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ON THE REACTION SEQUENCE OF THE K^+-DEPENDENT ACETYL PHOSPHATASE ACTIVITY OF THE NA^+ PUMP*

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SUMMARY

1. Literature reports differ whether Na^+ or K^+ is required for formation of an acid-stable intermediate in acetyl [^{32}]phosphate hydrolysis by K^+–phosphatase (acyl phosphate phosphohydrolase, EC 3.6.1.7) preparations. We have reinvestigated this question by using detergent treatment and gel filtration at neutral pH to isolate labeled protein.

2. Binding of ^{32}P to the enzyme is stimulated by Mg^{2+}, and decreased by K^+. No Na^+ requirement is seen, although Na^+ partially prevents discharge of labeling by K^+. These effects are consistent with the corresponding ion effects on acetyl phosphate hydrolysis by the enzyme.

3. K^+-phosphatase inhibitors such as ATP, ADP, P_i and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) cause corresponding decreases in the labeling. Similar effects of DTNB on the Na^+-dependent ADP–ATP exchange activity suggest involvement of the same intermediate.

4. Labeling by [^{32}P]orthophosphate is affected differently by K^+, a stimulation being observed.

5. Ouabain increases labeling by both substrates. That derived from acetyl phosphate remains K^+ dischargeable, whereas that from orthophosphate is less affected.

6. No transfer of ^{32}P from acetyl phosphate to ADP, nor ^{14}C from acetate to acetyl phosphate, could be demonstrated under a variety of ionic conditions.

INTRODUCTION

Membrane preparations containing the (Na^+,K^+)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) invariably also contain a K^+-phosphatase (acyl phosphate phosphohydrolase, EC 3.6.1.7) activity in constant proportion[1]. The common K^+ requirement and particularly the sensitivity to cardiac glycosides of both these activities[2,3] is

Abbreviations: (Na^+,K^+)-ATPase, the membrane adenosine 5′-triphosphate phosphohydrolase (EC 3.6.1.3) requiring simultaneously Na^+, K^+ and Mg^{2+} for full activity; K^+-phosphatase, the membrane acyl phosphate phosphohydrolase (EC 3.6.1.7) requiring both K^+ and Mg^{2+} for full activity; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

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strong presumptive evidence that both are enzymic manifestations of the Na^+ pump. Even the most highly purified (Na^+,K^+)-ATPase preparations contain undiminished K^+-phosphatase activity[4,5]. The lack of a Na^+ requirement for the K^+-phosphatase (indeed, inhibition by Na^+ is observed) suggests that this activity reflects the terminal stages of the reaction sequence of the (Na^+,K^+)-ATPase.

A few investigators have studied the reaction sequence of the K^+-phosphatase using radioactive acetyl phosphate as substrate. HEMS[6] has reported that [^{3}H]acetyl phosphate does not label the enzyme. On the other hand, other laboratories have reported either a Mg^{2+}, Na^+-dependent[7,8] or a Mg^{2+}, K^+-dependent[9] labeling of K^+-phosphatase preparations by acetyl [^{32}P]phosphate using acid precipitation to isolate the labeled enzyme.

Because of the above discrepancies in the literature regarding Na^+ and K^+ effects on labeling, we have reinvestigated the question using an alternative isolation procedure. NAGANO *et al.*[10] showed previously that one can use detergent to stop the labeling reaction (using [γ-^{32}P]ATP), followed by isolation of labeled protein by gel filtration at neutral pH. We have confirmed their findings for the Na^+-dependent phosphorylation of the enzyme by [γ-^{32}P]ATP using the detergent method and have extended this procedure to the acetyl [^{32}P]phosphate labeling of the enzyme. Our results show that labeling of the phosphatase measured in this manner is neither Na^+- nor K^+-stimulated, but requires only Mg^{2+}. As reported by other laboratories for the acid-stable labeling[7,8], the presence of K^+ discharges the Mg^{2+}-dependent detergent-stable labeling. These findings permit the postulation of a more reasonable reaction sequence for the K^+-phosphatase.

