-adenosine triphosphatase required 12 mM-

Table 4. *Adenosine-triphosphatase activities of membrane preparations from kidney-cortex*

The 800*g* fraction was prepared as described in the text and the membrane preparation was obtained by application of Neville's (1960) method to kidney cortex, with the modification described by Emmelot & Bos (1962). The crude membrane preparation was that resulting from the replacement of Neville's 1–2 mM-$NaHCO_3$ medium, pH 7·5, by the medium containing sucrose (0·25M), imidazole (10 mM) and EDTA (2 mM), adjusted to pH 7·9 with 5N-HCl, and the omission of the density-gradient-centrifugation procedure. The initial rates of ATP hydrolysis at 37° were measured in the presence of samples of the membrane preparation (0·05–0·12 mg. dry wt./ml.), the crude membrane preparation (0·21–0·31 mg. dry wt./ml.) and the 800*g* fraction (0·15–0·35 mg. dry wt./ml.), in media A and B (see Table 2 for composition).

	Adenosine-triphosphatase activity (μmoles of H^+ ion liberated/mg. dry wt./hr.)								
	Membrane fraction			800*g* fraction			Crude membrane fraction		
Medium ...	A	B	A – B	A	B	A – B			
Expt. no.	(Total)	(Ouabain-insensitive)	(Ouabain-sensitive)	(Total)	(Ouabain-insensitive)	(Ouabain-sensitive)	(Total)	(Ouabain-insensitive)	(Ouabain-sensitive)
I	40·9	33·3	7·6	—	—	—	—	—	—
II	56·4	43·8	12·6	—	—	—	—	—	—
III	66·5	59·5	7·0	18·7	9·9	8·8	—	—	—
IV	—	—	—	—	—	—	14·5	4·5	10·0
V	—	—	—	7·3	3·0	4·3	13·0	3·5	9·5

Table 5. *Effects of freezing and thawing and of ultrasound on the adenosine-triphosphatase activity of the 800g fraction*

Samples of the 800*g* fraction from kidney-cortex homogenate were (Expt. I) frozen by immersion in liquid air and then thawed at 37° or (Expt. II) exposed to ultrasonic vibrations for various lengths of time at 0°. The adenosine-triphosphatase activities of these samples were measured at 37° in media A and B (see Table 2 for composition) with 0·21–0·35 mg. dry wt. of tissue/ml.

		Adenosine-triphosphatase activity (μmoles of H^+ ion liberated/mg. dry wt./hr.)			
	Medium ...	A	B	A – B	100 (A – B)/A
	No. of times frozen and thawed	(Total)	(Ouabain-insensitive)	(Ouabain-sensitive)	(% inhibition due to ouabain)
I	0	22·0	8·6	13·4	61
	1	29·5	17·8	11·7	40
	2	31·0	19·6	11·4	34
	3	30·8	17·8	13·0	42
	Time exposed to ultrasound (min.)				
II	0	19·7	8·2	11·5	58
	1	42·6	28·2	14·4	34
	2	40·4	24·0	16·4	41
	4	23·6	15·9	7·7	33
	10	19·9	11·7	8·2	41

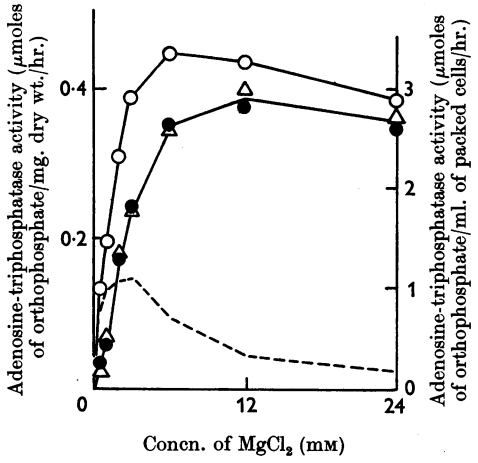


Fig. 5. Effects of various concentrations of Mg^{2+} ions on the adenosine-triphosphatase activity of erythrocyte-membrane fragments. The adenosine-triphosphatase activity of samples of fragmented erythrocyte membranes was measured, as described in the Methods section, in media containing (final concn.): ○, NaCl (100 mM), KCl (20 mM), ATP (3 mM), imidazole–HCl buffer, pH 7·5 (25 mM); △, NaCl (100 mM), ATP (3 mM), imidazole–HCl buffer, pH 7·5 (25 mM); ●, the same as ○ with ouabain (0·1 mM) present, and the concentrations of $MgCl_2$ indicated. The values given are means from three to seven experiments and the broken line shows the difference between the other two lines, thus giving the (NaK)-adenosine-triphosphatase activity.

