Comparison of some Minor Activities Accompanying a Preparation of Sodium-plus-Potassium Ion-Stimulated Adenosine Triphosphatase from Pig Brain

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1. An ATPase (adenosine triphosphatase) preparation obtained from pig brain microsomes by treatment with sodium iodide showed four apparently different ouabain-sensitive activities under various conditions. They were (a) ouabain-sensitive Mg^{2+} -stimulated ATPase, (b) K^+ -stimulated ATPase, (c) (Na^+, K^+) -stimulated ATPase and (d) Na^+ -stimulated ATPase activities. 2. These activities showed the same substrate specificity, ATP being preferentially hydrolysed and CTP slightly. AMP was not hydrolysed. 3. These activities were inhibited by low concentration of ouabain. The concentration producing 50% inhibition was $0.1 \mu M$ for ouabain-sensitive Mg^{2+} -stimulated ATPase, $0.2 \mu M$ for K^+ -stimulated ATPase, $0.1 \mu M$ for (Na^+, K^+) -stimulated ATPase and $0.003 \mu M$ for Na^+ -stimulated ATPase activity. 4. The ouabain-sensitive ATPase activities were inactivated by *N*-ethylmaleimide but the insensitive ATPase activity was not. 5. The three ouabain-sensitive ATPase activities were inhibited about 50% by $1 mM$ - Ca^{2+} , whereas the ouabain-sensitive Mg^{2+} -stimulated ATPase activity was activated by the same concentration of Ca^{2+} . The preparation was treated with ultrasonics at 20 kcy./sec. The 2 min. ultrasonic treatment inactivated the ATPase activities by 50%. 7. The temperature coefficient Q_{10} was 6.6 for K^+ -stimulated ATPase activity, 3.7 for (Na^+, K^+) -stimulated ATPase and 2.6 for Na^+ -stimulated ATPase. 8. Organic solvents inactivated the ATPase activities, to which treatment the K^+ -stimulated ATPase was the most resistant. 9. The phosphorylation of the enzyme preparation became less dependent on Na^+ with decreasing pH. This Na^+ -independent phosphorylation at low pH was sensitive to K^+ and hydroxylamine as well as the Na^+ -dependent phosphorylation at neutral pH.

It has been reported by Fujita, Nagano, Mizuno, Nakao & Nakao (1967) that, besides an ATPase* activity that requires the simultaneous addition of Na^+ and K^+ , three other ouabain-sensitive ATPase activities are detected in a partially purified (Na^+, K^+) -ATPase preparation when suitable assay conditions are selected. They are (a) ouabain-sensitive (Mg^{2+}) -ATPase, (b) (K^+) -ATPase and (c) (Na^+) -ATPase activities.

Despite differences in pH-activity relationships among these activities, the fact that they occur in a partially purified enzyme preparation suggested that they might be different expressions of a single enzyme system. In the present work effects of inhibitors such as ouabain, *N*-ethylmaleimide, alcohols and Ca^{2+} and of some other factors

including ultrasonic treatment and incubation temperature were examined in some detail. Effects of pH and Na^+ concentration on the phosphorylation of the ATPase preparation by $[\gamma\text{-}^{32}P]\text{ATP}$ were also examined.

METHODS

ATPase preparation. The enzyme preparation was obtained by NaI treatment of a microsomal fraction (Nakao, Tashima, Nagano & Nakao, 1965) from pig brain by using the minor modification described by Fujita *et al.* (1967).

ATPase activities. (Mg^{2+}) -ATPase activity (column A, Table 1) was measured with a reaction mixture containing (final concentrations): $0.1 M$ -buffer, $0.5 mM$ -tris-EDTA, $5 mM$ - $MgCl_2$, $3 mM$ -tris-ATP (pH adjusted to that of the buffer in use) and about $250 \mu g.$ of protein of the enzyme preparation, in a total volume of $1.0 ml.$ This mixture is referred to below as ' (Mg^{2+}) -ATPase assay mixture'. Incubations were carried out at 37° for 30 min. The mixture

* Abbreviations: ATPase, adenosine triphosphatase; (Na^+, K^+) -ATPase etc., Na^+ + K^+ -stimulated adenosine triphosphatase etc.

Table 1. *Ouabain-sensitive (Mg²⁺)-ATPase, (K⁺)-ATPase and (Na⁺)-ATPase activities*

The reaction mixture contained (final concentrations): 0.1 M-buffer (tris-acetic acid for pH 5.3, tris-maleic acid for pH 6.0 and tris-HCl for pH 7.6 and 9.0), 0.5 mM-EDTA, 5 mM-MgCl₂, 200 μ g. of protein of the enzyme preparation and when indicated 0.1 mM-ouabain, 1 mM-KCl or 100 mM-NaCl, in a total volume of 1.0 ml. The incubation was carried out at 37° for 30 min. Designation of the activities: A-B, ouabain-sensitive (Mg²⁺)-ATPase activity; C-A, (K⁺)-ATPase activity; D-A, (Na⁺)-ATPase activity. The specific activity in the presence of 14 mM-KCl and 140 mM-NaCl at pH 7.6 was 95 μ moles of P_i liberated/mg. of protein/hr.

pH	Further addition ...	P _i liberated (μ moles/mg. of protein/hr.)			
		A	B	C	D
	None		Ouabain	KCl	NaCl
5.3		2.0	0.8	3.3	2.0
6.0		1.9	0.7	2.2	2.3
7.6		1.8	1.6	1.8	3.7
9.0		1.5	1.5	1.5	3.3

was deproteinized and the liberated P_i was assayed as described by Fujita *et al.* (1967).