METHODS

Isolation of (Na^+,K^+)-ATPase

Bovine brain was obtained immediately after slaughter and kept chilled. The gray matter was removed, weighed and homogenized for 1 min at low speed in a Waring blendor in 5 vol. of a medium consisting of 0.25 M mannitol, 0.03 M Tris, pH 7.4, at 0° and 1 mM EDTA. The resulting homogenate was then centrifuged and the fraction sedimenting between 150 000 and 6 000 000 $\times$g$\cdot$min isolated. The pellet sedimenting at 150 000 $\times$g$\cdot$min was rehomogenized in the same volume of buffer and the microsomal fraction again isolated, this process being repeated twice. The isolated microsomes were suspended by hand in water with a Teflon homogenizer, the protein content determined[11], and then frozen in suitable aliquots.

As needed, enzyme preparations were thawed and resuspended with a Teflon homogenizer. The suspension was treated by the method of NAKAO *et al.*[12] to remove more than 95% of the (Na^+,K^+)-independent activity, along with 70% of the total protein, resulting in a membranous enzyme preparation with a (Na^+,K^+)-ATPase specific activity of 40–50 μmoles/mg per h. The ratio of K^+-phosphatase activity to (Na^+,K^+)-ATPase activity was 0.74 with a standard deviation of 0.16 ($n = 20$).

Enzyme assays.

K^+-phosphatase activity was assayed in 1 ml of a medium containing 25 mM Tris, pH 7.4, at 37°, 0.4–0.6 mg protein, 4–6 mM acetyl phosphate (Tris salt), 5 mM $MgCl_2$ and 15 mM KCl. A blank omitting the KCl was subtracted to determine the

K^+-dependent activity. This activity agreed well with the ouabain-sensitive phosphatase activity. Disappearance of acetyl phosphate was followed by the method of LIPMANN AND TUTTLE[13], the assay being terminated with hydroxylamine reagent after 3 min. The loss of acetyl phosphate was linear with time and protein concentration in the range used in these studies.

(Na^+,K^+)-ATPase activity was measured in the same buffer system except that 100 mM NaCl was also present and 5 mM ATP was the substrate. A blank omitting both Na^+ and K^+ was subtracted before calculating the Na^+,K^+-dependent activity, which was equal to the ouabain-sensitive activity. Phosphate release was measured by the method of GOMORI[14] and was linear with time and protein concentration in the range used in these studies.

ADP–ATP exchange activity was measured by the method of GLAZE AND WADKINS[15]. Each tube contained 5 mM ADP and 3 mM ATP and there was no significant change in these concentrations during the course of the reaction. All other conditons were as for the (Na^+,K^+)-ATPase assay, except K^+ was omitted. A Na^+-free control was used to estimate the Na^+-independent activity.

Labeling studies.

The standard reaction mixture of 1 ml contained 1.2 mg of protein and various concentrations of other species and radioactive substrate. Na^+ and K^+, when added, were at 100 mM and 15 mM, respectively. Incubation was usually for 10 sec, always at 37°. The reaction was initiated with the radioactive substrate and terminated by adding 1.0 ml of 0.2 M lauryl sulfate, Tris salt, pH 7.4. The clarified mixture was placed immediately on a Sephadex G-50 column (1 cm × 90 cm) and the protein separated from low molecular weight labeled material by elution at room temperature with water. Fractions were collected and aliquots taken for duplicate protein assay and counting on a gas-flow counter. Protein was recovered quantitatively and the total incorporation of ^{32}P into this fraction was divided by the amount of protein to calculate the specific radioactivity.

MATERIALS

Carrier-free $H_3{}^{32}PO_4$ was purchased from New England Nuclear Corporation. The dilithium salt of acetyl phosphate was obtained from Worthington Biochemical Company and converted to the Tris salt by passage over Dowex-50–Tris. Miles Laboratories supplied disodium ATP which was also converted to the Tris salt. Sephadex G-50 and Trizma base (Tris) were from Sigma Chemical Company. Sodium lauryl sulfate was from Eastman Organic Chemicals, and was converted to the Tris salt. Mannitol, trichloroacetic acid, EDTA and other chemicals used were reagent grade.

Synthesis of acetyl [^{32}P]phosphate and [^{32}P]orthophosphate.

Carrier-free $^{32}P_i$ in 2 mM HCl was made 0.1 M in HCl and heated to 100° for 10 min to hydrolyze any pyrophosphate present. It was then applied to Dowex-1-OH^- (200–400 mesh, 1.3 mequiv/ml, 0.45 cm × 7 cm) and eluted with 0.1 M HCl to remove traces of radioactive impurities which remain on the column. The radioactive material was collected and evaporated to dryness at temperatures below 50°. The residue was taken up in 5 ml distilled water and evaporated to dryness five times to

remove the HCl. The final residue was taken up in 5 ml distilled water and an aliquot counted. If used for labeling studies, it was mixed with unlabeled orthophosphate converted to the Tris salt, pH 7.4.

