MOLECULAR PHARMACOLOGY, 6, 99-107

Ouabain-Dependent Incorporation of ^{32}P from *p*-Nitrophenyl Phosphate into a Microsomal Phosphatase

CHARLES E. INTURRISI[1] AND ELWOOD TITUS

Laboratory of Chemical Pharmacology, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014

(Received November 10, 1969)

SUMMARY

The microsomal fraction of beef brain that contains both ($Na^+ + K^+$)-dependent ATPase and K^+-dependent phosphatase activities can, in the presence of ouabain, be labeled with ^{32}P from *p*-nitrophenyl phosphate-[^{32}P]. The ouabain-dependent labeling requires Mg^{++}, but not Na^+ or K^+. When a steady-state level of ouabain-dependent labeling is maintained with a saturating concentration of *p*-nitrophenyl phosphate-[^{32}P], the specific activity of the phosphoprotein can be reduced by addition of unlabeled *p*-nitrophenyl phosphate or P_i. If, however, $^{32}P_i$ of the same specific activity as the labeled *p*-nitrophenyl phosphate is added, there is no increase in the absolute amount of phosphoprotein labeled by the ouabain-dependent mechanism. The conditions that support ouabain-dependent labeling from *p*-nitrophenyl phosphate-[^{32}P] also permit labeling by $^{32}P_i$. However, the amount of P_i generated by hydrolysis of *p*-nitrophenyl phosphate cannot account for the labeling observed. These studies suggest that ouabain places the phosphatase in a configuration that allows *p*-nitrophenyl phosphate and P_i to label the same site on the enzyme.

INTRODUCTION

Many animal cells utilize the chemical energy derived from the hydrolysis of adenosine triphosphate to accumulate K^+ and expel Na^+ against transmembrane concentration gradients (1). An ATPase which requires both Na^+ and K^+ for its activity can be obtained from the membranes or microsomal fractions of numerous tissues. Since this hydrolytic enzyme is a component of the ion transport system (2), there is considerable interest in the mechanism by which the monovalent cations initiate its action. One of the properties which the intact transport system, as measured in the red cell, has in common with the microsomal ($Na^+ + K^+$)-ATPase[2] is inhibition by cardiac glycosides such as ouabain (1, 2). The manner in which ouabain alters the hydrolysis of ATP is therefore also under intensive investigation.

Mechanistic studies on ATPase have been greatly aided by the recognition that the enzymatic reaction occurs in at least two steps, an initial Na^+-induced transfer of the terminal phosphate of AT^{32}P to microsomal protein, and a subsequent K^+-induced dephosphorylation (3).

This differentiation of steps stems largely

[1] Formerly Research Associate in the Pharmacology and Toxicology Program at the National Institute of General Medical Sciences. Present address: Department of Pharmacology, Cornell University Medical College, New York, New York 10021.

[2] The abbreviation used is: ($Na^+ + K^+$)-ATPase, adenosine triphosphatase requiring both $Na^+ + K^+$.

from the observation that selective inhibitors such as *N*-ethylmaleimide and oligomycin permit the Na^+-dependent accumulation of phosphoprotein while blocking the production of P_i by (Na^+ + K^+)-ATPase (3, 4).

Recent observations have suggested that it might be possible to study the terminal K^+-dependent step independently by using substrates that do not react with the Na^+-dependent phosphotransferase step but are attacked by the K^+-requiring component. Thus, microsomal preparations that contain the (Na^+ + K^+)-ATPase invariably contain a neutral phosphatase activity which requires K^+, but not Na^+, to hydrolyze substrates, such as acetyl phosphate (5–7), carbamyl phosphate (8), and *p*-nitrophenyl phosphate (9–13). Much evidence has been presented to suggest that the K^+-dependent phosphatase represents the K^+ step of the (Na^+ + K^+)-ATPase (1). The inhibition by ouabain of both the (Na^+ + K^+)-ATPase and the K^+-dependent phosphatase is consistent with the view that the site of action of cardiac glycosides is at the K^+ step (1, 2).

The observations reported here were made as part of an attempt to label phosphoprotein intermediates in the final K^+-dependent step by introducing radioactive phosphorus from *p*-nitrophenyl phosphate-[^{32}P]. A ouabain-dependent phosphorylation has been observed, and the conditions under which this occurs are examined in the present report. The results suggest that *p*-nitrophenyl phosphate and P_i label a common site.