The 'ouabain-sensitive (Mg²⁺)-ATPase activity' was the difference between the activities measured at pH 6 with the (Mg²⁺)-ATPase assay mixture in the absence (column A, Table 1) and in the presence (column B, Table 1) of 0.1 mM-ouabain. The optimum pH was at 6.0 (Fujita *et al.* 1967).

'(K⁺)-ATPase activity' was defined as the activity increment over that of (Mg²⁺)-ATPase when 1 mM-KCl was incorporated into the (Mg²⁺)-ATPase assay mixture buffered at pH 5.3, which was the optimum pH of this activity.

'(Na⁺,K⁺)-ATPase activity' was assayed, as stated by Nakao *et al.* (1965), in the presence of 14 mM-KCl and 140 mM-NaCl added to the (Mg²⁺)-ATPase assay mixture at pH 7.7 and with 10 μ g. of protein of the enzyme preparation.

'(Na⁺)-ATPase activity' was measured in the (Mg²⁺)-ATPase assay mixture containing 0.1 M-NaCl (column D—column A, Table 1). The optimum pH was between 7.6 and 9.0.

The time-course of the reactions for the four ATPase activities was linear over a period of 1 hr. at 37°.

Buffers, tris-ATP and [γ -³²P]ATP. N-Acetic acid was adjusted with m-tris to pH 4.9 and 5.3. m-Maleic acid was adjusted with m-tris to pH 6.0. m-Tris was adjusted with n-HCl to pH 7.7 and 9.0. Disodium ATP (100 mM) was desalted by passage through a Dowex 50 column (H⁺ form) and adjusted with m-tris to the pH of the buffer in the reaction mixture. The final concentration of tris-ATP was adjusted to 30 mM. [γ -³²P]ATP was prepared according to the method described by Glynn & Chappell (1964).

Other procedures. These are described in the legends to the Tables and Figures.

RESULTS

Effect of Na⁺/K⁺ ratio on pH-activity curves. It was reported by Fujita *et al.* (1967) that Mg²⁺-

Fig. 1. Effect of high Na⁺/K⁺ ratio on pH-activity curves. The ATPase preparation (240 μ g. of protein) was incubated with 0.1 M buffer (tris-acetic acid for pH 5.0–5.5; tris-maleic acid for pH 5.5–7.0; tris-HCl for pH 7.0–9.0), 0.5 mM-tris-EDTA, 5 mM-MgCl₂, 10 mM-NaCl and 3 mM-tris-ATP (pH adjusted to that of the buffer in use). ●, 50 μ M-KCl; □, 35 μ M-KCl; △, 10 μ M-KCl; ○, no KCl added. The (Mg²⁺)-ATPase activity was subtracted from the total activity.

dependent hydrolysis of ATP by the sodium iodide-treated enzyme preparation was accelerated by the addition of Na⁺ alone above pH 7, whereas K⁺ stimulated hydrolysis in the pH range below neutrality. In the present work the effect on the pH-activity curve of varying the Na⁺/K⁺ ratio in reaction mixtures containing fixed concentrations of added Mg²⁺ was examined. (Mg²⁺)-ATPase activity at each indicated pH was subtracted from the total activity at that pH. Stepwise decreases in K⁺ concentration in a reaction mixture containing Mg²⁺, Na⁺ (fixed at 10 mM) and K⁺ resulted in stepwise decreases in the ATPase activity (Fig. 1). The decrease was greater at neutral than at alkaline pH. When the concentration of added K⁺ was lowered below 10 μ M at a fixed Na⁺ concentration of 10 mM, further removal of K⁺ showed only slight effect on the activity, the pH-activity curve converging to a final pattern. Thus, at an added Na⁺/K⁺ ratio greater than 10³:1, univalent cation-activated ATPase retained its activity mainly in a pH range from neutrality to alkalinity.