For acetyl[^{32}P]phosphate synthesis[16], the carrier-free stock $^{32}P_i$ was mixed with 25 μl 1.0 M potassium phosphate, pH 7.4, and evaporated to dryness. The residue was dissolved in 100 μl distilled water, 40 μl pyridine was added and the mixture placed on ice. 10 μl acetic anhydride was added, followed after 2 min by 25 μl 4 M LiOH, and then by 1.15 ml absolute ethanol. This mixture was shaken for 1 h in the cold on a wrist-action shaker, and then filtered through a sintered glass filter, trapping the precipitated dilithium acetyl[^{32}P]phosphate. This precipitate was washed twice with 1.15 ml portions of ethanol and dried at room temperature in a vacuum desiccator. It was taken up in 3 ml of 0.1 M Tris, pH 7.6 at 37°, placed on a 0.45 cm × 7 cm Dowex-50–Tris column, and eluted in the cold with distilled water. After adjusting the pH to 7–8 with Tris, the concentration of acetyl phosphate was determined as the hydroxamate and the total phosphate and ^{32}P contents measured. Yields ranged from 80–100% and the product showed negligible contamination with orthophosphate. The infrared spectrum of the unadulterated radioactive acetyl phosphate showed the absence of 1580-cm^{-1} and 860-cm^{-1} peaks attributable to acetate and inorganic orthophosphate, respectively.

Synthesis of [γ-^{32}P]ATP.

Carrier-free $^{32}P_i$ was used as purchased in the procedure of GLYNN AND CHAPPELL[30] to make [γ-$^{32}P_i$]ATP. This material was neutralized with Tris base and most of the salt removed by passage through a column of Sephadex G-10.

RESULTS

Ion effects on labeling by acetyl [^{32}P]phosphate.

The effects of Mg^{2+}, Na^+ and K^+ on labeling of (Na^+,K^+)-ATPase preparations by acetyl [^{32}P]phosphate are shown in Table I. Numerous other experiments gave essentially the same results, although the total Mg^{2+}-dependent labeling varies somewhat with both enzyme activity and substrate concentration*. The binding of ^{32}P is unaffected by Na^+ or K^+, but is stimulated 1.6-fold by the addition of Mg^{2+}. The Mg^{2+}-dependent labeling is discharged or prevented by the presence of K^+ in the incubation medium. This K^+-dischargeable labeling has been previously reported by two other laboratories using acid precipitation to isolate the labeled membranes[7,8], but was not found by SACHS *et al.*[9], who showed a K^+-stimulated labeling.

Contrary to the previous reports cited[7,8], using our method of assay we do not see a stimulatory effect of Na^+ on the binding of ^{32}P to the enzyme preparation. Rather Na^+ seems to partially reverse the effect of K^+. Since we were concerned that our preparations might contain sufficient endogenous Na^+ to saturate a Na^+-dependent labeling process**, we prepared enzyme by a second procedure, substituting Tris

* Independent experiments show that extent of labeling is being measured, since total incorporation is constant between 5 and 45 sec. Despite variations in extent of labeling from preparation to preparation, the ion effects described remain reproducible. Addition of a large excess of unlabeled orthophosphate to the detergent used to stop the reaction did not decrease the extent of labeling.

** This possibility is unlikely, since exogenous Na^+ markedly stimulates phosphorylation of these same enzyme preparations by [γ-^{32}P]ATP (see DISCUSSION).

iodide for NaI in the enrichment procedure [12]. The labeling pattern observed with this enzyme preparation resembled closely that seen in Table I. A Mg^{2+}-dependent labeling was again seen, while the value in the presence of both Mg^{2+} and Na^+ was 74 % that with Mg^{2+} alone.

Labeling by [^{32}P]orthophosphate.

