The Role of Bound Potassium Ions in the Hydrolysis of Low Concentrations of Adenosine Triphosphate by Preparations of Membrane Fragments from Ox Brain Cerebral Cortex

BY P. S. G. GOLDFARB AND R. RODNIGHT

*Department of Biochemistry, Institute of Psychiatry
(British Postgraduate Medical Federation, University of London),
DeCrespigny Park, London S.E.5, U.K.*

(Received 21 May 1970)

1. The intrinsic Na^+ , K^+ , Mg^{2+} and Ca^{2+} contents of a preparation of membrane fragments from ox brain were determined by emission flame photometry. 2. Centrifugal washing of the preparation with imidazole-buffered EDTA solutions decreased the bound Na^+ from 90 ± 20 to 24 ± 12 , the bound K^+ from 27 ± 3 to 7 ± 2 , the bound Mg^{2+} from 20 ± 2 to 3 ± 1 and the bound calcium from 8 ± 1 to <1 nmol/mg of protein. 3. The activities of the $\text{Na}^+ + \text{K}^+ + \text{Mg}^{2+}$ -stimulated adenosine triphosphatase and the Na^+ -dependent reaction forming bound phosphate were compared in the unwashed and washed preparations at an ATP concentration of $2.5 \mu\text{M}$ (ATP/protein ratio $12.5 \text{ pmol}/\mu\text{g}$). 4. The Na^+ -dependent hydrolysis of ATP as well as the plateau concentration of bound phosphate and the rate of dephosphorylation were decreased in the washed preparation. The time-course of formation and decline of bound phosphate was fully restored by the addition of $2.5 \mu\text{M}$ -magnesium chloride and $2 \mu\text{M}$ -potassium chloride. Addition of $2.5 \mu\text{M}$ -magnesium chloride alone fully restored the plateau concentration of bound phosphate, but the rate of dephosphorylation was only slightly increased. Na^+ -dependent ATP hydrolysis was partly restored with $2.5 \mu\text{M}$ -magnesium chloride; addition of K^+ in the range 2 – $10 \mu\text{M}$ -potassium chloride then further restored hydrolysis but not to the control rate. 5. Pretreatment of the washed preparation at 0°C with 0.5 nmol of K^+ /mg of protein so that the final added K^+ in the reaction mixture was $0.1 \mu\text{M}$ restored the Na^+ -dependent hydrolysis of ATP and the time-course of the reaction forming bound phosphate. 6. The binding of [^{42}K]potassium chloride by the washed membrane preparation was examined. Binding in a solution containing 10 nmol of K^+ /mg of protein was linear over a period of 20 min and was inhibited by Na^+ . Half-maximal inhibition of $^{42}\text{K}^+$ -binding required a 100 -fold excess of sodium chloride. 7. It was concluded (a) that a significant fraction of the apparent Na^+ -dependent hydrolysis of ATP observed in the unwashed preparation is due to activation by bound K^+ and Mg^{2+} of the $\text{Na}^+ + \text{K}^+ + \text{Mg}^{2+}$ -stimulated adenosine triphosphatase system and (b) that the enzyme system is able to bind K^+ from a solution of $0.5 \mu\text{M}$ -potassium chloride.

The Mg^{2+} -ATPase* enzyme activity of cell membrane fragments from brain is stimulated by Na^+ . Compared with that given under saturation conditions by Na^+ and K^+ together the extent of the stimulation by Na^+ is small, but it is reproducible and is inhibited by ouabain. In the early studies by

* Abbreviations: Mg^{2+} -ATPase, Mg^{2+} -stimulated adenosine triphosphatase; $\text{Na}^+, \text{Mg}^{2+}$ -ATPase, $\text{Na}^+ + \text{Mg}^{2+}$ -stimulated adenosine triphosphatase; $\text{Na}^+, \text{K}^+, \text{Mg}^{2+}$ -ATPase, $\text{Na}^+ + \text{K}^+ + \text{Mg}^{2+}$ -stimulated adenosine triphosphatase.

Skou (1962), where ATPase activity was measured in a 30 min incubation period with a substrate/enzyme ratio of approx. 300 nmol of ATP/ μg of membrane protein, the addition of 1 mM-sodium chloride increased the rate of hydrolysis 1.8 -fold. At very low substrate/enzyme ratios (in the range 10 – 100 pmol of ATP/ μg of protein) the apparent stimulation is about two- to five-fold (Rodnight, Carrera & Goldfarb, 1969) and may be even greater since under these conditions the ATP-utilizing systems in the membrane are far from saturated and