magnesium chloride for maximal activity and the fall in activity in the presence of higher concentrations of Mg^{2+} ions was slight. A consequence of these different responses to Mg^{2+} ion concentration was that the proportion of the adenosine-triphosphatase activity that was sensitive to Na^+ plus K^+ ions, and to ouabain, could be varied from about 80 % in the presence of 0·5 mM-magnesium chloride to about 10 % in the presence of 12–24 mM-magnesium chloride.

To try to obtain easily measurable adenosine-triphosphatase activity and a large response to Na^+ plus K^+ ions, the later experiments with elinin were performed with media containing ATP (3 mM) and magnesium chloride (1 mM).

Erythrocyte-membrane adenosine triphosphatase and the lipoprotein elinin. Before attempting to prepare a sample of elinin the effect on the adenosine-triphosphatase activity of extracting dried stroma with ether at 34° was examined (stage 3 in Scheme 1). The results indicated that this procedure had no effect, the activity of the extracted stroma being the same as that of dried stroma that had been stored *in vacuo* at 4° during the ether extraction (Expt. A in Table 6). A sample of elinin was prepared and its adenosine-triphosphatase activity compared with that of a sample of the original dried stroma (Expt. B in Table 6). The elinin showed only 15 % of the activity of the original stroma and was not inhibited by ouabain. (Also, the absolute changes in orthophosphate

Table 6. *Adenosine-triphosphatase activity of elinin and of various fractions of erythrocyte membranes*

The various fractions were prepared from 3–4-week-old human blood by the procedures outlined in Scheme 1. Samples of the fractions (up to 2 mg. dry wt. of tissue/ml.) were incubated for 15–60 min. at 37° in media I and II and the rates of orthophosphate liberation from ATP measured. Medium I contained (final concn.) ATP (3 mM), $MgCl_2$ (1 mM), NaCl (100 mM), KCl (20 mM) and imidazole (25 mM), and medium II contained in addition ouabain (0·1 mM); both media were adjusted to pH 7·5 with 5N-HCl.

	Adenosine-triphosphatase activity (μm-moles of orthophosphate liberated/mg. dry wt./hr.)					
Expt. no.	A		B		C	
Medium	I	II	I	II	I	II
Fraction	(Total)	(Ouabain-insensitive)	(Total)	(Ouabain-insensitive)	(Total)	(Ouabain-insensitive)
Dried stroma	40	20	100	80	45	20
Extracted stroma	40	15	—	—	40	20
Sediment 1	—	—	—	—	25	20
Sediment 2	—	—	—	—	15	15
Elinin	—	—	15	15	—	—

concentrations during the incubations with elinin were near the limit that could be estimated accurately.)

Examination of intermediate stages in the preparative procedure revealed that the first solubilization of extracted stroma at pH 9 (stages 4 and 5 in Scheme 1) decreased the activity of the (NaK)-adenosine triphosphatase by 75 % (Expt. C in Table 6). Repetition of this process (stage 6 in Scheme 1) removed the (NaK)-adenosine-triphosphatase activity completely, giving a preparation with low (Mg)-adenosine-triphosphatase activity only (Expt. C in Table 6). Elinin, prepared in this way, therefore does not contain the (NaK)-adenosine triphosphatase, although the extracted stroma does.

DISCUSSION

Membrane adenosine-triphosphatase activity. The present results add to the evidence, direct and indirect, that the adenosine-triphosphatase activity that requires Mg^{2+}, Na^+ and K^+ ions is associated with membrane material and is involved in the process of active cation transport.