METHODS

Enzyme preparation. Beef brain (Na^+ + K^+)-ATPase was prepared as described by Schoner *et al.* (14). This is essentially a microsomal preparation, but the particles are exposed to sodium deoxycholate and 2,3-dimercaptopropanol and passed over a column of glass beads in processing. Preparation 1, which was used in all experiments except those of Fig. 3, had a K^+-dependent *p*-nitrophenyl phosphatase activity, determined as described by Inturrisi and Titus (4), of 0.258 μmole/min/mg of protein. The activity of preparation 2, used to obtain the data in Fig. 3, was 0.178 μmole/min/mg of protein.

Rat kidney microsomes,[3] prepared by the method of Skou (15), possessed a K^+-dependent *p*-nitrophenyl phosphatase activity of 0.227 μmole/min/mg of protein.

Incorporation of ^{32}P into microsomes. The steady-state level of ^{32}P in microsomes was determined after incubation at 37° for 2 min in a medium of 1.25 mM $MgCl_2$, 25 mM Tris-HCl (pH 7.4), and various concentrations of disodium *p*-nitrophenyl phosphate-[^{32}P] (specific activity, 4.5–5.7 × 10^6 cpm/μmole; Amersham/Searle). The reaction was initiated by the addition of 1.5–1.8 mg of microsomal protein to the incubation medium in 13-ml polycarbonate centrifuge tubes and stopped by the addition of 5 ml of ice-cold 15% (w/v) trichloracetic acid. The labeled protein was recovered by a modification of the procedure of Post *et al.* (16). The trichloracetic acid suspension was centrifuged for 20 min at 10,000 rpm in the SM 24 rotor of a Sorvall RC-2 refrigerated centrifuge. The trichloracetic acid precipitate was resuspended with the aid of a glass rod in 5 ml of 10% trichloracetic acid containing 4–20 mM unlabeled disodium *p*-nitrophenyl phosphate and 50 mM KH_2PO_4 and centrifuged for 15 min as above. The precipitate was resuspended and washed twice with 5% trichloracetic acid. When ^{32}P incorporation from Tris-phosphate-[^{32}P] (prepared from carrier-free orthophosphate purchased from Tracerlab) was to be determined, unlabeled 0.2 M H_3PO_4 was added to all the trichloroacetic acid wash solutions. The precipitate was dissolved in 0.6 ml of 3% Na_2CO_3–0.4% NaOH and transferred to a 13-ml glass centrifuge tube. The tubes were covered and heated for 10–15 min on a boiling water bath and allowed to cool, and a 0.05-ml aliquot was taken for protein determination. Another 0.50-ml aliquot was transferred to a counting vial and mixed with 0.05 ml of 6 N HCl, followed by 20 ml of the scintillator fluid described by Cho and Curry (17). All determinations were made in triplicate; the mean values are reported. Blanks were measured by adding the trichloracetic acid

[3] Generously supplied by Dr. Colin F. Chignell.

before the microsomes or by adding microsomes which had been boiled for 5 min at 100°. The recovery of protein was 65–70% as measured by the method of Lowry *et al.* (18). A 1-ml aliquot of the first trichloracetic acid supernatant was counted before and after addition of charcoal to estimate $^{32}P_i$ released from *p*-nitrophenyl phosphate-[^{32}P]. The method was adapted from that described for nucleotides by Crane and Lipmann (19).

Preparation of "ouabain-enzyme." Between 15 and 20 mg of microsomes were incubated with 2.5 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.4), and 0.5 mM ouabain for 30 min at 37°. The microsomes were then labeled immediately (unwashed) or washed as described by Albers *et al.* (20) prior to labeling. In the washed microsomes more than 95% of the K^+-dependent *p*-nitrophenyl phosphatase and (Na^+ + K^+)-ATPase activities were inhibited when measured as described by Inturrisi and Titus (4). In the absence of ouabain, the 30-min incubation and subsequent washing did not decrease phosphatase or ATPase activity.

RESULTS

Incorporation of ^{32}P from p-nitrophenyl phosphate-[^{32}P] into microsomes. The results of attempts to demonstrate a cation-dependent phosphoprotein intermediate in the *p*-nitrophenyl phosphatase reaction are given in Table 1. Potassium was without effect. Sodium, either alone or in combination with ATP or oligomycin, could not increase the level of ^{32}P in microsomes incubated under conditions that favor Na^+-dependent phosphorylation by AT^{32}P (3, 16, 21). In other experiments, increasing the Na^+ concentration by 30-mM increments up to 120 mM was without effect.