presumably compete for the available ATP. Accompanying this $\text{Na}^+, \text{Mg}^{2+}$ -ATPase activity in membrane fractions there occurs another $\text{Na}^+ + \text{Mg}^{2+}$ -dependent reaction, namely the transfer of the γ -phosphate of ATP to protein-bound acyl groups to form labile bound phosphate. This reaction is now generally held to constitute an initial step in ATP hydrolysis by the $\text{Na}^+, \text{K}^+, \text{Mg}^{2+}$ -ATPase system (see Rodnight, 1970, and references cited therein). Now it is significant that the bound phosphate formed in the presence of Na^+ and Mg^{2+} is turning over, as shown by its rapid decline to zero both on addition of non-radioactive ATP to the system labelled with ^{32}P and as ATP hydrolysis approaches completion (Rodnight, Hems & Lavin, 1966; Rodnight, 1970), although according to the widely accepted scheme for the involvement of protein-bound phosphate in the action of the $\text{Na}^+, \text{K}^+, \text{Mg}^{2+}$ -ATPase system turnover should only occur in the presence of K^+ as well as Na^+ in the reaction mixture. With Na^+ alone the rate of loss of bound phosphate should be no greater than expected from non-enzymic hydrolysis. In the acid-denatured enzyme system (Hems & Rodnight, 1966) this is many times slower than that found in the intact system.

The stimulation of ATP hydrolysis by Na^+ and the $\text{Na}^+ + \text{Mg}^{2+}$ -dependent turnover of bound phosphate has been interpreted by some workers (Skou, 1965; Skou & Hilberg, 1969; Fujita *et al.* 1967, 1968) in terms of an enzyme activity that is closely related to, but not necessarily identical with, the $\text{Na}^+, \text{K}^+, \text{Mg}^{2+}$ -ATPase system. In discussing the subject Skou (1965) and Skou & Hilberg (1969) have expressed doubts as to the proposed role of K^+ in the multi-stage mechanism. Instead they suggest there may be two routes of ATP hydrolysis catalysed by univalent cations: an Na^+ -stimulated route and an $\text{Na}^+ + \text{K}^+$ -stimulated route, with only the former route involving covalent binding of phosphate to the enzyme system. This view implies that the bound phosphate is intrinsically unstable and does not require a univalent cation other than Na^+ for its hydrolysis; rather than increasing turnover it was suggested that K^+ may discharge the bound phosphate by converting the enzyme into a form not involving a phosphorylated intermediate. Fujita *et al.* (1967, 1968) also consider that the Na^+ -stimulated activity is closely related to the $\text{Na}^+, \text{K}^+, \text{Mg}^{2+}$ -ATPase system, but propose that under certain conditions (e.g. high pH) Na^+ can substitute for K^+ in the hydrolytic step.

In the present paper we examine the possibility that the Na^+ -stimulated activity is due to activation of the $\text{Na}^+, \text{K}^+, \text{Mg}^{2+}$ -ATPase system by K^+ bound to the membrane in a form available to the enzyme. A preliminary report of this work has appeared (Goldfarb & Rodnight, 1968).

MATERIALS

All materials used were of analytical grade or of the highest purity available. Solutions were made up in freshly deionized water that had been distilled from glass. K^+ -free NaCl was obtained from Johnson, Matthey and Co. Ltd., London E.C.1, U.K. EDTA and ATP (disodium salt) were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. The tris salt of ATP was prepared by exchange chromatography on Dowex 50 (X12; H^+ form) and stored at -20°C and 50 mM concentration. The mixed ion-exchange resin AG-501 (X8), consisting of equal parts of Dowex 50 (H^+ form) and Dowex 1 (OH^- form), was obtained from Micro-Bio Laboratories, London W.11, U.K.

Carrier-free $\text{H}_3^{32}\text{PO}_4$ and ^{42}KCl were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [γ - ^{32}P]ATP was prepared as described by Rodnight *et al.* (1966).

METHODS

Preparation and treatment of membrane fraction. The procedure for obtaining a partially purified membrane fraction from ox cerebral cortex was described by Rodnight (1970). The preparation was subjected to a number of treatments in attempts to minimize its content of endogenous cations. The treatments usually involved repeated resuspension and centrifugal washing of the preparation with various solutions, as illustrated by the following procedure with imidazole-buffered EDTA as the washing agent. The surfaces of all equipment coming into contact with the membrane preparation were thoroughly rinsed with freshly prepared deionized water before use. A sample of the preparation (20 mg) was resuspended at 2°C in 16 ml of 4 mM-imidazole-HCl buffer, pH 7.2, containing 5 mM-tris-EDTA added at the same pH (imidazole-buffered EDTA), in a glass-Teflon homogenizer (A-type from A. H. Thomas Inc., Philadelphia, Pa., U.S.A.; clearance 0.16–0.24 mm on the diameter). The resuspended material was poured into two Spinco no. 50 centrifuge tubes and sealed with caps that had been scrubbed and rinsed in deionized water. The caps were disassembled and rinsed before each of the following washes. The pellets obtained by spinning for $10^6\text{g}\cdot\text{min}$ were resuspended as before and given two further centrifugal washes in the same medium, followed by two washes in 4 mM-imidazole-HCl buffer, pH 7.2. The final pellet was suspended in 2 ml of the imidazole buffer and stored in 0.5 ml portions at -20°C .