The most direct evidence on location has been provided by investigations of fragmented erythrocyte membranes (Post, Merritt, Kinsolving & Albright, 1960; Dunham & Glynn, 1961), neuronal membranes of the vestibular nucleus (Cummins & Hyden, 1962), a cell-membrane fraction of liver homogenate (Emmelot & Bos, 1962) and a fraction containing endoplasmic reticulum from kidney homogenate (Landon & Norris, 1963). That the enzyme system possesses the spatial orientation required for active transport has been shown by the directional effects of Na^+ and K^+ ions on ATP hydrolysis in reconstituted erythrocyte 'ghosts' (Glynn, 1962; Whittam, 1962*b*). Under more physiological conditions, indirect evidence has been provided by the observations that orthophosphate production in erythrocytes (Laris & Letchworth, 1962) and in intact crab nerve (Baker, 1963) is stimulated by K^+ ions from the outside of the cell membranes and by Na^+ ions from the inside. In cells more complex than erythrocytes, the various microsomal fractions from the several tissues investigated must have contained some endoplasmic reticulum (Landon & Norris, 1963), and, in brain, glia and nerve-cell membranes (Cummins & Hyden, 1962), so the observed (NaK)-adenosine-triphosphatase activities of these fractions also could be associated with membrane fragments (Skou, 1957, 1960, 1962; Deul & McIlwain, 1961; Bonting, Simon & Hawkins, 1961; Järnefelt, 1961, 1962; Aldridge, 1962; Schwartz, 1962; Schwartz, Bachelard & McIlwain, 1962; Yoshida & Fujisawa, 1962; Auditore & Murray, 1962; Lee & Yu, 1963). The localization of adenosine-triphosphatase activity in kidney-cell membranes has further been amply demonstrated by histochemical techniques (Spater, Novikoff & Masek, 1958; Novikoff, Drucker, Shin & Goldfischer, 1961; Ashworth, Luibel & Stewart, 1963). Novikoff *et al.* (1961) reported that Na^+ ions, K^+ ions and ouabain had no effects on this adenosine-triphosphatase activity, but since the tissue was fixed in a medium containing Ca^{2+} ions, and since the incubation medium apparently contained 10 mM-Mg^{2+} ion with only 0·8 mM-ATP, the failure to observe stimulation of the activity by Na^+ plus K^+ ions might well have been the result of adverse experimental conditions (see Fig. 4, and Wheeler & Whittam, 1962).

The 800*g* fraction from kidney-cortex homogenate that we have used for most investigations contains part of the 'membrane fraction' as prepared according to Neville's (1960) method. Kin-

solving *et al.* (1963) showed by electron microscopy that their '1500*g* fraction' of kidney-cortex homogenate, which must have contained both our 'membrane' and '800*g*' fractions combined, contained a variety of well-preserved membranes; also, their second fraction was clearly almost identical with our 'crude membrane fraction'. Thus there is substantial evidence to implicate cell-membrane fragments in the (NaK)-adenosine-triphosphatase activity of these various heavy fractions from kidney-cortex homogenates. However, although the proportions of the ouabain-sensitive to ouabain-insensitive components of the adenosine-triphosphatase activity in our different preparations varied considerably, the purest 'membrane fraction', instead of showing an increase in ouabain-sensitive activity, actually showed a decrease. It seems likely that the (NaK)-adenosine-triphosphatase activity is dependent on some structural integrity of the membranes that may be destroyed by the fractionation and fragmentation procedures.

Distribution of adenosine-triphosphatase activity. Most of the (NaK)-adenosine-triphosphatase activity in kidney-cortex homogenate was distributed between the nuclear fraction (about 50 %) and the microsomal fraction (about 37 %). Bonting, Caravaggio & Hawkins (1962) also found that these two fractions accounted for most of this component of the adenosine-triphosphatase activity of liver and brain homogenates, but the proportions in the two fractions were different. Thus the nuclear fraction from liver homogenate contained about 93 % of the total (NaK)-adenosine-triphosphatase activity and the microsomal fraction only about 4 %, whereas the distribution was about 33 and 57 % respectively for brain homogenates. Kidney cortex obviously is intermediate between brain and liver, as far as this distribution is concerned, and these differences presumably reflect the different distributions of membrane fragments in homogenates of these tissues.