Because $^{32}P_i$ derived from acetyl [^{32}P]phosphate hydrolysis might produce the labeling seen, we measured the binding of radioactive orthophosphate to the protein under the same conditions. Fig.1 compares the labeling by both substrates over a similar concentration range. In contrast to the labeling by acetyl [^{32}P]phosphate, which is decreased by K^+, $^{32}P_i$ labeling is actually increased by this ion. Since the time of reaction with acetyl [^{32}P]phosphate never exceeded 10 sec, the actual concentrations of orthophosphate present were far below those of Fig. 1. Hence, labeling by orthophosphate is unlikely to account for the Mg^{2+}-dependent labeling with acetyl phosphate. Furthermore, the effect of K^+ on the amount of [^{32}P]-labeled enzyme produced differs for the two substrates.

Effect of K^+-phosphatase inhibitors.

Table II shows the effects of certain inhibitors of the K^+-phosphatase on the Mg^{2+}-dependent labeling of the enzyme by acetyl [^{32}P]phosphate. Incubation of the K^+ phosphatase with 4.5 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) at 37° comple-

TABLE I

EFFECT OF Mg^{2+}, Na^+ AND K^+ ON LABELING OF (Na^+, K^+)-ATPase PREPARATIONS BY ACETYL [^{32}P]PHOSPHATE

Enzyme was incubated with acetyl [^{32}P]phosphate at 37°. The reaction was terminated and amount of bound label determined as described under METHODS. Each experiment used a different enzyme preparation. All values are expressed relative to the Mg^{2+}-containing sample.

Expt.	Mg^{2+} (*mM*)	*Acetyl phosphate* (*mM*)	*Ions present*	*Relative amount of labeling*
1	1.0	0.45	Mg^{2+}	100**
			Mg^{2+}, K^+	71.5
			Mg^{2+}, Na^+	100.4
			Mg^{2+}, Na^+, K^+	83.4
2	0.5	0.5	Mg^{2+}	100**
			Mg^{2+}, K^+	70.1
			Mg^{2+}, Na^+	96.8
			Mg^{2+}, Na^+, K^+	98.3
3	5.0	1–3	Mg^{2+}	100** ($n = 8$)
			Mg^{2+}, K^+	54.6 ± 8.2* ($n = 4$)
			Mg^{2+}, Na^+	88.8 ± 7.8* ($n = 4$)
			Na^+	68.6 ± 13.6* ($n = 4$)
			K^+	62.4 ± 10.3* ($n = 4$)
			None	62.9 ± 6.1* ($n = 3$)

* Values are mean ± S.D.

** Labeling in the presence of Mg^{2+} varied between 14 and 22 pmoles ^{32}P per mg protein with 0.5 mM acetyl [^{32}P]phosphate and 250–500 pmoles/mg protein at higher substrate concentrations. The enzyme used in Expt. 3 was considerably more active than that used in either Expt. 1 or 2.

tely inactivates it in parallel with the (Na^+,K^+)-ATPase (Table III). Under these same conditions, the Na^+-dependent ADP–ATP exchange activity of the enzyme is also completely inhibited (Fig. 2). Inhibition of each enzymatic activity is reversed by addition of dithiothreitol (results not shown). Results to be reported elsewhere show that the Na^+-dependent labeling of the enzyme by [γ-^{32}P]ATP is also blocked by DTNB.

The inhibition of overall K^+-phosphatase activity by ATP and ADP has been reported previously[17], as has that by orthophosphate[18]. Both nucleotides inhibit the Mg^{2+}-dependent labeling by acetyl [^{32}P]phosphate. It appears that a portion of the non specific labeling is also blocked by ADP and ATP. The effect of orthophosphate is

TABLE II

EFFECT OF SEVERAL K^+-PHOSPHATASE INHIBITORS ON LABELING BY ACETYL [^{32}P]PHOSPHATE

Enzyme was incubated with 0.05 mM substrate for 5 sec at 37°. In the DTNB experiment, enzyme was incubated 10 min with DTNB before carrying out labeling. All samples contained 5 mM Mg^{2+}.

Expt.	*Additions or deletions*	*Labeling relative to control*
1	None	100
	+ 4.5 mM DTNB	68
	+ 4.5 mM DTNB, $-Mg^{2+}$	61
2	None	100
	+ 5 mM MgATP	41
	+ 5 mM MgADP	35
	+ 5 mM MgATP, + 100 mM Na^+	35
3	None	100*
	+ 13 mM P_i (Tris)	93
4	None	100**
	+ 15 mM P_i (Tris)	81

* 10 sec, 1.6 mM substrate.
** 10 sec, 2.3 mM substrate.