In a few experiments the content of endogenous cations in the preparation was decreased by treatment with a mixed ion-exchange resin. The resin, AG-501 (X8; 200–400 mesh), was repeatedly washed by decantation with deionized water to remove fine particles. To 20 ml of the preparation suspended in 4 mM-imidazole-HCl buffer, pH 7.2, 12 ml of settled resin was added in six equal portions. The treatment was carried out in a stoppered cylinder held in crushed ice and the contents were mixed by inversion several times after each addition of resin. The pH of the suspension rose from 7.25 to 7.58. The resin was allowed to settle and the rest of the suspension was sampled for cations, decanted and spun down in the Spinco no. 50 head at $10^6\text{g}\cdot\text{min}$.

Determination of bound phosphate and ATPase activity. The procedures given by Rodnight *et al.* (1966) and in the preceding paper (Rodnight, 1970) were followed, except that the basal medium did not contain $MgCl_2$. As before, the standard concentration of NaCl in the medium was 50 mM.

Determination of cations. These were determined with the Unicam SP. 90 Atomic Absorption Spectrophotometer in the absorption mode for Mg^{2+} and Ca^{2+} , and in the emission mode for Na^+ and K^+ . The tissue fractions were diluted to 0.5 mg of protein/ml with deionized water and the cations determined without further treatment. Standard curves for $MgCl_2$ in deionized water were linear in the range 1–50 μM and for NaCl and KCl in the range 1–100 μM and were not affected by the inclusion of bovine plasma albumin (0.5 mg/ml) in the solution. The use of internal $MgCl_2$, $CaCl_2$, NaCl and KCl standards in the diluted buffer controls and in controls containing known amounts of membrane preparation showed that there was no interference with the cation determinations from these sources.

Determination of protein. The method of Miller (1959) was used.

Exposure of membrane material to [^{42}K]potassium chloride. The experiments were carried out at 0°C in Spinco no. 65 tubes rinsed with deionized water. To 1 ml of membrane preparation (1 mg of protein/ml of 4 mM-imidazole-HCl buffer, pH 7.2) ^{42}KCl (specific radioactivity 350 d.p.m./nmol) was added in a minimal volume to give a final concentration of 5–50 μM . In certain experiments NaCl was added together with the ^{42}KCl in the range 10 μM –50 mM. The tubes were kept at 0°C for the requisite time and the contents then diluted to 15 ml with cold 4 mM-imidazole-HCl buffer. The tubes were sealed with carefully pre-rinsed caps and then spun for 2.5×10^6 g·min. The supernatant was decanted, and the remaining drops were rapidly washed out with imidazole buffer without disturbing the pellet, which was then suspended in 6 ml of 0.5 M-NaOH. Counts due to ^{42}K were determined by Čerenkov radiation in a Packard Tri-Carb spectrometer with an efficiency of 70%. Dilution of the reaction mixture 15-fold before centrifuging ensured that the radioactivity in the pellet due to unbound $^{42}K^+$ was decreased to an insignificant value in relation to the total radioactivity counted.

RESULTS

Cation contents of the membrane preparations

These are given in Table 1. The high content of K^+ in the crude preparation obtained by Rodnight

et al. (1966) was greatly decreased by the fractionation procedure described by Rodnight (1970), but there was little change in Na^+ content. Most of the K^+ removed at this stage is probably unbound K^+ from the cytoplasm. Centrifugal washing by the standard procedure with imidazole buffer followed by imidazole-buffered EDTA further decreased the K^+ content by 75%; also, the Na^+ content was decreased by a similar amount and most of the Mg^{2+} and Ca^{2+} was removed. The process was most effective when the protein concentration was maintained in the range 1–2 mg/ml; at higher concentrations the intrinsic cations were removed much more slowly (Fig. 1), whereas in more dilute suspensions the Na^+, K^+, Mg^{2+} -ATPase system tended to lose activity.

The resin treatment was only applied in a few experiments, but gave the lowest final values for intrinsic cations, particularly for K^+ . The treatment was rather difficult to control and unless applied very carefully resulted in an irreversible loss of enzyme activity.

Enzymic properties of treated preparations

Time-course of formation of bound phosphate and ATP hydrolysis. Time-courses for washed and unwashed preparations and the effect of adding low concentrations of Mg^{2+} and K^+ to washed material are shown in Fig. 2; all media contained 50 mM-sodium chloride, but in the control series Mg^{2+} was absent. With the unwashed material both time-courses were identical with those observed in the presence of 2.5 μM -magnesium chloride (Rodnight, 1970). In the washed preparations the plateau concentration and the rate of decline of bound phosphate were both markedly decreased, as was the rate of ATP hydrolysis. The resin-treated preparations gave the same result.