It is possible that part or all of the (NaK)-adenosine-triphosphatase activity exhibited by the mitochondrial fraction from kidney cortex was caused by contamination by other fractions, since each fraction was washed once only. However, even if all this activity was mitochondrial, it is obvious that almost 90 % of the total (NaK)-adenosine-triphosphatase activity in kidney-cortex homogenate was extramitochondrial Moreover, about 80 % of the (Mg)-adenosine-triphosphatase activity also was extramitochondrial, and the results of Bonting *et al.* (1962) show that in liver and brain homogenates even less of the adenosine-triphosphatase activity is associated with the mitochondria. Thus it appears that, if the connexion between active cation transport and respiration in these tissues is mediated by adenosine-triphosphatase activity that determines the availability of ADP or orthophosphate (Whittam, 1961, 1962*a*; Whittam & Willis, 1963), then this control of respiration is due to ADP or orthophosphate produced mainly outside the mitochondria (see also Blond & Whittam, 1964).

The difficulties of locating membrane material in tissue homogenates are not encountered with erythrocytes, so they are particularly suitable for investigations of the nature of the enzyme system that hydrolyses ATP. Since thoroughly washed and fragmented erythrocyte membranes exhibit (NaK)-adenosine-triphosphatase activity, this activity is undoubtedly associated with the cell membrane. The removal of some lipid from this membrane by ether extraction during the first stages in the preparation of elinin had no effect on the adenosine-triphosphatase activity (Table 6), so that the latter must be associated with the remaining components of the membrane. The loss of activity found during the solubilization and washing processes involved in the isolation of elinin might indicate that substantial membrane integrity is required for the enzymic activity: certainly the most active preparations obtained were 'ghosts' washed free of most of the haemoglobin and fragmented by freezing and thawing. The soluble preparation of elinin was without (NaK)-adenosine-triphosphatase activity. However, in view of the rather drastic treatment involved in the isolation of elinin, the present evidence suggests that actual fragments of membranes are essential for the adenosine-triphosphatase activity.

Substrate specificity. There is some disagreement in the literature on the question of the specificity of adenosine triphosphatases, though some of the discrepancies could be due to differences between tissues. Thus hydrolysis of ITP as well as ATP was caused by a crab nerve enzyme (Skou, 1960) and a brain microsomal enzyme (Järnefelt, 1962); we found the same with the kidney-cortex preparation. However, under conditions that were optimum for ATP hydrolysis in our system, the rate of ITP hydrolysis was less than that of ATP and, in agreement with Skou's (1960) findings, the proportion of activity dependent on Na^+ plus K^+ ions was much lower with ITP as substrate than with ATP. In contrast, Post *et al.* (1960) reported that fragmented erythrocyte membranes did not hydrolyse ITP, and Kinsolving *et al.* (1963) also concluded that ITP was not a substrate for their kidney-cortex preparation. The hydrolysis of ADP, again slow compared with that of ATP, that we observed might have been caused by adenylate kinase. The hydrolysis of pyrophosphate was considered unimportant because the rate was low and variable and was not stimulated by Na^+ plus K^+ ions (Table 1).

Effect of Ca^{2+} *ions.* There are reports that Ca^{2+} ions can replace Mg^{2+} ions in activating the ouabain-insensitive component of adenosine triphosphatases (e.g. Emmelot & Bos, 1962; Taylor, 1962; Aldridge, 1962; Järnefelt, 1962; Lee & Yu, 1963). Usually, however, the effect of Ca^{2+} ions in the presence of Mg^{2+}, K^+ and Na^+ ions has been investigated, when Ca^{2+} ions have always been found to inhibit the (NaK)-adenosine-triphosphatase activity (Skou, 1957, 1960; Dunham & Glynn, 1961; Deul & McIlwain, 1961; Aldridge, 1962; Järnefelt, 1962; Taylor, 1962; Lee & Yu, 1963; Kinsolving *et al.* 1963). Our findings show that Ca^{2+} ions could replace Mg^{2+} ions to give the ouabain-insensitive adenosine-triphosphatase activity, but that no (NaK)-adenosine-triphosphatase activity occurred in the absence of Mg^{2+} ions. Moreover, Ca^{2+} ions inhibited the ouabain-sensitive activity requiring Mg^{2+}, Na^+ and K^+ ions, in agreement with the findings listed above. These observations raise the question of the site of action of Ca^{2+} ions: whether it is intracellular or extracellular. Inhibition of K^+ ion influx in human erythrocytes by internal but not by external Ca^{2+} ions has been reported (Rummel, Seifen & Baldauf, 1963). Ca^{2+} ions in the medium also have little effect on the active transport of Na^+ and K^+ ions in kidney-cortex slices (Kleinzeller & Cort, 1960) or on their initial rate of respiration (Cutting & McCance, 1947; Robinson, 1949; Krebs, 1950). However, Ca^{2+} ions cause a large decrease in the rate of respiration of kidney-cortex homogenates, making it likely that the effects of Ca^{2+} ions are elicited only when the ion is intracellular (Blond & Whittam, 1964).