The pH-activity curve in the presence of 10 mM-

Fig. 2. Effect of relatively low Na⁺/K⁺ ratio on pH-activity curves. The ATPase preparation (240 μg. of protein) was incubated with 3mm-tris-ATP as described in the legend to Fig. 1 except that the concentration of K⁺ was fixed at 1mm, whereas that of Na⁺ was varied as indicated: ▲, 1.2mm-NaCl; ●, 1.0mm-NaCl; □, 0.8mm-NaCl; △, 0.5mm-NaCl; ○, with or without 0.1mm-NaCl added. ■—■, Activity (right-hand ordinate) in the presence of 14mm-KCl and 140mm-NaCl, i.e. the optimum condition for the (Na⁺,K⁺)-ATPase activity of the present preparation, assayed with 12 μg. of protein. The activity in the absence of both Na⁺ and K⁺ was subtracted from the total activity.

sodium chloride alone was monophasic with a shoulder at pH 7.3; this became a definite peak different from that at pH 8.0, with a trough appearing between them, when the concentration of added K⁺ was 35 μM and the Na⁺/K⁺ ratio was 290:1 (Fig. 1). The pH-activity curve in the presence of 14mm-potassium chloride and 140mm-sodium chloride, the optimum condition for the (Na⁺,K⁺)-ATPase activity of the sodium iodide-treated preparation, showed no such depression at any neutral pH and a single pH optimum at 7.6 (broken line in Fig. 2).

Gradual removal of Na⁺, on the other hand, from a reaction mixture containing Mg²⁺, Na⁺ and K⁺ (fixed at 1mm) also resulted in activity decrease (Fig. 2). The activity reached a minimum at a Na⁺ concentration lower than 0.1mm or at a Na⁺/K⁺ ratio smaller than 1:10, but under these conditions activity was only seen below pH 7.

Substrate specificity. It was reported by Nakao *et al.* (1965) that an (Na⁺,K⁺)-ATPase preparation

from rabbit brain hydrolysed ATP preferentially, CTP slightly, but none of the other nucleoside triphosphates. In the present experiment the substrate specificity of the four ouabain-sensitive ATPase activities was examined (Table 2). They are in agreement with those reported in the above paper for (Na⁺,K⁺)-ATPase activity of an enzyme prepared from rabbit brain by treatment with sodium iodide. There are also no gross discrepancies among the four activities. However, the present preparation from pig brain seems to be contaminated by adenylate kinase, probably owing to the larger scale of the preparative procedure. The preparation obtained from rabbit brain by a similar method, but on a smaller scale with a glass-Teflon homogenizer, showed no activity with ADP (Nakao *et al.* 1965). It was also noticed that AMP and β-glycerophosphate were not hydrolysed to any extent. The hydrolysis of *p*-nitrophenyl phosphate in the absence of univalent cations was detected largely at low pH. A univalent cation-activated hydrolysis of this compound was detected at higher pH. The cation-activated phosphatase activity requires a concentration of K⁺ or Na⁺ higher than 10mm or 100mm respectively (Fujita *et al.* 1966; Nagai, Izumi & Yoshida, 1966; Albers & Kovals, 1966; Bader, Sen & Post, 1966).

Effect of ouabain. It is well known that the cardiac glycoside ouabain inhibits cation transport in intact cells and (Na⁺,K⁺)-ATPase activity of subcellular preparations from various sources at low concentration. In the previous work (Fujita *et al.* 1967) it was demonstrated that a ouabain-sensitive (Mg²⁺)-ATPase activity was detectable at relatively low pH. In that work, however, the effect of the inhibitor at low concentration was examined only at 0.1mm. Here the inhibition was investigated in more detail by varying the concentration of the inhibitor (Table 3). The data confirm that part of (Mg²⁺)-ATPase activity at low pH (6.0 in this case) is highly sensitive to the glycoside. The inhibition seemed to reach a maximum at the ouabain concentration of 0.1mm and 50% inhibition at about 0.1 μM. Knowledge of the contents of any contaminating Na⁺ and K⁺ in the present preparation is important in the interpretation of the ouabain-sensitive (Mg²⁺)-ATPase activity. To investigate this, the enzyme preparation (protein content 190mg.) was first dried by heating in a Pyrex Kjeldahl flask and 1ml. of concentrated nitric acid was added at intervals of 1hr. until no dark substances remained. The flask was rinsed thoroughly with 2ml. of water. A portion (0.8ml.) was taken for measurement of sodium content with a Na⁺-sensitive glass electrode (Hitachi-Horiba Seisakusho, Tokyo, Japan) connected to a pH-meter and recorder. The total volume of the solution under measurement was 2ml., containing 0.8ml. of

Table 2. *Substrate specificity under various conditions*

The enzyme preparation was incubated with each substrate at 3mM concentration (desalted and neutralized with tris base) in the (Mg²⁺)-ATPase assay mixture described in the Methods section with the exception that ATP was omitted when other substrates were tested and indicated amounts of Na⁺ or K⁺ or both were added in the form of NaCl and KCl. The (Na⁺,K⁺)-ATPase activity at pH 7.7 was assayed with the amount of the enzyme one-twentieth of that used for the other activities. The values in this column therefore, when they are below 5, should not be taken to be as accurate as those in the other columns. *p*-NPP, *p*-nitrophenyl phosphate; β -GlyP, β -glycerophosphate.