TABLE III

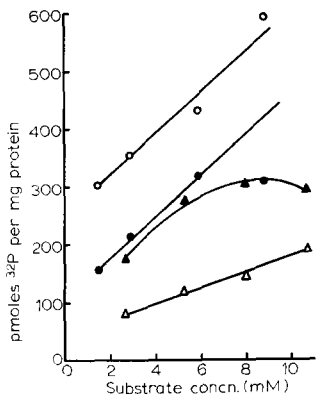
PARALLEL INHIBITION OF K^+-PHOSPHATASE AND (Na^+, K^+)-ATPase BY DTNB

The time shown in the first column is the duration of exposure to DTNB prior to addition of substrate. All samples contained DTNB during the additional 3 min required to measure activity remaining (which represents av. activity over that interval). DTNB concn., 4.5 mM.

Time (min)	*Relative activity**	
	(Na^+, K^+)-ATPase	*K^+-phosphatase*
0	21	24
1	17	19
5	0.3	4
10	0	0

* Untreated control taken as 100.

somewhat less than expected. The concentrations of orthophosphate used in Table II inhibit the K^+-phosphatase about 80 % when assayed at 5 mM acetyl phosphate concentration.



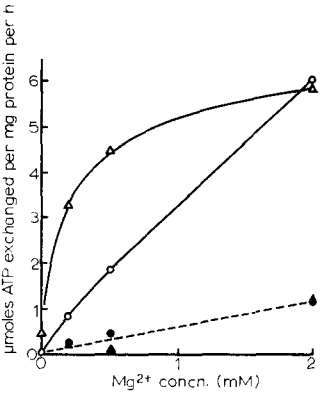


Fig. 1. Comparison of labeling of enzyme preparation by acetyl [^{32}P]phosphate and [^{32}P]orthophosphate. Enzyme was incubated for 10 sec at 37° with the radioactive substrate and the [^{32}P]enzyme analyzed as described under METHODS. Circles, acetyl [^{32}P]phosphate; triangles, [^{32}P]orthophosphate. Open symbols, Mg^{2+}; filled symbols, Mg^{2+}, K^+.

Fig. 2. Effect of DTNB on Na^+-dependent ADP–ATP exchange activity. Enzyme was incubated 10 min with and without 4.5 mM DTNB. The ADP–ATP substrate mixture was added and incubation continued for 16 min. Open symbols, control; filled symbols, with DTNB. Triangles, Mg^{2+}, Na^+; circles, Mg^{2+} only.

TABLE IV

EFFECT OF OUABAIN ON LABELING OF K^+-PHOSPHATASE BY ACETYL [^{32}P]PHOSPHATE

Enzyme was preincubated 30 min at 25° with 10^{-3} M ouabain in the absence of K^+. K^+ was added to the samples indicated and incubation continued for an additional 3 min at 37°. Labeling was then carried out as described under METHODS. In Expt. 3, values are mean ± S.D. (n = 3). Each experiment utilized a different enzyme preparation. N.D., not determined.

Expt.	*Ouabain*	*Relative labeling*		
		No added cations	*Mg^{2+}*	*Mg^{2+}, K^+*
1	—	N.D.	100	68.6
	+	N.D.	370	258
2	—	64.5	100	N.D.
	+	69.7	228	N.D.
3	—	N.D.	100	65.6 ± 6.7
	+	N.D.	411 ± 29	153 ± 38

Exchange experiments.

The reversibility of labeling by acetyl [^{32}P]phosphate was tested in two ways: (1) We looked for a [^{14}C]acetate–acetyl phosphate exchange reaction; and (2) we attempted to demonstrate transfer of ^{32}P from acetyl phosphate to ADP to form [γ-^{32}P]ATP. Neither of these reactions occurred to any detectable extent in media of

various ionic compositions. SACHS *et al.*[9] previously reported the absence of an acetyl phosphate–ATP exchange in similar preparations.

Effect of ouabain.

The effect of ouabain on the labeling of the enzyme by acetyl [^{32}P]phosphate is shown in Table IV. Curiously, a very large increase in the amount of bound radioactivity is observed, but this labeling is still partially decreased by K^+. The Mg^{2+}-independent labeling is unaffected by ouabain. These results differ from those seen with $^{32}P_i$ (Table V): With this substrate ouabain-treated enzyme shows an enhanced Mg^{2+}-dependent binding but the ability of K^+ to increase or decrease this labeling varies with the substrate concentration (columns 4 and 5, Table V).