Effect of oligomycin. The action of oligomycin on (NaK)-adenosine triphosphatases has not been widely examined, but 60–90 % inhibition has been reported for brain microsomal preparations (Järnefelt, 1962; Jöbsis & Vreman, 1963; van Croningen & Slater, 1963), particulate fractions of the electric organ (Glynn, 1963; Jöbsis & Vreman, 1963) and fragmented erythrocyte membranes (van Croningen & Slater, 1963). Jöbsis & Vreman (1963) compared their findings on the inhibition by oligomycin of this enzymic activity with work on the inhibition of mitochondrial adenosine triphosphatases by this antibiotic (Lardy, Johnson & McMurray, 1958; Huijing & Slater, 1961; Jöbsis, 1963), and postulated that in the presence of Na^+, K^+ and Mg^{2+} ions the hydrolysis of ATP might involve a high-energy intermediate similar to one of those formed during oxidative phosphorylation. This view is rendered tenable by van Rossum's (1962) findings that active transport of Na^+ and K^+ ions in rat-liver slices was partially inhibited by oligomycin, whether the transport depended on respiration or on glycolysis, and by the similar inhibitions of Na^+ and K^+ ion transport in erythrocytes (Glynn, 1963; R. Whittam & A. Blake, unpublished work). The mechanism of membrane hydrolysis of ATP assumes a new importance as the intermediates involved may be the same as those participating in the generation of ATP. Indications of a complex reaction are shown by the direct demonstration of a phosphorylated intermediate being involved in the hydrolysis of ATP (Judah, Ahmed & McLean, 1962; Charnock & Post, 1963; Albers, Fahn & Koval, 1963). However, van Croningen & Slater (1963) have pointed out that the concentration of oligomycin required for half-maximal inhibition of a (NaK)-adenosine triphosphatase in brain microsomes was about 100 times that required for the adenosine triphosphatase of brain mitochondria, so a direct relationship has yet to be proved between the actions of oligomycin on mitochondrial and cell-membrane phenomena.

Phlorrhizin and phloretin. The mode of action of phlorrhizin in inhibiting glucose transport across renal tubules and small intestinal mucosa is unknown. Phlorrhizin also inhibits energy metabolism in kidney and intestine but the concentrations required are higher (10 μM–1·0 mM) than those necessary to inhibit glucose transport (1–10 μM) (see Lotspeich, 1961). Considering all the evidence available, Lotspeich (1961) concluded that, in low concentrations, phlorrhizin blocks sugar transport by binding at cell-membrane carrier sites. The effects of these agents on cation transport seem not to have been examined. However, it has been shown that the presence of Na^+ ions is required for the active transport of some sugars by intestine *in vitro* (Riklis & Quastel, 1958*a*; Csaky & Thale, 1960; Bihler & Crane, 1962; and see Crane, 1962) and that such active transport in isolated surviving guinea-pig intestine can be stimulated when K^+ ions are also present, whereas 10 μM-phlorrhizin inhibits this stimulation (Riklis & Quastel, 1958*a*, *b*). Moreover, Kleinzeller & Kotyk (1961) found that the active transport of galactose by kidney-cortex slices required the presence of Na^+ and K^+ ions and that both ouabain and phlorrhizin inhibited this transport: hence the relevance of the actions of phlorrhizin and phloretin on the kidney adenosine-triphosphatase activity. Both compounds (at 10 μM–1 mM) inhibited the (NaK)-adenosine-triphosphatase activity (Table 2), and it is attractive to suppose that the inhibition by these compounds indicates a connexion between the (NaK)-adenosine triphosphatase and glucose transport.

Further evidence that the (NaK)-adenosine triphosphatase may be involved in more than the transport of Na^+ and K^+ ions is the finding that both phloretin and phlorrhizin affect the active accumulation of certain amino acids by slices of rat-kidney cortex (Segal, Blair & Rosenberg, 1963).