Effects of Mg^{2+} and K^+ . Addition of 2.5 μM -magnesium chloride to the reaction mixture increased ATP hydrolysis and restored the plateau concentration of bound phosphate obtained by using the washed material to the control value; the rate of dephosphorylation, however, was only marginally increased. The latter was fully restored by

Table 1. Cation contents of membrane preparations

Details of the treatment applied are given in the Methods section. The crude preparation was that described by Rodnight *et al.* (1966). Subfractionation of the crude material was carried out as described by Rodnight (1970). Results are expressed as means where less than six observations are available; otherwise means $\pm$ S.D. are quoted.

Membrane preparation	Treatment	Cation content (nmol/mg of protein)			
		Na^+	K^+	Mg^{2+}	Ca^{2+}
Crude	None	100	120	—	—
Subfractionated	None	90 ± 20	27 ± 3	20 ± 2	8 ± 1
Subfractionated	Centrifugally washed	24 ± 12	7 ± 2	3 ± 1	< 1
Subfractionated	Resin-treated	20	2	4	< 1

adding $2\mu\text{M}$ -potassium chloride, although this concentration of K^+ failed to bring ATP hydrolysis back to the control rate (Fig. 2). Hydrolysis could be increased (though not to the original rate) by adding more K^+ , but only at a concentration ($10\mu\text{M}$ -potassium chloride) that also decreased the plateau concentration of bound phosphate (Table 2a).

The results presented in Fig. 2 are the means of two identical experiments with the same membrane preparation in which the determinations were done in duplicate. Different preparations subjected to the washing procedure gave exactly the same pattern and an estimate of the reliability of observations was obtained by averaging the results from 12 preparations. A *t* test then showed that the increase

in the 10s concentration of bound phosphate given by the addition of $2.5\mu\text{M}$ -magnesium chloride and the decrease in the 20s concentration produced by the further addition of $2\mu\text{M}$ -potassium chloride were both significant ($P < 0.01$). On the other hand the difference between the 10 and 20s concentrations of bound phosphate with the washed material after the addition of Mg^{2+} and between the 20s concentrations with the control material and with the washed material after addition of Mg^{2+} and K^+ were not significant ($P > 0.1$).

Sensitivity of washed preparation to Na^+ . The formation of bound phosphate in the washed preparation was more sensitive to low concentrations of Na^+ than in the control unwashed material; the approximate values for half-maximal stimulation were 1mM - and 2.5mM -sodium chloride respectively. ATP hydrolysis, on the other hand, was decreased in the washed preparation at all concentrations of Na^+ (Fig. 3). These observations are consistent with the conclusion that a fraction of the intrinsic K^+ removed by the washing procedure competes with Na^+ for the K^+ -binding site in the control material.

Pretreatment of washed material with K^+ . In an attempt to relate the reactivation obtained by addition of K^+ to the reaction mixture to the quantity of K^+ rebound by the washed material, experiments were carried in which a washed preparation was pretreated with very low concentrations of K^+ for 30 min at 0°C . The amounts of K^+ chosen were such that if none were bound the final concentration of unbound K^+ in the reaction mixture after

Fig. 1. Effect of protein concentration on the release of cations from membrane fragments washed by the standard procedure. The protein concentration was: (a) 2.4mg/ml ; (b) 1.2mg/ml . $\circ$, Na^+ ; $\triangle$, K^+ ; $\square$, Mg^{2+} . Other details are given in the Methods section.

Fig. 2. Time-course of (a) the formation of bound phosphate and of (b) ATP hydrolysis in control unwashed preparation and after washing by the standard procedure. The basal reaction mixture contained 30mM -tris-HCl buffer, $\text{pH } 7.5$, $2.5\mu\text{M}$ -ATP, 50mM -NaCl and 0.5mg of protein in a final volume of 2.5ml . $\bullet$, Unwashed control preparation; $\circ$, washed preparation; $\triangle$, washed preparation with further addition of $2.5\mu\text{M}$ - MgCl_2 ; $\square$, washed preparation with further addition of $2.5\mu\text{M}$ - MgCl_2 and $2\mu\text{M}$ -KCl. Other details are given in the Methods section.

Table 2. *Reactivation of the washed membrane preparation by K^+*

The subfractionated preparation of Table 1 was washed by the standard procedure. The basal reaction mixture contained 50mM-NaCl and 2.5 μ M- $MgCl_2$. Pretreatment with K^+ was carried out at 0°C in a volume of 1 ml as described in the text. Different preparations were used in parts (a) and (b) of the table.

