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ACETYL PHOSPHATE AS A SUBSTITUTE FOR ATP IN $(Na^+ + K^+)$-DEPENDENT ATPase

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SUMMARY

1. In the presence of Mg^{2+} and Na^+, acetyl phosphate replaced ATP as an agent for phosphorylating Na^+- and K^+-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3).

2. The phospho-enzymes produced by both substrates were similar in the following respects: (a) electrophoretic mobility and chemical reactivity of phosphopeptides released by proteolytic digestion of the denatured enzyme, (b) the quantity of phospho-enzyme, (c) the concentration of Na^+ for half-maximal phosphorylation, (d) a requirement for Mg^{2+}, (e) the half-life at 0°. Furthermore, each substrate inhibited phosphorylation by the other, and the inhibitor ouabain enhanced the inhibitory effect of acetyl phosphate.

3. In the presence of Mg^{2+} and K^+, acetyl phosphate, unlike ATP, also phosphorylated the enzyme. The quantity of phospho-enzyme was less and its turnover was faster. K^+ appeared to accelerate both phosphorylation and dephosphorylation.

4. The same active site may be an intermediate in both the Na^+- and K^+-dependent ATPase activity and the K^+-dependent acetyl phosphatase activity of this enzyme system in functionally different conformations of an active center.

INTRODUCTION

The hydrolysis of ATP by membrane-bound $(Na^+ + K^+)$-ATPase (ATP phosphohydrolase, EC 3.6.1.3) involves at least two steps. In the presence of Na^+ and Mg^{2+} the terminal phosphate group of ATP is transferred to an acceptor in the membrane to form a phospho-enzyme; when K^+ is added, this phospho-enzyme is rapidly hydrolyzed[1–4]. The active site is part of a protein, and the phosphate group is an acyl phosphate[5–7].

Because an acyl phosphate bond is hydrolyzed during the reaction, BADER AND SEN[8] tested acetyl phosphate as a substrate for the $(Na^+ + K^+)$-ATPase of guinea pig kidney. They found an acetyl phosphatase activity which required Mg^{2+} and K^+,

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and was blocked by ouabain and other inhibitors of $(Na^+ + K^+)$-ATPase. The pH optimum of this activity was the same as for $(Na^+ + K^+)$-ATPase, and cations which substitute for K^+ in $(Na^+ + K^+)$-ATPase also replaced K^+ in stimulating acetyl phosphate hydrolysis. Na^+, however, could not replace K^+. They suggested that "the K^+-dependent acetyl phosphatase is the same as the K^+-dependent part of the $(Na^+ + K^+)$-ATPase system which hydrolyzes the phosphorylated intermediate". Similar suggestions have been made by others to explain a K^+-dependent *p*-nitrophenyl phosphatase activity found in association with $(Na^+ + K^+)$-ATPase preparations[9,10].

We set out to test the hypothesis that acetyl phosphate substitutes for the phospho-enzyme as substrate for a K^+-dependent hydrolytic reaction in the reaction sequence of $(Na^+ + K^+)$-ATPase. The results indicate that the interaction of acetyl phosphate with the system is more complex than this hypothesis suggests. Acetyl phosphate was found to substitute for ATP in forming the phospho-enzyme, with loss of specificity with respect to the monovalent cation requirement of this reaction. A preliminary report of this work has appeared[11].

METHODS

The $(Na^+ + K^+)$-ATPase of guinea pig kidney membranes and terminally labeled [^{32}P]ATP were prepared as described by POST AND SEN[12].

Unlabeled dilithium acetyl phosphate (Sigma Chemical Co., St. Louis, Mo.) was converted to the Tris salt by passage through a column of AG 50W-X8 cation-exchange resin (Bio-Rad Laboratories, Richmond, Calif.) in the Tris form at 0°. This material was 70 to 80 % pure as assayed by the method of LIPMAN AND TUTTLE[13]. Acetyl [^{32}P]phosphate was prepared by a modification of Method B of STADTMAN[14]. The product was converted to the Tris salt. The specific activity was about 10^7 counts/min per μmole. Acetyl phosphate accounted for as little as 70 % and as much as 85 % of the total phosphate in these preparations. Total phosphate was determined after hydrolyzing the acetyl phosphate by boiling for 30 min and measuring P_i (see ref. 12). The difference between the total phosphate and the acetyl phosphate was contributed by P_i. This was verified by subjecting aliquots to high-voltage paper electrophoresis as described below. Radioautography revealed two spots, corresponding in mobility to acetyl phosphate and P_i. When these spots were cut out and counted, the purity based on counts agreed with the purity determined chemically.