Substrate (3mM)	Further addition ...	P _i liberated (μ moles/mg. of protein/hr.)							
		pH 6.0		pH 5.3		pH 7.7		pH 9.0	
		Ouabain				KCl (14mM) +		NaCl	
		None	(0.1mM)	KCl (1.0mM)	None	NaCl (140mM)	None	(140mM)	None
ATP		2.5	1.4	4.0	2.7	69	2.6	5.3	2.2
CTP		1.7	1.0	1.8	1.8	16	2.3	3.9	2.4
ITP		1.7	1.7	1.8	1.7	5	2.7	3.6	2.6
GTP		1.2	1.3	1.5	1.4	(4)	1.8	2.0	1.7
UTP		0.8	0.8	0.9	0.9	(3)	1.4	1.9	1.4
ADP		1.2	1.0	1.6	1.6	15	2.1	3.0	1.9
AMP		0.1	0.1	0.1	0.1	(1)	0.1	0.1	0.1
<i>p</i> -NPP		2.6	2.5	4.3	4.3	(1)	0.4	2.7	1.5
β -GlyP		0.3	0.3	0.2	0.2	(3)	0.1	0.1	0.1

Table 3. *Effect of various concentrations of ouabain on the ATPase activities*

The ATPase preparation was incubated in the (Mg²⁺)-ATPase assay mixture (see the Methods section) with and without NaCl, KCl and ouabain as indicated in the Table.

-log (Concn. of ouabain)	Further addition ...	P _i liberated (μ moles/mg. of protein/hr.)							
		pH 6.0		pH 5.3		pH 7.7		pH 9.0	
						KCl (14mM) +		NaCl (100mM)	
		None	KCl (1.0mM)	None	None	NaCl (140mM)	None	NaCl (100mM)	None
No addition		2.0	2.8	1.8		87	—	3.9	1.6
9.0		2.1	2.9	1.8		95	—	2.9	1.5
8.0		—	2.3	1.6		72	—	2.4	1.4
7.0		1.3	2.0	1.4		58	—	1.7	1.5
6.0		1.1	1.4	1.2		9.2	—	1.5	1.4
5.0		0.9	1.1	1.1		1.6	1.6	1.4	1.4
4.0		0.9	1.0	0.9		1.6	1.6	1.4	1.4

sample and 10mM-tris-hydrochloric acid buffer, pH 7.7. Another 1ml. portion of the digest was diluted to 5ml. with water, and potassium concentration measured with a flame photometer (Advanced Instruments Inc., Cambridge, Mass., U.S.A.). These measurements gave a value 0.5 μ g. atom/g. of protein for the content of both sodium and potassium.

The (Mg²⁺)-ATPase was sensitive to ouabain at pH 5.3 as it is at pH 6.0. (K⁺)-ATPase activity was also inhibited by ouabain, the concentration required for half-maximum inhibition being 0.2 μ M. (Na⁺,K⁺)-ATPase activity at pH 7.7 required 0.1 μ M-ouabain for half-maximum inhibition. Of the four activities the most sensitive to ouabain is

the (Na⁺)-ATPase activity at pH 9.0. The half-maximum inhibition concentration of the inhibitor for activity at this pH was 0.003 μ M.

These four ouabain-sensitive ATPase activities showed a general parallelism in their inhibition curves. However, a difference in the extent of inhibition between them was not negligible in that (K⁺)-ATPase activity was the most resistant, and (Na⁺)-ATPase the most sensitive, to ouabain. Their *K_i* values differed 150-fold.

Effect of N-ethylmaleimide treatment. It was first shown by Skou (1963) that (Na⁺,K⁺)-ATPase was inactivated by *N*-ethylmaleimide. The K⁺-activated *p*-nitrophenyl phosphatase activity accompanying the (Na⁺,K⁺)-ATPase

Table 4. *Effect of N-ethylmaleimide on ATPase activities*

The enzyme preparation (3.6mg. of protein) was incubated with 0.1M-tris-HCl buffer, pH7.7, and various concentrations of *N*-ethylmaleimide, in a total volume of 1.5ml. at 37° for 30min. The mixtures were then chilled in an ice-water bath and a 0.1 ml. sample of each mixture was taken for the ATPase assay. The assay was carried out with the (Mg²⁺)-ATPase assay mixture (see the Methods section) with the exception that NaCl, KCl or ouabain was added as indicated.

-log (Concn. of <i>N</i> -ethylmaleimide)	Further addition	...	P _i liberated (μmoles/mg. of protein/hr.)						
			pH 6.0		pH 5.3		pH 7.7	pH 9.0	
			None	Ouabain (0.1 mM)	KCl (1.0 mM)	None	KCl (14 mM) + NaCl (140 mM)	NaCl (100 mM)	None
No addition			1.8	0.8	3.3	2.0	80	2.7	1.0
5.0			1.9	1.0	3.5	2.1	84	2.4	0.9
4.0			1.4	0.9	2.6	1.6	15	1.0	0.8
3.0			0.8	0.8	1.4	1.1	1.5	0.9	0.9
2.0			1.0	0.9	1.2	1.4	1.1	0.7	0.8

Table 5. *Effect of Ca²⁺ on the ouabain-sensitive ATPase activities*

Each ATPase activity was assayed as described in the Methods section except that CaCl₂ was added to the indicated final concentrations when indicated.