Membrane preparation	Pretreatment with K^+ (nmol/mg of protein)	K^+ present in reaction mixture (nmol/ml)	Bound phosphate (pmol/mg of protein)		ATP hydrolysed (%)	
			At 10s	At 20s	At 10s	At 20s
(a) Unwashed	None	0	148,146	112,118	55,55	95,96
Washed	None	0	148,148	144,142	35,35	68,69
Washed	None	2	150,146	116,114	38,41	75,74
Washed	None	5	146,148	116,116	41,43	80,80
Washed	None	10	124,126	100,100	40,44	84,86
(b) Unwashed	None	0	164,164	124,122	55,54	95,95
Washed	None	0	164,162	162,158	38,37	72,72
Washed	0.5	0.1	160,166	140,138	44,45	75,76
Washed	1.0	0.2	164,164	124,130	45,45	81,79
Washed	2.5	0.5	166,162	120,118	49,47	92,92

Fig. 3. Sensitivity to Na^+ concentration of (a) the formation of bound phosphate and of (b) ATP hydrolysis in the washed and unwashed preparation. The reaction mixture contained 2.5 μ M- $MgCl_2$. ●, Control preparation; ○, washed preparation. The reaction time was 10s. Other details are given in the Methods section.

dilution of the protein would be five- to ten-fold less than that required to reactivate the system when added directly to the reaction mixture. Pretreatment under these conditions was effective in restoring both the rate of dephosphorylation and ATP hydrolysis (Table 2b); some reactivation was even evident when the equivalent K^+ concentration in the reaction mixture was as low as 0.1 μ M. The greater efficacy of K^+ added in this fashion in restoring the ATPase rate without lowering the plateau concentration of bound phosphate than when added directly to the reaction mixture suggests that a time factor was involved in the binding of these very low amounts of K^+ .

It is noteworthy that although only 1–2 nmol of K^+ /mg of protein was required to reactivate the system some 20 nmol/mg was removed by the washing procedure. Clearly, therefore, only a small proportion of the K^+ bound in the unwashed material is available to the enzyme.

Further attempts to decrease the residual bound K^+ .

The treated material retained about 50% of its original Na^+ -ATPase activity, suggesting that it still contained sufficient intrinsic K^+ to activate the enzyme. It was not possible to decrease this residual K^+ by further washing with imidazole buffer; in fact, as shown in Fig. 1, minimum K^+ concentrations were reached in the washing procedure after the third wash. The washing procedure was therefore modified in a variety of ways as indicated in Table 3 and below. None of these modifications was successful in further diminishing the residual K^+ , although they did modify the properties of the preparation in other respects.

First we found that washing with a relatively high concentration of sodium chloride was no more effective in removing K^+ than washing with a Na^+ -free medium (a and b in Table 3). This suggests that the residual fraction is bound in a highly specific fashion that precludes displacement of any significant fraction of it by high concentration of Na^+ . Analysis of the cation contents of preparations

treated by the method of Nakao, Tashima, Nagano & Nakao (1965), a procedure involving exposure of the membrane fragments to a very high concentration of Na^+ (2M-sodium iodide) followed by washing with imidazole buffer, support this conclusion. The K^+ content of three different preparations treated in this way was 4–6 nmol/mg of protein, whereas the Na^+ content was increased to 100–140 nmol/mg of protein (R. Rodnight, D. Carrera & P. S. G. Goldfarb, unpublished work).

Secondly, we postulated that since ATP hydro-

lysis in the intact cell results in K^+ transport the intrinsic K^+ that is presumed to be activating the enzyme might be released to the medium from the membrane fragments during ATP hydrolysis. The preparation was therefore exposed to Na_2ATP either before washing or by substituting a solution containing ATP and sodium chloride for the third wash. The K^+ contents of preparations treated in this new way were either higher or the same as obtained by the standard washing procedure (*c* in Table 3). Enzymic properties in these ATP-treated preparations were also similar to those of the standard washed preparations, showing that the procedure had not removed a quantitatively significant fraction of the intrinsic K^+ bound at or near the site of enzyme action.

The membrane preparation is known from electron microscopy to contain vesicular structures probably formed by the curling up of membrane fragments on disruption of the cell. We therefore considered the possibility that a fraction of the intrinsic K^+ was occluded in such vesicles and therefore inaccessible to the washing reagents. If this were the case disruption of the membrane structure might be expected to release the occluded cation. However, two disrupting agents, sodium deoxycholate and urea, were ineffective in this regard; both treatments gave preparations containing about half the original bound K^+ content (*d* and *e* in Table 3). The deoxycholate treatment released about 20% of the enzymically inactive membrane protein in a soluble form, resulting in a raised plateau concentration of bound phosphate compared with the control value (Fig. 4). In other respects the treated material had similar properties

Table 3. Attempts to release bound K^+ from the membrane preparation by modified washing reagents.

The usual centrifugal washing procedure (see the Methods section) was followed, except that the composition of the third (or for *c* the first) wash was modified as follows: (*a*) 20 mM-NaCl in 4 mM-imidazole-HCl buffer, pH 7.2; (*b*) 1 mM-MgATP and 20 mM-NaCl in 4 mM-imidazole-HCl buffer, pH 7.2; (*c*) 10 mM- Na_2ATP in 4 mM-imidazole-HCl buffer, pH 7.2; (*d*) 0.1% sodium deoxycholate in 4 mM-imidazole-HCl buffer, pH 7.2; (*e*) 1 M-urea and 2.5 mM- Na_2ATP in 4 mM-imidazole-HCl buffer, pH 7.2.