Phloretin diminished the accumulation, whereas phlorrhizin enhanced it, apparently by inhibiting the efflux of amino acids from the intracellular fluid. Again, the net uptake of glycine by ascites-tumour cells is inhibited by lack of Na^+ and K^+ ions, or by the presence of ouabain (Riggs, Walker & Christensen, 1958; Bittner & Heinz, 1963). Therefore Na^+ and K^+ ions seem to be associated with movements of sugars and amino acids alike, implying that the (NaK)-adenosine triphosphatase may be a key enzyme for active transport generally.

Effect of thiol reagents. The inhibition of (NaK)-adenosine-triphosphatase activity by thiol reagents has been reported for a brain microsomal preparation (Skou, 1963), fragmented erythrocyte membranes (Weed & Berg, 1963), a particulate fraction from cardiac muscle (Schwartz, 1963), a heavy-microsomal fraction from kidney (Frazer, 1963) and a cell-debris fraction from kidney (Taylor, 1963). It is difficult to compare these results in detail because of the variety of experimental conditions and concentrations of reagents employed. However, the predominating feature appears to be a preferential inhibition of (NaK)-adenosine-triphosphatase activity by most of the reagents, as we found with *p*-chloromercuribenzoate (Table 2). The exception seems to be *N*-ethylmaleimide, which inhibited the (Mg)-adenosine triphosphatase rather more than the (NaK)-adenosine triphosphatase of both Skou's (1963) and our preparations, though in our system the amount of inhibition was small (Table 2). Weed & Berg (1963) reported that this reagent had no effect on the erythrocyte-membrane adenosine-triphosphatase activity.

These results are of particular interest with regard to the kidney preparations because of the possibility of mercurial diuretics producing their effect through inhibition of (NaK)-adenosine-triphosphatase activity. Frazer (1963) concluded that the (NaK)-adenosine triphosphatase is probably not directly involved in diuretic responses, but Taylor (1963) argued that it might be, Further, as a result of simultaneous microscopic and enzymic studies on kidneys from rats that had been injected with mersalyl, Goth, Holman & O'Dell (1950) decided that the observed decrease in adenosine-triphosphatase activity was a result and not a cause of renal injury. However, they measured only the (Mg)-adenosine-triphosphatase activity. A relevant observation is the correlation between rates of K^+ and Na^+ ion movement across rat diaphragm and the number of ionizable thiol groups present (Ungar & Romano, 1963). These scattered findings taken together suggest that the inhibition of (NaK)-adenosine-triphosphatase activity by thiol reagents provides some evidence for a close linkage between the enzymic activity and membrane transport phenomena of physiological significance.

The two components of adenosine-triphosphatase activity. A feature of the results presented above is the different response of the two components of adenosine-triphosphatase activity [the (Mg)-adenosine triphosphatase and the (NaK)-adenosine triphosphatase] to a number of preparative treatments and to various inhibitors. Most of the latter had a preferential, if not exclusive, effect on the (NaK)-adenosine-triphosphatase activity, and this component of the erythrocyte-membrane adenosine triphosphatase was preferentially activated by low concentrations of Mg^{2+} ions. On the other hand, the several preparations obtained by different methods from kidney cortex usually varied in their (Mg)-adenosine-triphosphatase activity, but not in their (NaK)-adenosine-triphosphatase activity, when the various preparations were made from the same homogenate. From these findings and investigations of other tissues it seems that the ratio of (NaK)-adenosine-triphosphatase activity to (Mg)-adenosine-triphosphatase activity can be altered by a range of procedures that may affect one or both of these components. These procedures include freezing and thawing, exposure to ultrasound, variation in osmotic strength of the preparative media (Yoshida & Fujisawa, 1962), treatment with detergents (Skou, 1962; Schwartz *et al.* 1962; van Croningen & Slater, 1963; Frazer, 1963), aging in a suitable medium (Kinsolving *et al.* 1963) and dialysis (Landon & Norris, 1963). The Mg^{2+} ion:ATP concentration ratio in the incubation media, as well as the Na^+ ion:K^+ ion ratio, is particularly important in determining the ratio of the two components of the adenosine-triphosphatase activity (Figs. 4 and 5), and this factor appears to be dependent on the tissue (compare the results given above for the erythrocyte and kidney preparations, and see Skou, 1960, 1962). In view of these observations it becomes obvious that great care must be exercised in making quantitative comparisons of adenosine-triphosphatase activity with other processes such as cation transport.