Concn. of Ca ²⁺ ...	...	P _i liberated (μmoles/mg. of protein/hr.)		
		1 mM	5 mM	10 mM
Control	1.05	1.55	1.22	0.85
Ouabain-sensitive (Mg ²⁺)-ATPase	1.25	0.75	0.18	—
(K ⁺)-ATPase	87	49	12.3	—
(Na ⁺ ,K ⁺)-ATPase	2.55	1.1	0.4	—

preparation was also shown to be sensitive to *N*-ethylmaleimide (Fujita *et al.* 1966). The effect of *N*-ethylmaleimide treatment on the ouabain-sensitive (Mg²⁺)-ATPase, (K⁺)-ATPase and (Na⁺)-ATPase activities as well as (Na⁺,K⁺)-ATPase activity is shown in Table 4. It was found that the four ATPase activities were all sensitive to the reagent, the inhibition being almost complete with 1mM-*N*-ethylmaleimide, whereas ouabain-insensitive activity was not inactivated by *N*-ethylmaleimide.

Effect of Ca²⁺. It is known that (Na⁺,K⁺)-ATPase activity is strongly inhibited by relatively low concentrations of Ca²⁺ (Skou, 1957). The effect of Ca²⁺ on the four ouabain-sensitive ATPase activities is presented in Table 5. As expected, (K⁺)-ATPase, (Na⁺,K⁺)-ATPase and (Na⁺)-ATPase activities were strongly inhibited by Ca²⁺. At 1mM-Ca²⁺ concentration activities were inhibited by 40, 44 and 57% respectively; 5mM-Ca²⁺ inhibited about 85%. However, the ouabain-sensitive

Table 6. *Effect of ultrasonic treatment on ATPase activities*

The enzyme preparation (3.9mg. of protein) was suspended in 1.5ml. of water. The tip of a 20kcyc./sec. Sonicator (type 4120; Kaijo-denki Co. Ltd., Tokyo, Japan) was immersed in the suspension in a plastic centrifuge tube (internal diam. 14 mm.) cooled in ice-water. The ultrasonic treatment was performed for the indicated time with a 1min. break interposed every 2min. Of the suspension, 0.1ml. was taken for assay of each ATPase activity. The assay was carried out as described in the Methods section.

Time of ultrasonic treatment ...	...	P _i liberated (μmoles/mg. of protein/hr.)			
		Control	2 min.	5 min.	15 min.
Ouabain-sensitive (Mg ²⁺)-ATPase	0.72	0.32	0.20	0.18	
(K ⁺)-ATPase	0.78	0.45	0.30	0.15	
(Na ⁺ ,K ⁺)-ATPase	67	32	19	8	
(Na ⁺)-ATPase	1.25	0.75	0.48	0.20	

(Mg²⁺)-ATPase showed an unexpected behaviour against Ca²⁺. As shown in Table 5, it was not inhibited by 1mM- and 5mM-Ca²⁺; on the contrary, it was definitely activated by 1mM-Ca²⁺; 10mM-Ca²⁺ inhibited it by 20%.

Effect of ultrasonic treatment. To determine whether or not the four ouabain-sensitive ATPase activities were susceptible to the same degree of ultrasonic treatment, the preparation was exposed to ultrasonics (20kcyc./sec.) for 2, 5 and 15min. The results are presented in Table 6. All four activities were decreased to half the control values after 2min. ultrasonic treatment. However, there were no gross discrepancies among the four activities with regard to the inactivation.

Effect of incubation temperature. It has been reported (Ahmed & Judah, 1965; Nagano *et al.*

1967) that the stimulatory effect of K^+ on (Na^+, K^+) -ATPase activity was diminished more rapidly than that of Na^+ as the incubation temperature was lowered. The four ouabain-sensitive ATPase activities were therefore assayed at 37° , 27° and 17° (Table 7). The ratio of the activity at 37° to that at 27° was 6.0 for the ouabain-sensitive (Mg^{2+}) -ATPase, 6.6 for (K^+) -ATPase, 3.7 for (Na^+, K^+) -ATPase and 2.6 for (Na^+) -ATPase activity.

Table 7. *Temperature-dependence of ATPase activities*

Each ATPase activity was assayed as described in the Methods section with the exception of the variations of incubation temperature indicated in the Table.

Incubation temp. ...	P_i liberated (μ moles/mg. of protein/hr.)		
	37°	27°	17°
Ouabain-sensitive (Mg^{2+}) -ATPase	0.90	0.15	0.02
(K^+) -ATPase	1.18	0.18	0.02
(Na^+, K^+) -ATPase	87	23	4.7
(Na^+) -ATPase	2.82	1.00	0.35

Table 8. *Effect of various concentrations of ethanol on the ATPase activities*

Each ATPase activity was measured as described in the Methods section except that the indicated concentrations of ethanol were added to the reaction mixture. The reaction was started by the addition of the enzyme preparation.