TABLE V

EFFECT OF OUABAIN ON LABELING BY [^{32}P]ORTHOPHOSPHATE

Experimental procedure as in Table IV. Mg^{2+} concn., 5 mM.

$^{32}P_i$ concn. (mM)	*pmoles ^{32}P per mg protein*			
	Control		*Ouabain added*	
	Mg^{2+}	*Mg^{2+}, K^+*	*Mg^{2+}*	*Mg^{2+}, K^+*
2.7	79	176	138	185
5.3	119	277	189	215
8.0	145	304	234	221
10.7	191	299	260	214

DISCUSSION

As previously reported using the acid precipitation method[7,8], we find a K^+-dischargeable labeling of (Na^+, K^+)-ATPase preparations by acetyl [^{32}P]phosphate, but do not confirm the results of SACHS *et al.*[9] Our results do not discriminate between K^+ inhibition of labeling and a K^+-stimulated dephosphorylation, but the latter possibility seems more likely. In contrast to the previous reports[7,8], we do not find any Na^+-dependence of the labeling with this substrate. The lack of a Na^+ requirement contrasts strongly with the definite Na^+ stimulation of labeling by [γ-^{32}P]ATP seen in these same preparations using the detergent-gel filtration method (Table VI, refs. 10 and 28). Furthermore, use of Tris iodide to prepare enriched enzyme[12], which should deplete any endogenous Na^+ in the membrane preparation, produces no change in the labeling pattern (see text). Therefore, while it is possible that the effect of Na^+ may differ when the intermediate is trapped by the two different methods, it is nevertheless interesting that the ion effects observed in our studies are more easily reconciled to the known ion requirements of the enzyme. The K^+-phosphatase has no apparent Na^+ requirement, in fact being somewhat inhibited by added Na^+. Our particular enzyme preparation shows Na^+ inhibition to a degree comparable to the depression of the acetyl [^{32}P]phosphate labeling by the same concentration of Na^+.

Little is known about the Mg^{2+}-independent, K^+-stable labeling. It is not affected by either alkali metal ions or by ouabain, and on these grounds may probably be attributed to some process not involving the Na^+ pump. This fraction is also

TABLE VI

EFFECT OF Mg^{2+}, Na^+ AND K^+ ON LABELING OF (Na^+,K^+)-ATPase PREPARATIONS BY [γ-^{32}P]ATP

Enzyme was incubated with [γ-^{32}P]ATP at 37° and analyzed for bound radioactivity as described under METHODS. Expts. 1 and 2 represent different enzyme preparations. Numerous other enzyme preparations gave essentially the same results.

Expt.	*ATP* (μM)	*Ions present*	*Relative labeling**
1	15	Mg^{2+}	51.8 ± 9.9 (n = 5)
		Mg^{2+}, Na^+	100**
		Mg^{2+}, Na^+, K^+	37.9 ± 1.6 (n = 3)
2	34	Mg^{2+}	59.1, 64.7
		Mg^{2+}, Na^+	100
		Mg^{2+}, Na^+, K^+	31.6, 42.0
		Mg^{2+}, K^+	28.8, 26.9

* Mg^{2+}, Na^+-containing samples taken as 100.

** Labeling in the presence of Mg^{2+} and Na^+ averaged 62.4 pmoles/mg protein in Expt. 1 and 108.4 pmoles/mg protein in Expt. 2.

apparently stable to 0.5 M hydroxylamine, whereas the K^+-dischargeable fraction is not (unpublished results). The K^+-stable labeling is unaffected by the addition of unlabeled orthophosphate to the detergent used to stop the reaction, and apparently does not saturate easily when the reaction is carried out with added amounts of unlabeled substrate (Fig. 1). This behavoir contrasts with that of the K^+- dischargeable labeling, which is already apparently saturated at 2 mM acetyl phosphate. The inability to saturate the system with substrate in the presence of K^+ suggests some sort of physical occlusion process, not involving specific binding sites. A similar effect is seen with K^+-independent labeling by $^{32}P_i$.