All cations were added as the chloride salts.

Incorporation of ^{32}P into membranes

The phosphorylation reactions, with either [^{32}P]ATP or acetyl [^{32}P]phosphate as substrate, were carried out at 0° as described by POST *et al.* [3].The reaction mixture had a volume of 1 ml and was buffered to pH 7.6 with 10 mM imidazole–glycylglycine. Other additions were indicated in the text. Membranes (1–5 mg protein) were incubated with the radioactive substrate for about 10 sec. The reaction was stopped by adding 35 ml of ice-cold 0.25 M trichloroacetic acid containing 0.6 mM ATP for experiments with [^{32}P]ATP, or 3 mM acetyl phosphate and 50 mM H_3PO_4 for experiments with acetyl [^{32}P]phosphate. The denatured membranes were washed 3 times with ice-cold 0.3 M trichloroacetic acid. In experiments with [^{32}P]ATP the acid-washed membranes were dispersed in a fresh mixture of chloroform–methanol–formic

acid (4:2:1, by vol.). One aliquot was counted in a thin-window gas-flow counter, and a second was assayed for protein by the method of LOWRY *et al.*[15].

Peptic digestion and paper electrophoresis

Phosphorylation by acetyl phosphate had two components, one of which was stimulated by cations, and one of which was not. This latter component showed wide variation, and was at times large enough to obscure the stimulatory effect of cations. Since it had been previously reported that the cation-dependent phosphorylation seen with [^{32}P]ATP as substrate is released in the form of radioactive phosphopeptides after digestion with pepsin[1,6,7], we tried this technique to separate our two components of phosphorylation. In a representative experiment using acetyl [^{32}P]phosphate as substrate, the radioactivity incorporated into the membranes in the presence of Na^+ and Mg^{2+} was about twice as great as with Mg^{2+} alone. The increment was released upon peptic digestion, and the radioactivity which remained bound to the membranes was the same in both cases. We consistently found that the radioactivity released by pepsin depended on the ionic conditions under which the phosphorylation reaction was carried out. For this reason, membranes which had been phosphorylated with acetyl [^{32}P]phosphate were routinely digested with pepsin, and the soluble phospho-peptides were collected and counted. Pepsin digestion was carried out as described by BADER *et al.*[7]. In some experiments an aliquot of each digest was counted directly, and in others an aliquot was subjected to high-voltage paper electrophoresis[7] to separate the phospho-peptides from P_i. The phospho-peptides, located on the paper by radioautography, were cut out and counted. The reproducibility of the method was satisfactory, and corrections for variable recovery proved unnecessary. When radioactivity in seven identical peptic digests was compared in one experiment, the coefficient of variation was $\pm$ 4 %.

Other procedures

Digestion of membranes with pronase (Calbiochem, Los Angeles, Calif.) was carried out as described by BADER *et al.*[16]. Treatment with performic acid was carried out as described by BADER *et al.*[7]. Treatment with *N*-ethylmaleimide was done according to BADER *et al.*[16].

RESULTS

If acetyl phosphate and the phosphate group at the active site of the $(Na^+ + K^+)$-ATPase system were hydrolyzed in a common active center, they might compete with one another. This could be seen as an inhibition of the hydrolysis of the phospho-enzyme in the presence of acetyl phosphate. To test this, we formed the phospho-enzyme from [^{32}P]ATP and measured the rate of loss of radioactivity from the membranes following a chase of unlabeled ATP, with and without acetyl phosphate. Acetyl phosphate, at a final concentration of 10 mM, had no observable effect on hydrolysis of the phospho-enzyme, even with the further addition of 1 mM K^+.