SUMMARY

1. The effects of some inhibitors of various membrane and other cellular processes on Na^+ ion-plus-K^+ ion-dependent adenosine-triphosphatase activity have been investigated by using a cell-debris fraction from rabbit kidney-cortex homogenate. This component of the enzymic activity was selectively inhibited by ouabain and also by Ca^{2+} ions, phlorrhizin, phloretin and oligomycin. The thiol reagent *p*-chloromercuribenzoate preferentially inhibited this component but, like *N*-ethylmaleimide, also inhibited that part of the

adenosine-triphosphatase activity which was not dependent on Na^+ and K^+ ions.

2. The distribution of the enzymic activity among subcellular fractions of kidney-cortex homogenate was determined and its association with membrane-containing fractions examined. The enzymic activity was distributed mainly between the cell-debris and microsomal fractions. A crude membrane fraction showed the highest proportion of Na^+ ion-plus-K^+ ion-dependent activity, whereas a more purified fraction contained only a small proportion of this component.

3. A partial fractionation of human erythrocyte membranes was attempted by monitoring the adenosine-triphosphatase activity of the material obtained during the various stages in the isolation of the main lipoprotein component of the membranes. The activity dependent on Na^+ and K^+ ions was lost during the procedures involving solubilization and salting out at alkaline pH.

4. Evidence is presented that the Na^+ ion-plus-K^+ ion-dependent adenosine-triphosphatase activity is essentially a membrane enzyme system and is probably involved in membrane transport phenomena.

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Studies in Immunochemistry

23. SOME OBSERVATIONS ON THE HETEROGENEITY OF PREPARATIONS OF HUMAN BLOOD-GROUP-SPECIFIC SUBSTANCES*

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In man, blood-group-specific substances occur in saliva, gastric juice, ovarian-cyst fluids, meconium etc. These sources of the blood-group substances, however, frequently contain considerable quantities of other constituents, mainly proteins, which are most readily removed by digestion with pepsin at pH 1·5–2·0, or by extracting the freeze-dried materials with 90 % or 95 % (w/v) phenol. After extraction with phenol, the group-specific substances, which have been characterized as mucopolysaccharides, remain as phenol-insoluble residues (Morgan & King, 1943), the water-soluble components of which, after further purification, yield the specific blood-group substances in a high state of purity (Gibbons, Morgan & Gibbons, 1955; Pusztai & Morgan, 1961*b*). The examination of a considerable number of blood-group substances prepared in this manner showed that they have a remarkably similar amino acid composition, irrespective of the serological specificity of the substances within the ABO and Lewis blood-group systems (Pusztai & Morgan, 1963). In a few instances, however, substances that had been similarly purified were found to differ significantly in amino acid composition, and it seemed possible that the specific mucopolysaccharides obtained from cyst fluids by the procedures described could sometimes be contaminated with extraneous protein or glycoprotein contained in the cyst fluid. Examination of the specific substances and some of these apparently abnormal substances by immunoelectrophoresis with an anti-(human plasma protein) serum, however, gave no evidence to support the suggestion that these mucopolysaccharides were contaminated with a protein or glycoprotein constituent derived from the plasma.

In an attempt to explain the different amino acid composition that was occasionally found in thoroughly purified preparations of group-specific mucopolysaccharides, a number of selected preparations were examined for their behaviour on diethylaminoethylcellulose columns, and the opportunity was taken to determine the amount and observe the distribution of sialic acid in a few of the substances. The results are given in this paper.

* Part 22: Pusztai & Morgan (1963).

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EXPERIMENTAL

Blood-group substances. These were obtained from ovarian-cyst fluids by the methods of Morgan & King (1943) and Gibbons *et al.* (1955), and were purified further by fractionation with saturated ammonium sulphate at 60°, thorough dialysis, fractionation from water by ethanol, centrifuging at 30000*g* for 1 hr. and drying from the frozen state (Pusztai & Morgan, 1961*b*). The substances obtained were examined in the Svedberg oil-driven ultracentrifuge and by moving-boundary electrophoresis, as described by Kekwick (1950). In some instances, materials obtained at different stages in the fractionation process were examined in greater detail by Dr J. M. Creeth, in a Spinco model E ultracentrifuge.