The labeled protein derived from $^{32}P_i$ does not show a K^+-dischargeable component, but rather an increase in bound ^{32}P in the presence of this ion. Other workers have found amounts of acid-stable labeling with this substrate similar to those found here[19,20]. LINDENMAYER *et al.*[21] first reported a K^+-stimulated, acid-stable labeling using carrier-free $^{32}P_i$, but this labeling was abolished by adding carrier orthophosphate.

These results are consistent with the following scheme depicting the reaction sequence of the (Na^+,K^+)-ATPase.

$$ATP + E \overset{Mg^{2+},\,Na^+}{\rightleftharpoons} E_1\text{-}P + ADP \quad (1)$$

$$E_1\text{-}P \rightleftharpoons E_2\text{-}P \quad (2)$$

$$E_2\text{-}P \overset{K^+}{\rightleftharpoons} E + P_i \quad (3)$$

$$\text{Acetyl phosphate} + E \xrightarrow{Mg^{2+}} E_2\text{-}P + \text{acetate} \quad (4)$$

Reactions 1–3 have been postulated by numerous investigators as an overall reaction sequence for the (Na^+,K^+)-ATPase. Reaction 4 has simply been added to explain the observations here. In this latter reaction acetyl [^{32}P]phosphate is seen to phosphorylate the enzyme in the presence of Mg^{2+} to produce E_2–P, that phosphorylated form incapable of participating in ADP–ATP exchange[22]. This reaction is considered to

be irreversible because of the failure to observe [^{14}C]acetate–acetyl phosphate exchange under our experimental conditions (see text). The lack of reaction of E_2–P with ADP that we also find (text) agrees with the results of KUME AND POST[22] regarding the reversibility of Reaction 2. However, a dashed line is indicated for reversal of Reaction 2 since other workers[22–25] can reverse the pump under special conditions using intact erythrocyte ghosts, where defined interior and exterior compartments can be maintained experimentally.

Our results with K^+ using the two radioactive substrates suggest that this ion simply brings Reaction 3 into play, and that this reaction is somewhat readily reversible: A discharge of E_2–P occurs, but does not proceed to completion. Conversely, partial synthesis of E_2–P from E and P_i is allowed in the presence of K^+. Na^+ competes with K^+ for this step, but is not effective catalytically. Apparently the rate of the forward reaction is greater than that of the reverse, since unlabeled orthophosphate does not lower the extent of labeling very dramatically. In addition, orthophosphate has been shown to be competitive with *p*-nitrophenyl phosphate as substrate for the K^+-phosphatase[18], accounting for its effect on overall enzyme rate. Since we are measuring the extent of an irreversible labeling process, the level of labeling seen will not necessarily reflect the competitive inhibition by orthophosphate of its rate.

The effect of DTNB on labeling is consistent with, but does not prove the view that acetyl phosphate phosphorylates the enzyme. This is supported by the fact that DTNB blocks both ADP–ATP exchange activity and phosphorylation by [γ-^{32}P]ATP of the enzyme. If the phosphorylated intermediate derived from ATP and acetyl phosphate is indeed one and the same[8], one would then expect DTNB to inhibit its formation in either case, as observed here.

The stimulation of acetyl [^{32}P]phosphate labeling by ouabain, with retention of its K^+-sensitivity, is somewhat harder to understand. It is noteworthy that the amount of labeling in the presence of Mg^{2+}, K^+ and ouabain is generally higher than that in the presence of Mg^{2+} alone. Ouabain appears to stabilize the labeled enzyme regardless of whether orthophosphate or acetyl phosphate is the substrate. SEN *et al.*[29] have previously reported the stabilization of the phosphorylated form of the enzyme by ouabain[29]. However, under our conditions, blockage of the K^+-dependent discharge is not complete, even though overall K^+-phosphatase activity seems to be completely inhibited. On must remember that with the labeling reaction we are measuring a small amount of intermediate (of the order of pmoles), whereas overall enzyme rate measurements involve 10^6 times as much material being handled by the system. Since the turnover of the labeled enzyme seems to be so rapid (labeling is already at a maximum in 5 sec at 37°), a small 'leak' caused by K^+ will rapidly dissipate the intermediate even though very little enzymatic activity is occurring.

The effect of K^+ on labeling by $^{32}P_i$ in the presence of ouabain is somewhat variable, with a stimulation at low concentrations and a partial discharge at high concentrations (Table V). This latter observation may relate to the reported discrepancies in the literature regarding the role of K^+ in this process[19,21,26].