Concn. of ethanol ...	Control	P_i (μ moles/mg. of protein/hr.)		
		1%	5%	10%
Ouabain-sensitive (Mg^{2+}) -ATPase	1.22	0.85	0.55	0.25
(K^+) -ATPase	1.82	1.68	1.30	0.42
(Na^+, K^+) -ATPase	91	94	36	17
(Na^+) -ATPase	2.62	1.88	0.22	0.08

Effect of alcohols. It is well known that organic solvents inhibit (Na^+, K^+) -ATPase activity. The effect of ethanol on the four ATPase activities was investigated at several concentrations of the alcohol (Table 8). Ethanol was added to the reaction mixture and the reaction was started by the addition of the enzyme. It was found that all four ATPase activities were inhibited by ethanol. However, there were slight but definite differences in the inhibition of the four activities. When the alcohol was added to the reaction mixture to a final concentration of 5%, the inhibitions produced were 29% for (K^+) -ATPase, 60% for (Na^+, K^+) -ATPase, 67% for ouabain-sensitive (Mg^{2+}) -ATPase and 91% for (Na^+) -ATPase activities. This order of sensitivity to ethanol was observed with several other alcohols, as is evident from the results of the experiment presented in Table 9, in which effects of 5% ethanol, 2% propanol, 0.5% butanol and 5% acetone were compared. It was also noticed that, as the number of alkyl carbon atoms of an alcohol increased, the inhibitory effect of the alcohol also increased. As expected, alcohols are not the sole group of organic solvents that inhibit the (Na^+, K^+) -ATPase. Acetone also inhibited the activities to similar extents. The (Na^+, K^+) -ATPase was extremely sensitive to sodium dodecyl sulphate (detailed results not presented); 0.005% in final concentration completely inhibited the activity.

Phosphorylation of the enzyme preparation with $[^{32}P]ATP$. The dependence on Na^+ of ^{32}P incorporation by the enzyme preparation from $[\gamma\text{-}^{32}P]ATP$ was examined at various pH values (Fig. 3). The values in Fig. 3 represent the total radioactivity of the phosphorylated enzyme preparation. The maximum incorporation was obtained at 100–140 mM- Na^+ at pH 8.1 and Na^+ -independent incorporation was 13% of the total. This value is decreased to 8% if the incorporation at zero time is subtracted from the total incorporation. At pH 7.7 the incorporation curve was similar, but a higher Na^+ concentration (400 mM) was required for a maximum incorporation. At pH 4.9 the pattern of stimulation was different;

Table 9. *Effect of alcohols and acetone on the ATPase activities*

Each ATPase activity was assayed as described in the Methods section with the exception that ethanol, propanol, butanol or acetone was added to the reaction mixture at the indicated concentrations. The reaction was started by the addition of the enzyme preparation.

	P_i liberated (μ moles/mg. of protein/hr.)				
	Control	Ethanol (5%)	Propanol (2%)	Butanol (0.5%)	Acetone (5%)
Ouabain-sensitive (Mg^{2+}) -ATPase	1.12	0.38	0.50	0.72	0.70
(K^+) -ATPase	1.32	1.20	0.80	1.02	1.25
(Na^+, K^+) -ATPase	84	44	37	64	53
(Na^+) -ATPase	2.78	0.50	0.40	0.52	0.60

Fig. 3. Effect of Na⁺ concentration on the phosphorylation of the enzyme at various pH values. The phosphorylation of the enzyme was performed by essentially the same method as described by Nagano *et al.* (1965). The reaction mixture contained (final concentrations): 50 mM-buffer, pH as indicated in the Figure (tris-acetic acid for the reaction at pH 4.9, □; tris-maleic acid for pH 6.0, ●; tris-HCl for pH 7.7, ○, and pH 8.1, △), 5 mM-MgCl₂, 0.5 mM-tris-EDTA, 1.0 mg. of protein of the enzyme preparation and 20 μl. of 1.4 mM- γ -³²P]ATP (1.4 × 10⁵ counts/min.) prepared according to the method of Glynn & Chappell (1964), in a total volume of 1.0 ml. The reaction was started by the addition of [³²P]ATP into the reaction mixture pre-cooled in an ice-water bath and stopped by the rapid injection of 10 ml. of cold 0.5 N-HClO₄ after 15 sec. incubation with continuous shaking. The tubes were centrifuged in a swinging-bucket-type rotor at 1500 g for 15 min. under refrigeration. The supernatant was discarded as perfectly as possible: remaining drops were removed with a piece of blotting paper. Fresh cold 0.5 N-HClO₄ containing 5 mM-P_i was added and the pellet was dispersed as thoroughly as possible with a mechanical vibrator. This washing procedure by centrifugation and resuspension was repeated four times. The pellet was finally suspended in about 1 ml. of water and transferred to a planchet and dried. The planchet was counted under a Geiger-Müller tube.

the level of Na⁺-insensitive incorporation was increased over the corresponding incorporation at pH 7.7 and Na⁺ had no stimulatory effect as far as 0.1 M.