Modification of treatment	Cation content (nmol/mg of protein)		
	Na^+	K^+	Mg^{2+}
(a)	44	8	5
(b)	98	18	12
(c)	36	4	5
(d)	70	12	2
(e)	72	12	4

Fig. 4. Time-course of (*a*) the formation of bound phosphate and of (*b*) ATP hydrolysis in a preparation washed with sodium deoxycholate (see *d* in Table 3). ●, Control preparation; ○, washed preparation; △, washed preparation with further addition of $2.5 \mu\text{M-MgCl}_2$; □, washed preparation with further addition of $2.5 \mu\text{M-MgCl}_2$ and $2 \mu\text{M-KCl}$. Other details are given in the Methods section.

to the standard washed preparation, except that the addition of Mg^{2+} increased the rate of ATP hydrolysis compared with the control material. The further addition of $2\mu M$ - K^+ restored the rate of dephosphorylation but again failed to stimulate hydrolysis beyond that given by Mg^{2+} . The urea treatment was applied in the presence of Na_2ATP to protect the enzyme system from denaturation (Cooper & McIlwain, 1967). The extents of formation of bound phosphate and ATP hydrolysis were similar to those observed in the standard washed preparation, but no reactivation occurred when Mg^{2+} and K^+ were added to the reaction mixture.

In several instances a clear correlation was evident between the amount of residual K^+ and the enzymic properties of the treated material. For example, the resin-treated preparation, which contained only 2nmol of K^+ /mg of protein as compared with 7nmol/mg in the standard washed preparation, was only slightly reactivated by the addition of $2\mu M$ -potassium chloride to the reaction mixture; also, in the type of reactivation experiment described in Table 2(b), full restoration of activity in this preparation required pretreatment with 1.5 nmol of K^+ /mg. Conversely, enzyme activities in the preparation treated by method (c) (Table 3), which for some unexplained reason retained 70% of the original bound K^+ , were fully restored by the addition of Mg^{2+} alone.

Binding of $^{42}K^+$ by the washed membrane preparation

The experiment of Table 2(b) suggested that the binding of K^+ by the membrane preparation was a time-dependent process. To investigate this process further some preliminary investigations on the binding of radioactive K^+ to the standard washed preparation were carried out. In these experiments the conditions under which $^{42}K^+$ binding was observed were initially chosen to resemble, as far as possible, those of the experiments of Table 2(b). However, it was not possible to obtain [^{42}K]potassium chloride of high enough specific radioactivity to study radioactive K^+ uptake below a concentration of $5\mu M$ -potassium chloride (equivalent to 5nmol of K^+ /mg of protein).

Time-course of $^{42}K^+$ binding. The use of the dilution and centrifugation technique described in the Methods section introduced some question as to the exact point at which radioactivity ceased to be incorporated into the sedimented material. To obtain an estimate of this, [^{42}K]potassium chloride at 10nmol/mg of protein was added to the system at a series of intervals before dilution and centrifugation, and the $^{42}K^+$ bound was plotted on an arbitrary time-scale of 30min (Fig. 5). By extrapolation it was found that the latest time at which

Fig. 5. Time-course of the binding of $^{42}K^+$ by the washed membrane preparation. ^{42}KCl (10nmol/mg of protein) was added at 20, 10, 5 and 0min before dilution. The uptake was measured in a volume of 1ml at $0^\circ C$. Other details are given in the Methods section. The points are the means of duplicates performed in the same experiment and the line drawn through them is the calculated regression line. At A the sample was diluted to 15ml; at B centrifugation was started.

some binding could still be taking place was approx. 2min after centrifugation started; binding was probably decreased to a very low rate by the dilution of the reaction mixture in view of the concentration-dependence of the uptake described below. Over the time-period before dilution the binding was linear and at the longest time-interval observed (27min) about 20% of the added $^{42}K^+$ had been bound.

Effect of Na^+ on $^{42}K^+$ binding. The washed preparation was exposed for 30min to a fixed amount of $^{42}K^+$ (10nmol/mg of protein) in the presence of three concentrations of Na^+ . With the two cations in equimolar concentrations there was virtually no competition for the K^+ -binding sites, the $^{42}K^+$ bound being only slightly lower than the value obtained in the absence of Na^+ (Fig. 6a). When the Na^+ concentration was increased 100-fold, $^{42}K^+$ binding was inhibited by about 50% and at 5000 nmol of Na^+ /mg of protein the K^+ binding was nearly abolished; complete inhibition required 6300nmol/mg. By re-plotting the results on a straight logarithmic scale (Fig. 6b) it is seen that the fall in K^+ binding became progressively larger as the Na^+ concentration was increased and that the decrease was especially steep above 1mM-sodium chloride. This is probably due to existence of Na^+ -specific sites in the membrane and only when these are saturated does competition with K^+ for the K^+ -binding sites become appreciable. A similar conclusion was reached from kinetic considerations by Kanazawa, Saito & Tonomura (1967).