Acetyl phosphate inhibition of phosphorylation by [^{32}P]ATP

Since acetyl phosphate is a competitive inhibitor of $(Na^+ + K^+)$-ATPase[8], it appeared that it might act at an earlier step in the reaction sequence to inhibit

phosphorylation by ATP. In order to investigate this possibility, we incubated membranes with unlabeled acetyl phosphate, Na^+ and Mg^{2+} for 10 sec before adding [^{32}P]-ATP. The result was a marked reduction in both the rate and extent of phosphorylation (Fig. 1, Curve A). When Na^+ was omitted during the pre-incubation, but was added along with the [^{32}P]ATP to start the reaction, there was no longer any observable inhibition of the rate of formation of phospho-enzyme, although the extent of phosphorylation was still somewhat reduced (Fig. 1, Curve B). In other experiments, not shown, we found that Mg^{2+} was also required during the pre-incubation with acetyl phosphate in order to obtain this inhibition in rate. In addition, results similar to those described above were observed with acetyl phosphate concentrations as low as 1 mM and [^{32}P]ATP concentrations as high as 0.2 mM. The reduction in the extent of phosphorylation may be due to competition between acetyl phosphate and ATP for binding. The cation-dependent reduction in the rate of phospho-enzyme formation, on the other hand, suggested that acetyl phosphate could have phosphorylated the (Na^+ — K^+)-ATPase system in the presence of Na^+ and Mg^{2+}. Formation of unlabeled phospho-enzyme with acetyl phosphate would delay incorporation of ^{32}P from [^{32}P]ATP because of the slow turnover of the phospho-enzyme in the absence of K^+ (ref. 3). This slow turnover would gradually release free sites for reaction with [^{32}P]ATP.

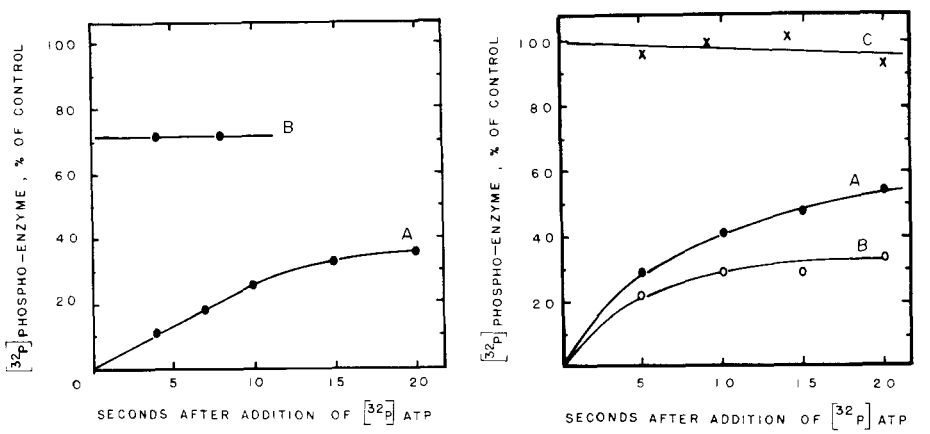

Fig. 1. Acetyl phosphate inhibition of phosphorylation by [^{32}P]ATP. Amount of phospho-enzyme is expressed as % of a stable control level formed in the presence of Na^+ and Mg^{2+} after incubation with [^{32}P]ATP for 5 sec. The radioactivity in a blank which contained K^+ and Mg^{2+} was subtracted from all values. The system contained: 0.04 mM [^{32}P]ATP; 16 mM Na^+ or 16 mM K^+; 8 mM Mg^{2+}. The temperature was 0°. Curve A, after a 10-sec pre-incubation with 7 mM acetyl phosphate (*plus* Na^+ and Mg^{2+}), [^{32}P]ATP was added, and the reaction was stopped at the times shown. Curve B, the conditions were the same as for Curve A except that Na^+ was not added during the pre-incubation with acetyl phosphate, but was added with [^{32}P]ATP to start the reaction at zero time.

Fig. 2. Inhibition by acetyl phosphate of phosphorylation by [^{32}P]ATP with and without ouabain. The system contained 4 mM Mg^{2+} and $2.5 \cdot 10^{-4}$ M ouabain. Other conditions are as given in Fig. 1. Curve A, membranes were pre-incubated with acetyl phosphate for 10 sec before adding [^{32}P]ATP at zero time; the reaction was then stopped at the times shown. Curve B, membranes were pre-incubated with ouabain for 10 sec, then acetyl phosphate was added; 10 sec later [^{32}P]ATP was added at zero time. Curve C, membranes were pre-incubated with ouabain and without acetyl phosphate for 10 sec before addition of [^{32}P]ATP at zero time.