Fig. 4 shows the pH-dependence of ³²P incorporation at different Na⁺ concentrations. At the Na⁺ concentration of 140 mM a maximum incorporation was found at about pH 8.0 and there was a shoulder at pH 5. The curve at other concentrations of Na⁺ were plotted from the data of

Fig. 4. Effect of pH on the phosphorylation of the enzyme at various concentrations of Na⁺. The data were taken from Fig. 3 except the experiment at the Na⁺ concentration of 140 mM (○). Other Na⁺ concentrations were: 0 mM (●); 0.3 mM (△); 1.0 mM (□); 3.0 mM (▲); 10 mM (■); 100 mM (▽).

Fig. 3. They show that, at pH values lower than 6, Na⁺-dependence of ³²P incorporation was much diminished.

It has been reported by several authors that the bulk of the ³²P incorporated by an ATPase preparation was releasable as P_i by hydroxylamine treatment (Nagano *et al.* 1965; Hokin, Sastry, Galsworthy & Yoda, 1965; Bader *et al.* 1966). To exclude the phosphorylation due to enzyme(s) other than the ouabain-sensitive ATPase, the enzyme preparation phosphorylated under various conditions was treated with hydroxylamine (Table 10).

In agreement with the data based on the total ³²P incorporation (Figs. 3 and 4) the preparations phosphorylated in the absence of Na⁺ released larger amounts of [³²P]P_i as the pH of the incubation was lowered, whereas those dependent on Na⁺ released greater amounts of [³²P]P_i as the incubation pH was raised.

The inclusion of K⁺ in the phosphorylation system

markedly diminished the Na^+ -dependent incorporation of ^{32}P at pH 7.7 (Nagano *et al.* 1967). In the present work it was found that K^+ inhibited the Na^+ -independent incorporation at pH 4.9 and that at pH 6.0 (Tables 10 and 11).

It seemed possible that the decreased ^{32}P incorporation in the presence of Na^+ at lower pH values might be due to incomplete phosphorylation

even after 15 sec. of incubation. The experiment in Table 11 showed that a maximum phosphorylation at pH 6.0 was attained in 15 sec., which was the incubation time for the experiments of Figs. 3 and 4 and Table 10. In a complete system including Na^+ and K^+ , phosphorylation reaches a saturation level within 0.5 sec. even at 0° (Nagano *et al.* 1967).

DISCUSSION

The identity or non-identity of the four ouabain-sensitive ATPase activities, discriminated on the basis of the various cations added to the reaction mixture, is discussed below. With regard to the cation conditions and the interpretation of the ouabain-sensitive (Mg^{2+})-ATPase activity, knowledge of the contents of any contaminating Na^+ and K^+ in the present preparation is important. As can be calculated from the data presented in the text, a reaction mixture containing 200 μg . of protein of the enzyme preparation in a total volume of 1 ml. gives the value of 0.1 μM as the final concentration of each of the endogenous sodium and potassium. However, low concentrations of Na^+ and K^+ of this order did not cause any detectable ATP hydrolysis dependent on both Na^+ and K^+ at pH 7.7. It was shown previously (Fujita *et al.* 1967) that the (K^+)-ATPase activity was first activated to a detectable extent when the concentration of K^+ was 100 μM , or 1000 times that due to the endogenous K^+ ; similarly the (Na^+)-ATPase activity was first detected when the concentration of added Na^+ was 1 mM, or 10000 times that of the endogenous Na^+ . These findings make it difficult to explain the ouabain-sensitive (Mg^{2+})-ATPase activity as due to contaminating Na^+ and K^+ . The effect of Ca^{2+} also suggests that this activity is a different entity; 1 mM- Ca^{2+} stimulated it, whereas the same concentration of Ca^{2+} inhibited the (K^+)-, (Na^+)- and (Na^+ , K^+)-ATPase activities. It is considered that further discussion of the ouabain-sensitive (Mg^{2+})-ATPase activity is not appropriate here.

The substrate-specificity, high sensitivity to ouabain, inactivation by *N*-ethylmaleimide or ultrasonics and inhibition by Ca^{2+} support a possible identity of the (K^+)-ATPase, (Na^+ , K^+)-

Table 10. *Effect of pH and cations on the enzyme phosphorylation (data based on the measurement of the hydroxylamine-sensitive phosphate)*

Phosphorylation was performed as described in the legend to Fig. 3. KCl (14 mM) was included in the phosphorylation system where indicated. The specific radioactivity of [^{32}P]ATP was 1.5×10^7 counts/min./ μmole . The precipitate was washed with 0.5 N- HClO_4 by centrifugation, suspended in 0.35 ml. of water and 0.3 ml. of 2 M-hydroxylamine (adjusted to pH 6.5 with NaOH) was added. The mixture was kept at 20° for 20 min. Then 0.12 ml. of 3 N- HClO_4 was added and the mixture was centrifuged. Of the supernatant 0.5 ml. was taken and 0.1 ml. of ammonium molybdate- H_2SO_4 mixture was added, followed by the addition of 1.5 ml. of 2-methylpropan-1-ol-benzene mixture. The mixture was stirred vigorously with a rotatory vibrator for 2 min. and centrifuged. The organic solvent layer (1.0 ml.) was transferred to a planchet and the radioactivity was measured after evaporation in the form of infinite thinness under a Geiger-Müller counter. A control experiment in which NaCl was substituted for hydroxylamine showed negligible liberation of P_i .