Fig. 6. Inhibition of the binding of $^{42}K^+$ by added Na^+ . The ^{42}KCl concentration was 10 nmol/mg of protein; $^{42}K^+$ bound was measured after 30 min exposure at 0°C. The three concentrations of NaCl used were 50 mM, 1 mM and 10 μ M. Other details are given in the Methods section. Points are the means of duplicates performed in the same experiment. Results are plotted (a) versus $\log(1/[Na^+])$ and (b) versus $\log[Na^+]$.

Fig. 7. Effect of temperature on $^{42}K^+$ binding by the membrane preparation. Bound $^{42}K^+$ was measured after 30 min exposure in the concentration range 5–50 nmol of K^+ /mg of protein. $\circ$, 0°C; Δ , 20°C; $\square$, 37°C. Other details are given in the Methods section.

Effect of temperature on $^{42}K^+$ binding. The uptake was examined at 0, 20 and 37°C at K^+ concentrations in the range 5–50 nmol/mg of protein. There was a small decrease in the binding with increase in temperature at all concentrations (Fig. 7). These findings suggest an opening-out of the protein structure of the membrane as the temperature was raised, with the bound K^+ being more easily displaced by H^+ or H_3O^+ .

DISCUSSION

These results demonstrate that a proportion of the Na^+, Mg^{2+} -ATPase activity in membrane preparations is due to intrinsic bound K^+ . Further, since we were unable to obtain a preparation completely free of bound K^+ , it still remains possible that the whole of the activity is K^+ - as well as Na^+ -dependent. It is difficult to compare our results with those of Fujita *et al.* (1968) since they were measuring activities in a system with an ATP/protein ratio about a 1000-fold greater than that used here. Under these saturation conditions intrinsic cations might be expected to have a less obvious effect, and in fact the rate of Na^+ -dependent activity reported by these authors was only some 3% of the activity observed in the presence of optimum Na^+ and K^+ in the medium. Further, the long incubation times used to measure Na^+ -dependent activity would also have allowed time for traces of K^+ in the reaction mixture to be bound by the preparation. In our experience it is extremely difficult to prepare reagents completely free of K^+ , and the ability of membrane fragments to bind K^+ from very low concentrations is demonstrated in the present study. We are therefore inclined to the view that the minor activities described by Fujita *et al.* (1968) could be due to intrinsic cations, despite the very low contents of these that they claim for their preparation.

Nature of the intrinsic K^+ . The ease with which the major part of the intrinsic K^+ could be removed by washing or resin treatment suggests that this represents a fraction that is relatively loosely bound to anionic sites in the membrane fragments by charge neutralization. The binding of Na^+ and K^+ to rat liver and kidney microsomes was considered to involve this type of bond, the process being compared with ion-exchange on synthetic resins of the weakly acidic type (Sanui & Pace, 1959). Competition between different cations for the sites and a high affinity for H^+ was observed (Sanui & Pace, 1964). Some degree of specificity, as would be expected from an ion-exchange model, was also demonstrated: K^+ , for instance, was bound more strongly than Na^+ . However, cation binding to membrane fragments is almost certainly much more complex than suggested by the simple resin model; it seems reasonable to assume a range of anionic sites on the membrane exhibiting differing affinities and specificities for cations. If this is true K^+ may be bound in a number of ways, not all of which will necessarily involve sites concerned with membrane function. In the present work, the ability of 2 μ M-potassium chloride to reactivate the ATPase system in the presence of 50 mM-sodium chloride (Table 2a) demonstrates the presence of sites with a remarkably high affinity for K^+ . The Na^+/K^+

ratio in the reaction mixture in this experiment is 2.5×10^4 or nearly five times the Na^+/K^+ ratio that completely inhibited $^{42}K^+$ binding (Fig. 6). It therefore appears that the high-affinity sites are only a very small proportion, undetectable in this experiment, of the total number available to the cation.

With regard to the tightly bound residual fraction of the intrinsic K^+ , the following possibilities have to be considered. (a) That it is bound by charge neutralization but for physical reasons remains inaccessible to displacement by other positively charged ions. This possibility has to be considered very seriously in view of the insoluble nature of the preparation; inaccessibility might be due to occlusion, as already mentioned, or to the covering of binding sites by the hydrophobic groups of lipids. Moreover, the mild disrupting agents used in the present work have not finally settled the question, since extensive disruption of the membrane structure was avoided in the interests of retaining enzyme activity. However, the fact that neither treatment gave preparations with lower K^+ contents than the standard washed preparation suggest that this explanation is unlikely to account for more than a small fraction of the residual K^+ . (b) That the K^+ is bound to sites with an exceptionally high affinity, which precludes its displacement by mass action. The chelation mechanism proposed by Saroff (1957) and Lewis & Saroff (1957) for the binding of Na^+ and K^+ to myosin has a high association constant for K^+ , but our results at present are inadequate to support this suggestion one way or the other. (c) That it comprises K^+ bound to high-affinity sites that equilibrates in the final washes with traces of K^+ present in the reagents and vessels. This could well account for part though hardly all of the residual K^+ . Thus the lowest concentration ($0.5 \mu M$) of K^+ needed to reactivate the ATPase system is of the same order as found in the washing agents, but the ratio of bound K^+ in the final preparation to contaminating K^+ in the final wash fluid is still about 10:1. Even if it is assumed that the membrane can take up or exchange all the free K^+ to which it is exposed during the washing process it would still only account for less than one-half of the bound residual fraction.