As a test of this interpretation the action of ouabain was investigated. In the presence of Na^+, ouabain does not react with the dephospho-enzyme but stabilizes the phospho-enzyme[17]. An enhancement by ouabain of acetyl phosphate inhibition was a further indication that acetyl phosphate had formed the phospho-enzyme (Fig. 2).

Phosphorylation of membranes by acetyl phosphate: Characteristics of the phosphorylated product

Substitution of acetyl [^{32}P]phosphate for [^{32}P]ATP in the labeling procedure yielded phosphorylated membranes. In order to identify this material we digested the radioactive, denatured membranes with pepsin and electrophoresed the solubilized phosphopeptides. These were similar in appearance and mobility to those obtained from [^{32}P]ATP (Fig. 3). The similarity persisted after treatment with performic acid alone, or with *N*-ethylmaleimide followed by performic acid. The changes resulting from these treatments are due to the presence of a sulfhydryl group (see ref. 16). Pronase digests of membranes phosphorylated by [^{32}P]ATP or acetyl [^{32}P]phosphate likewise gave similar patterns of phospho-peptides upon electrophoresis. Also, hydroxylamine split ^{32}P from the phospho-peptides derived from acetyl[^{32}P]phosphate just as it does from those formed by [^{32}P]-ATP[5–7].

The effect of Na^+ concentration on formation of the phospho-enzyme by acetyl

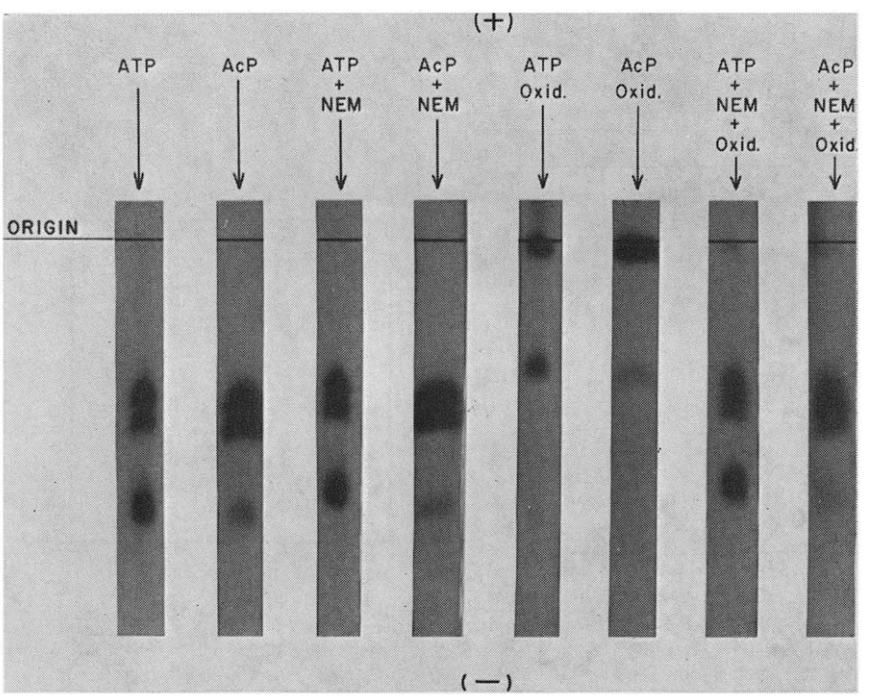

Fig. 3. Radioautogram of an electrophopherogram of soluble, radioactive peptides released after peptic digestion of membranes phosphorylated by [^{32}P]ATP or acetyl [^{32}P]phosphate. For phosphorylation by [^{32}P]ATP the system contained 16 mM Na^+ and 4 mM Mg^{2+}. For phosphorylation by acetyl [^{32}P]phosphate the system contained 160 mM Na^+ and 12 mM Mg^{2+}. Other conditions and procedures are given in METHODS. Abbreviations: NEM, *N*-ethylmaleimide; AcP, acetyl phosphate; Oxid. refers to oxidation by performic acid.