Phosphorylation system			[^{32}P]P _i liberated by hydroxylamine treatment of phosphorylated preparation (counts/min.)
pH	Concn. of Na ⁺ (mM)	Concn. of K ⁺ (mM)	
4.9	—	—	297
	—	14	33
6.0	140	—	476
	—	—	106
7.7	140	—	394
	—	—	24
	140	—	1124
	140	—	72*

* Enzyme, thermally denatured at 70° for 15 min.

Table 11. *Time-course of phosphorylation at pH 6.0*

The ATPase preparation was phosphorylated at pH 6.0 as described in the legend to Table 10 except that the incubation was prolonged to 60 sec. and the specific radioactivity of [γ - ^{32}P]ATP was 2.5×10^7 counts/min. by a Geiger-Müller counter used throughout the present work.

Phosphorylation system			[^{32}P]P _i liberated by hydroxylamine treatment (counts/min.)		
Concn. of Na ⁺ (mM)	Concn. of K ⁺ (mM)	Incubation time ...	0 sec.	15 sec.	60 sec.
140	—	...	110	1802	1980
140	14	...	—	415	712

ATPase and (Na⁺)-ATPase. The decreasing order of ouabain-sensitivity of the ATPase activities of the present preparation was: (Na⁺)-ATPase; (Na⁺,K⁺)-ATPase; (K⁺)-ATPase. This result is consistent with observations (Glynn, 1957; Dunham & Glynn, 1961; Post & Albright, 1961; Hoffman, 1962; Auditore, 1964; Schatzmann, 1965) that increased concentrations of K⁺ counteract the ouabain-inhibition of the (Na⁺,K⁺)-ATPase competitively or non-competitively and especially the finding (Matsui & Schwartz, 1966) that an increased Na⁺/K⁺ ratio results in increased ouabain-sensitivity.

The data on phosphorylation suggest that the preparation phosphorylated at low pH has the same property as that phosphorylated at neutral pH. This finding seems to support, not only the identity of the ouabain-sensitive ATPase activities measured under various conditions, but also the current hypothesis that the K⁺- and hydroxylamine-sensitive phosphorylated complex is a true intermediate of (Na⁺,K⁺)-ATPase reaction or one closely related to it (see Nagano *et al.* 1967), although some complications have been reported (Schoner, Kramer & Seubert, 1966).

The similarities listed above seem to favour the idea that the ouabain-sensitive activities reported in this paper arise from a single-enzyme system. The conspicuous differences in pH-activation of these ouabain-sensitive activities can be explained, provided that it is assumed that the Na⁺ site (Na⁺ carrier) has a higher pK than the K⁺ site, that Na⁺ replaces K⁺ at the K⁺ site at neutral to alkaline pH, and that H⁺ replaces Na⁺ at the Na⁺ site at low pH. Therefore the (Na⁺,K⁺)-ATPase activity arises when Na⁺ acts on the Na⁺ site and K⁺ on the K⁺ site, (K⁺)-ATPase activity when H⁺ acts on the Na⁺ site and K⁺ on the K⁺ site, and (Na⁺)-ATPase activity when Na⁺ acts on both Na⁺ and K⁺ sites. If this explanation is assumed, (K⁺)-ATPase may be represented more exactly as '(H⁺,K⁺)-ATPase' and (Na⁺)-ATPase as '(Na⁺,Na⁺)-ATPase', in analogy with the expression (Na⁺,K⁺)-ATPase.

It was reported by Fujita *et al.* (1967) that the Na⁺ affinity of (Na⁺)-ATPase activity increased as the pH of the reaction mixture increased. On the other hand, the effect of pH on the K⁺ affinity of (K⁺)-ATPase activity was not clear, because the activity decreased rapidly as the pH was raised above 6. However, this also supports the above assumption that H⁺ is necessary for (K⁺)-ATPase activity. It was also reported there that the inhibition of (K⁺)-ATPase by increased concentration of K⁺ became more marked above pH 6. This seems to reflect the competition between H⁺ and K⁺ at the Na⁺ site.

Davies & Keynes (1961) proposed a coupled Na⁺-K⁺ pump model that included a K⁺ carrier

with pK 4 and an Na⁺ carrier with pK 6. An apparent agreement between their scheme and our finding might suggest a physiological role of the present (Na⁺,K⁺)-ATPase. In unpublished experiments we have recently detected similar minor ATPase activities in an (Na⁺,K⁺)-ATPase preparation from rabbit kidney.

It is well known that H⁺ efflux and K⁺ influx are coupled in the fermenting yeast cell. It was also reported that yeast discriminates in the uptake of Na⁺ and K⁺ at different pH values, with K⁺ favoured at low pH, although a single carrier was assumed in this case for both ions (Armstrong & Rothstein, 1966). If it is permissible to discuss the present results in terms of analogy with yeast, it might be suggested that (K⁺)-ATPase activity reflects active K⁺-H⁺ exchange *in vivo* and that (Na⁺)-ATPase activity reflects Na⁺-Na⁺ exchange.

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