Nucleotide inhibition of acityl [^{32}P]phosphate labeling is independent of Na^+, an observation previously reported for overall K^+-phosphatase activity[8,27]. The unsuccessful exchange experiments attempted (text, ref. 9) appear to rule out turnover of a common intermediate as the means of depressing labeling.

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REFERENCES

1 H. BADER, R. L. POST AND G. H. BOND, *Biochim. Biophys. Acta*, 150 (1968) 41.
2 K. AHMED AND J. D. JUDAH, *Biochim. Biophys. Acta*, 93 (1964) 603.
3 M. FUJITA, T. NAKAO, Y. TASHIMA, N. MIZUNO, K. NAGANO AND M. NAKAO, *Biochim. Biophys. Acta*, 117 (1966) 42.
4 D. W. TOWLE AND J. H. COPENHAVER, JR., *Biochim. Biophys. Acta*, 203 (1970) 124.
5 S. UESUGI, N. C. DULAK, J. F. DIXON, T. D. HEXUM, J. L. DAHL, J. F. PERDUE AND L. E. HOKIN, *J. Biol. Chem.*, 246 (1971) 531.
6 D. A. HEMS, *J. Neurochem.*, 16 (1969) 277.
7 G. H. BOND, H. BADER AND R. L. POST, *Fed. Proc.*, 25 (1966) 567.
8 Y. ISRAEL AND E. TITUS, *Biochim. Biophys. Acta*, 139 (1967) 450.
9 G. SACHS, J. D. ROSE AND B. I. HIRSCHOWITZ, *Arch. Biochem. Biophys.*, 119 (1967) 277.
10 K. NAGANO, N. MIZUNO, M. FUJITA, Y. TASHIMA, T. NAKAO AND M. NAKAO, *Biochim. Biophys. Acta*, 143 (1967) 239.
11 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J Biol. Chem.*, 193 (1951) 265.
12 T. NAKAO, Y. TASHIMA, K. NAGANO AND M. NAKAO, *Biochem. Biophys. Res. Commun.*, 19 (1965) 755.
13 F. LIPMANN AND L. C. TUTTLE, *J. Biol. Chem.*, 159 (1945) 21.
14 G. GOMORI, *J. Lab. Clin. Med.*, 27 (1941–42) 955.
15 R. P. GLAZE AND C. L. WADKINS, *J. Biol. Chem.*, 242 (1967) 2139.
16 A. KORNBERG, S. R. KORNBERG AND E. S. SIMMS, *Biochim. Biophys. Acta*, 20 (1956) 215.
17 K. NAGAI AND H. YOSHIDA, *Biochim. Biophys. Acta*, 128 (1966) 410.
18 K. NAGAI, F. IZUMA AND H. YOSHIDA, *J. Biochem. Tokyo*, 59 (1966) 295.
19 R. W. ALBERS, G. J. KOVAL AND G. J. SIEGEL, *Mol. Pharmacol.*, 4 (1968) 324.
20 G. J. SIEGEL, G. J. KOVAL AND R. W. ALBERS, *J. Biol. Chem.*, 244 (1969) 3264.
21 G. E. LINDENMAYER, A. H. LAUGHTER AND A. SCHWARTZ, *Arch. Biochem. Biophys.*, 127 (1968) 187.
22 S. KUME AND R. L. POST, *Fed. Proc.*, 29 (1970) 539.
23 P. J. GARRAHAN AND I. M. GLYNN, *Nature*, 211 (1966) 1414.
24 A. F. LANT AND R. WHITTAM, *J. Physiol.*, 199 (1968) 457.
25 A. G. DAWSON AND R. WHITTAM, *Biochim. Biophys. Acta*, 203 (1970) 590.
26 G. E. LINDENMAYER AND A. SCHWARTZ, *Arch. Biochem. Biophys.*, 140 (1970) 371.
27 H. BADER AND A. K. SEN, *Biochim. Biophys. Acta*, 118 (1966) 116.
28 K. NAGANO, T. KANAZAWA, N. MIZUNO, Y. TASHIMA, T. NAKAO AND M. NAKAO, *Biochem. Biophys. Res. Commun.*, 19 (1965) 759.
29 A. K. SEN, T. TOBIN AND R. L. POST, *J. Biol. Chem.*, 244 (1969) 6596.
30 I. M. GLYNN AND J. B. CHAPPELL, *Biochem. J.*, 90 (1964) 147.