[^{32}P]phosphate is shown in Fig. 4. The amount of phospho-enzyme was a saturable function of Na^+ concentration, with 1.5 mM Na^+ giving a half-maximal effect. This value is the same as that found when [^{32}P]ATP is the substrate[3]. This similarity suggests that Na^+ acts at the same site in both cases. A little phospho-enzyme appeared in the absence of added Na^+.

These results indicated that ATP and acetyl phosphate can act at a common site in the ($Na^+ + K^+$)-ATPase system. We therefore compared the ability of ATP, ADP and several other nucleotides to inhibit phosphorylation by acetyl [^{32}P]phos-

TABLE I

THE EFFECT OF NUCLEOTIDES ON PHOSPHORYLATION BY ACETYL [^{32}P]PHOSPHATE

The concentration of all nucleotides was 0.2 mM. After a 10-sec pre-incubation with the unlabeled nucleotide, acetyl [^{32}P]phosphate was added to a concentration of 1.6 mM. The reaction was stopped after 10 sec. The system also contained 40 mM Na^+ and 8 mM Mg^{2+}. The temperature was 0°.

Nucleotide added (non-radioactive)	*Counts/min in 10 µl of peptic digest*
—	300
ATP	68
ADP	76
CTP	222
ITP	281
GTP	287
UTP	284
TTP	297

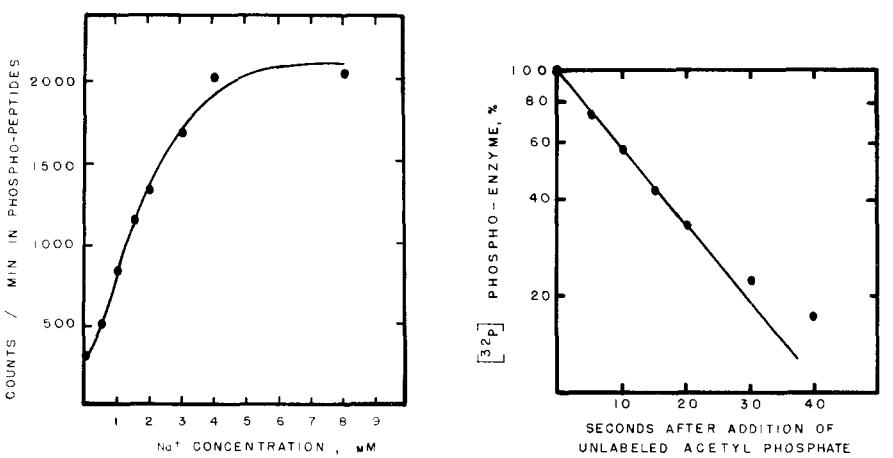

Fig. 4. Phosphorylation by acetyl [^{32}P]phosphate as a function of Na^+ concentration. Membranes were incubated with 1 mM acetyl [^{32}P]phosphate for 10 sec before addition of cold acid to stop the reaction. The system contained 4 mM Mg^{2+}. The temperature was 0°.

Fig. 5. Turnover of the radioactive material formed by acetyl [^{32}P phosphate. Membranes were incubated with 1 mM acetyl [^{32}P]phosphate for 10 sec. The system contained 10 mM Na^+ and 4 mM Mg^{2+}. The temperature was 0°. The volume was 1 ml. After 10 sec, 0.6 ml of 96 mM unlabeled acetyl phosphate was added to dilute the specific activity. At the times shown on the abscissa the reaction was stopped. The amount of phospho-enzyme was estimated from the isolated phospho-peptides and is expressed as % of that at zero time.

phate (Table I). ATP and ADP were effective, CTP inhibited slightly, and ITP, GTP, UTP and TTP had little or no effect. ATP probably inhibited by phosphorylating the enzyme. ADP may have displaced acetyl phosphate from the active center, since it has been reported to be a competitive inhibitor of $(Na^+ + K^+)$-ATPase[18]. Inhibition by CTP is explainable on the basis that it is the most effective nucleotide substrate next to ATP in a number of ATPase preparations [19,20].Other nucleotides probably have a lower affinity for the system than either ATP or ADP. FAHN *et al.*[21], for example, reported that ATP and ADP inhibited phosphorylation by [^{32}P]ATP, but that a number of other nucleotide triphosphates were without effect.