We have no information at present on the nature of the anionic sites binding cations in the membrane, but it is reasonable to assume that lipids as well as proteins are concerned. A protein binding K^+ from a micro-organism has been described (Damadian, 1969).

Activation of ATP utilization by bound Mg^{2+} and K^+ . The results also show that a fraction of the intrinsic Mg^{2+} in the unwashed preparation is available to the Na^+, K^+, Mg^{2+} -ATPase system. Since free ATP is a good complexing agent for Mg^{2+}

(Kosower, 1962) there is no need to postulate that the intrinsic Mg^{2+} is bound at or near the ATPase system; the high affinity of ATP for Mg^{2+} should permit the transitory formation of free $MgATP$, which is generally accepted to be the immediate substrate for the enzyme. Other workers have observed the displacement of bound Mg^{2+} by ATP in membrane preparations from brain (Ahmed & Judah, 1966) and liver (Sanui & Pace, 1964).

With K^+ it must be assumed that the active fraction of the intrinsic cation is bound at a site accessible to the active centre of the enzyme, since in the intact cell the system initiating K^+ transport is on the outside of the cell and therefore separated from ATP. The question then arises as to how ATP hydrolysis can continue in a K^+ -free medium once the K^+ bound to the enzyme has been displaced from the system by the counterpart of the transport mechanism remaining in the cell membrane fragments. It seems unlikely that it is released to the reaction mixture as free K^+ and then immediately rebound, since little reactivation was found in the presence of less than $1.0 \mu M$ -potassium chloride, unless the preparation was preincubated with K^+ . It must be assumed therefore that it remains in some way associated with the membrane, probably in the vicinity of the enzyme sites. Possibly in the disrupted membrane, where true spatial separation of the Na^+ and K^+ sites does not exist, special factors operate that enable the enzyme system to recapture K^+ displaced by ATP hydrolysis before it escapes to the surrounding fluid. Such factors, whatever they are, could be of importance in controlling the entry of K^+ into the intact cell.

We are grateful to the Medical Research Council for a Scholarship to P.S.G.G.

REFERENCES

- Ahmed, K. & Judah, J. D. (1966). *Biochim. biophys. Acta*, **112**, 58.
- Cooper, J. R. & McIlwain, H. (1967). *Biochem. J.* **102**, 675.
- Damadian, R. (1969). *J. gen. Physiol.* **54**, 110S.
- Fujita, M., Nagano, K., Mizuno, N., Tashima, Y., Nakao, T. & Nakao, M. (1967). *J. Biochem., Tokyo*, **61**, 473.
- Fujita, M., Nagano, K., Mizuno, N., Tashima, Y., Nakao, T. & Nakao, M. (1968). *Biochem. J.* **106**, 113.
- Goldfarb, P. S. G. & Rodnight, R. (1968). *Biochem. J.* **111**, 16P.
- Hems, D. A. & Rodnight, R. (1966). *Biochem. J.* **101**, 516.
- Kanazawa, T., Saito, M. & Tonomura, Y. (1967). *J. Biochem., Tokyo*, **61**, 555.
- Kosower, E. N. (1962). *Molecular Biochemistry*, p. 268. New York: McGraw-Hill Book Co.
- Lewis, M. S. & Saroff, H. A. (1957). *J. Am. chem. Soc.* **79**, 2112.
- Miller, G. L. (1959). *Analyt. Chem.* **31**, 964.

- Nakao, T., Tashima, Y., Nagano, K. & Nakao, M. (1965). *Biochem. biophys. Res. Commun.* **19**, 755.
- Rodnight, R. (1970). *Biochem. J.* **120**, 1.
- Rodnight, R., Carrera, D. & Goldfarb, P. S. G. (1969). *Abstr. 2nd int. Meet. International Society for Neurochemistry*, p. 345. Milan: Tamburini Editore.
- Rodnight, R., Hems, D. A. & Lavin, B. E. (1966). *Biochem. J.* **101**, 502.
- Saroff, H. A. (1957). *Archs Biochem. Biophys.* **71**, 194.
- Sanui, H. & Pace, N. (1959). *J. gen. Physiol.* **42**, 1325.
- Sanui, H. & Pace, N. (1964). *J. cell. comp. Physiol.* **65**, 27.
- Skou, J. C. (1962). *Biochim. biophys. Acta*, **58**, 314.
- Skou, J. C. (1965). *Physiol. Rev.* **45**, 596.
- Skou, J. C. & Hilberg, C. (1969). *Biochim. biophys. Acta*, **185**, 198.