Turnover of the phospho-enzyme formed by acetyl[^{32}P]phosphate was investigated next. The enzyme, which was first phosphorylated by acetyl [^{32}P]phosphate in the presence of Na^+ and Mg^{2+}, was chased with unlabeled acetyl phosphate. The radioactive phospho-enzyme disappeared exponentially with a half-life of about 12 sec (Fig. 5), which is essentially the same as the value reported for the phospho-enzyme formed by [^{32}P]ATP under similar conditions[3]. In another experiment, 1 mM K^+ was added along with the unlabeled acetyl phosphate. In this case 80 % of the radioactivity was released from the membranes within 3 sec. Therefore K^+-stimulated breakdown of this phospho-enzyme as it does the one formed by ATP[3].

The maximum extent of phosphorylation by ATP and acetyl phosphate was similar (Table II). The concentration of acetyl phosphate used in this experiment (2 mM) was saturating. The apparent K_m for acetyl phosphate in the phosphorylation reaction was about 0.2 mM.

These data support the conclusion that ATP and acetyl phosphate are both substrates in a $(Na^+ + Mg^{2+})$-stimulated phosphorylation of $(Na^+ + K^+)$-ATPase. It seemed unlikely, however, that this phospho-enzyme could be an intermediate in K^+-dependent acetyl phosphatase, since this activity shows no requirement for for Na^+ (ref. 8). We therefore investigated phosphorylation by acetyl phosphate in the presence of other cations.

Effect of cations on formation and turnover of the phospho-enzyme

Mg^{2+} was required for phosphorylation. When Mg^{2+} was omitted a small amount of phospho-enzyme appeared, but this was probably due to traces of Mg^{2+} in the reaction mixture. No phospho-enzyme was found in the presence of EDTA (Table III).

Phospho-enzyme appeared in the presence of K^+ and Mg^{2+} (Table III). This

TABLE II

COMPARISON OF THE EXTENT OF PHOSPHORYLATION BY [^{32}P]ATP WITH THAT BY ACETYL [^{32}P]-PHOSPHATE

Membranes were incubated with 20 mM Na^+, 4 mM Mg^{2+} and either 0.04 mM [^{32}P]ATP or 2 mM acetyl [^{32}P]phosphate at 0° for 10 sec. An aliquot of denatured, washed membranes was assayed for protein. The membranes were digested with pepsin and the digests were counted.

Substrate	*Extent of phosphorylation (pmoles ^{32}P per mg protein)*
[^{32}P]ATP	367
Acetyl [^{32}P]phosphate	344

finding was verified in other experiments. Na^+ caused a 3–5-fold increase in the level of intermediate, but if K^+ was also present, no stimulatory effect of Na^+ was evident (Table III).

Any material considered as a true intermediate in acetyl phosphatase activity, and which is maintained in the presence of K^+, must turn over rapidly since K^+ accelerates the hydrolysis of acetyl phosphate. We therefore estimated turnover in the presence of several combinations of cations, and the results are shown in Table IV.

TABLE III

EFFECT OF MONOVALENT AND DIVALENT CATIONS ON PHOSPHORYLATION BY ACETYL [^{32}P]PHOSPHATE

Membranes were incubated with 1 mM acetyl [^{32}P]phosphate for 10 sec before addition of cold acid. Expt. 1: 10 mM Na^+ and K^+. Expts. 2, 3 and 4: 20 mM Na^+ and K^+. Expts. 1, 3 and 4: 4 mM Mg^{2+}. Expt. 2: 8 mM Mg^{2+}. The concentration of $Tris_4EDTA$ was 40 mM. The temperature was 0°.

Additions	*Counts/min in phospho-peptides*			
	Expt. 1	*Expt. 2*	*Expt. 3*	*Expt. 4*
$Na^+ + Mg^{2+}$	2223	845	605	1550
$K^+ + Mg^{2+}$	547	251	236	351
$Na^+ + K^+ + Mg^{2+}$	--	255	--	
Na^+	416		--	
K^+	120		--	
$Na^+ + (Tris)_4EDTA$	0		--	
$K^+ + (Tris)_4EDTA$	0		--	
*$Na^+ + Mg^{2+}$	--		7	

* Acetyl [^{32}P]phosphate was heated for 30 min at 60°. This was sufficient to hydrolyze it.

TABLE IV

TURNOVER OF THE PHOSPHO-ENZYME FORMED FROM ACETYL [^{32}P]PHOSPHATE IN THE PRESENCE OF MONOVALENT AND DIVALENT CATIONS

After a 10-sec incubation of the membranes with 1 mM acetyl [^{32}P]phosphate, enough unlabeled acetyl phosphate was added to dilute the specific activity of the labeled substrate 60-fold. At the times shown, the reaction was stopped by addition of cold acid. All monovalent cations were present at 40 mM concentration. Expts. 1 and 2: 12 mM Mg^{2+}. Expts. 3 and 4: 8 mM Mg^{2+}. The temperature was 0°.

Expt. No.	*Additions*	*Initial counts/min in phosphopeptides*	*Counts/min in phospho-peptides after dilution of specific activity Time after dilution:*			
			3 sec	*4 sec*	*6 sec*	*10 sec*
1	$Na^+ + Mg^{2+}$	3881		2987		1803
	$K^+ + Mg^{2+}$	1080		83		
2	$K^+ + Mg^{2+}$	821		45		
3	$K^+ + Mg^{2+}$	287	31			
	$Li^+ + Mg^{2+}$	488	168	126		
	Mg^{2+}	247	186	139		
4	$K^+ + Mg^{2+}$	433	79			
	$Rb^+ + Mg^{2+}$	406	56			
	$Cs^+ + Mg^{2+}$	385	81			

In the presence of K^+ and Mg^{2+}, 80–95 % of the phospho-enzyme disappeared within 3–4 sec after a chase of unlabeled acetyl phosphate. Similar results were obtained when K^+ was replaced by Rb^+ or Cs^+, although with Li^+ turnover appeared to be slower. These results were consistent, since Rb^+, Cs^+ and Li^+ all substitute for K^+ in accelerating hydrolysis of the phospho-enzyme formed from [^{32}P]ATP[3]. They also replace K^+ in $(Na^+ + K^+)$-ATPase[22] and K^+-dependent acetyl phosphatase activities[8]. In each case Li^+ is the least effective substitute.

DISCUSSION

Acetyl phosphate transferred its phosphate group to $(Na^+ + K^+)$-ATPase. The phospho-enzyme formed in the presence of Na^+ and Mg^{2+} from either acetyl [^{32}P]phosphate or [^{32}P]ATP showed the following similarities: (1) electrophoretic mobilities and chemical reactivity of phospho-peptides prepared by proteolytic digestion of denatured membranes (Fig. 3 and ref. 30), (2) the quantity of phospho-enzyme (Table II), (3) the half-life of the phospho-enzyme (compare Fig. 5 in this paper with Fig. 15 in ref. 3), and (4) the concentration of Na^+ required for a half-maximal effect (compare Fig. 4 in this paper with Fig. 14 in ref. 3). Furthermore, pre-incubation with either phosphorylating agent impaired phosphorylation by the other (Figs. 1 and 2 and Table I), and ouabain, an inhibitor of $(Na^+ + K^+)$-ATPase, enhanced the inhibitory effect of pre-incubation with acetyl phosphate (Fig. 2). Nucleotides other than ATP and ADP were less effective in blocking phosphorylation by acetyl phosphate (Table I). It is thus possible that the same active site participates in both the K^+-dependent acetyl phosphatase and $(Na^+ + K^+)$-ATPase activities. This proposal is reasonable in light of evidence that this site participates in three separate activities in a flexible active center; namely, reversible phosphorylation from ATP in the presence of Mg^{2+} and Na^+, irreversible hydrolysis of the phosphate bond in the presence of K^+, and reversible phosphorylation from inorganic phosphate in the presence of Mg^{2+} and ouabain[23,24].

In contrast to [^{32}P]ATP, however, acetyl [^{32}P]phosphate phosphorylated $(Na^+ + K^+)$-ATPase in the presence of Mg^{2+} and K^+ without Na^+ (Tables III and IV), and the resulting phospho-enzyme turned over rapidly (Table IV). Since addition of Na^+ to a system containing K^+ and Mg^{2+} did not change the level of phospho-enzyme (Table III), it appears that K^+ may stimulate both formation and breakdown. The lack of a requirement for Na^+ in the hydrolysis of acetyl phosphate, as well as its failure to stimulate phosphorylation in the presence of K^+ could be explained in this way.

From the phosphorylation appearing in the presence of Mg^{2+} alone (Table IV) it would appear that a monovalent cation was not required for phosphorylation from acetyl phosphate. However, these preparations of acetyl phosphate contained as much as 30 % P_i, and subsequent experiments in this laboratory have shown that P_i phosphorylates the native enzyme in the presence of Mg^{2+}, without inorganic monovalent cations. Hydrolysis of this form of the phospho-enzyme was not accelerated by K^+. Furthermore, the turnover of the native phospho-enzyme formed from P_i corresponded to that observed in the presence of Mg^{2+} (Table IV). For this reason, part or all of the phospho-enzyme appearing in the absence of inorganic monovalent cations could have come from P_i.

Since turnover of the phospho-enzyme is believed to be linked to the transport

of Na^+ and K^+, and since our experiments demonstrated effects of both Na^+ and K^+ on formation and breakdown of this phospho-enzyme when formed from acetyl phosphate, it appeared that acetyl phosphate might substitute for ATP in driving the Na^+ pump. BRINLEY AND MULLINS[25], however, were unable to demonstrate Na^+ extrusion from squid axons internally perfused with acetyl phosphate; only ATP and dATP were effective. This apparent inability of acetyl phosphate to drive the pump may be due to a specific requirement for ATP at a second site other than that for phosphorylation. At this second site ATP may act as an effector, inducing a conformation in which both Na^+ and K^+ are necessary for hydrolysis[26–29], and both cations undergo translocation.

ISRAEL AND TITUS[30] examined the relationship between ($Na^+ + K^+$)-ATPase and K^+-dependent phosphatase. They confirmed our earlier observation[11] of phosphorylation by acetyl phosphate, and found the phospho-peptides produced to be similar electrophoretically to those formed from ATP. Nevertheless, kinetic experiments led them to conclude that "the K^+-activated acetyl phosphatase represents a different entity from the K^+-dependent activity which hydrolyzes the phosphorylated intermediate of the ($Na^+ + K^+$)-ATPase". Specifically, in a single reaction mixture they found that the concentration of ouabain required for half-maximal inhibition was $2.2 \cdot 10^{-6}$ M for K^+-dependent acetyl phosphatase and $4.0 \cdot 10^{-5}$ M for ($Na^+ + K^+$)-ATPase (their Fig. 1). This apparent difference in sensitivity to ouabain may be explainable on a technical consideration. For estimation of the ($Na^+ + K^+$)-ATPase activity, the incubation time was 4 min, but for K^+-dependent acetyl phosphatase it was 10 min. Because inhibition by cardioactive steroids develops slowly at low concentrations[31], it is possible that the longer incubation time permitted a greater inhibition of K^+-dependent acetyl phosphatase. This interpretation is supported by the fact that the concentration of ouabain required for half-maximal inhibition of ($Na^+ + K^+$)-ATPase in their experiment was 10 times higher than the value reported by BADER AND SEN[8]. ISRAEL AND TITUS[30] also observed selective inhibition of ($Na^+ + K^+$)-ATPase by *N*-ethylmaleimide or oligomycin. There is now further evidence that these agents restrict the conformational flexibility of the enzyme and impair only certain steps in the overall reaction sequence (see ref. 32), and so allow K^+-dependent phosphatase activity to persist when ($Na^+ + K^+$)-ATPase is inhibited. It is likely that ($Na^+ + K^+$)-ATPase and K^+-dependent acetyl phosphatase are aspects of a common system.

SACHS *et al.*[33] phosphorylated brain microsomes with acetyl [^{32}P]phosphate without isolating phospho-peptides. In a single experiment they observed stimulation by K^+ but not by Na^+. The reason for the discrepancy between their results and ours is not clear.

INTURRISI AND TITUS[34] recently studied phosphorylation of ($Na^+ + K^+$)-ATPase by *p*-nitrophenyl [^{32}P]phosphate and found phosphorylation only when the enzyme was inhibited by ouabain. They concluded that "ouabain places the phosphatase in a configuration that allows *p*-nitrophenyl phosphate and inorganic phosphate to label the same site on the enzyme". This phosphorylation bears no clear relationship to the K^+-dependent *p*-nitrophenyl phosphatase activity, and it may be that this substrate is hydrolyzed without the participation of a phosphorylated intermediate.

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