In the presence of 0.5 mM ouabain, a large increase in the incorporation of ^{32}P into beef brain microsomes was observed (Table 1). A somewhat smaller increase was seen in the labeling of rat kidney microsomes.

In the presence of Mg^{++}, ouabain, and *p*-nitrophenyl phosphate-[^{32}P] at 37°, the level of ^{32}P in microsomal protein reaches a steady state in 1 min, and this persists up to at least 7 min. The observations shown in Table 2 were therefore made 5 min after starting the reaction. Simple omission of Mg^{++} decreased the level of phosphorylation by 40%, and addition of EDTA to the Mg^{++}-free medium abolished the labeling. Mg^{++} is thus required for the reaction, and some of the activating cation is apparently firmly bound to the enzyme. In other experiments, not shown in Table 2, 0.062 mM Mg^{++} proved adequate for maximal labeling, and increases up to 12.5 mM did not further increase the level of phosphorylation.

When the temperature was reduced to 0°, ouabain-dependent labeling did not occur, even in incubations prolonged to 10 min. Since Albers *et al.* (20) have shown that both the inhibition of the (Na^+ + K^+)-ATPase by ouabain and the essentially irreversible binding of tritiated ouabain to electroplax enzyme are temperature-dependent, it seemed likely that failure to bind ouabain was responsible for the lack of phosphorylation at low temperature. "Ouabain-enzyme" was therefore prepared by

TABLE 1

Conditions for incorporation of ^{32}P into microsomes

Each tube contained 0.4 mM *p*-nitrophenyl phosphate-[^{32}P], 1.25 mM $MgCl_2$, 25 mM Tris-HCl (pH 7.4), and 1.5–1.8 mg of microsomes in a final volume of 1 ml. The additions were, as indicated, 30 mM Na^+, 0.1 mM ATP, 0.1 mM oligomycin, 1 mM K^+, and 0.5 mM ouabain. Incubation was conducted for 2 min at 37°. Reaction mixtures in experiment A contained 1.82×10^6 cpm/tube, and in experiment B, 1.46×10^6 cpm/tube.

Addition	^{32}P incorporation	
	cpm/mg protein	*μμmoles/mg protein*
A. Beef brain microsomes		
No addition	170	37
Na^+	217	46
Na^+ + ATP	192	42
ATP	107	23
Na^+ + oligomycin	223	49
K^+	97	22
Ouabain	2181	487
B. Rat kidney microsomes		
No addition	460	131
Na^+	213	59
Ouabain	1281	365

TABLE 2

Factors affecting ouabain-dependent incorporation of ^{32}P into beef brain microsomes

Final concentrations were 0.4 mM *p*-nitrophenyl phosphate-[^{32}P], 25 mM Tris-HCl (pH 7.4), and, as indicated, 0.5 mM ouabain, 1.25 mM $MgCl_2$, or 2 mM disodium EDTA. "Ouabain-enzyme" was prepared as described in METHODS.

Additions	Incubation		^{32}P incorporation
	Time	Temperature	
	min		*μμmoles/mg protein*
Untreated enzyme			
Mg^{++} + ouabain	5	37°	512
Ouabain	5	37°	309
EDTA + ouabain	5	37°	47
Mg^{++} + ouabain	10	0°	57
"Ouabain-enzyme"			
Mg^{++}	5	37°	569
Mg^{++}	5	0°	314
Mg^{++}	10	0°	317

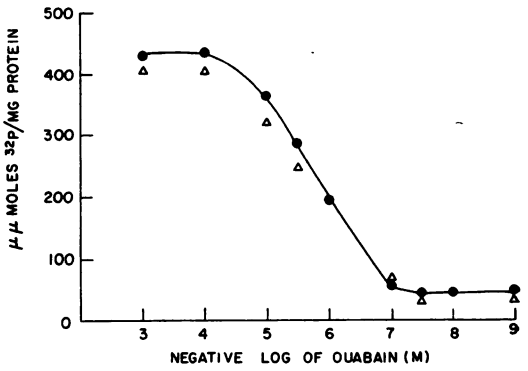

FIG. 1. *Effect of ouabain concentration on ouabain-dependent incorporation of ^{32}P into beef brain microsomes*

The conditions were as described in Table 1, with the concentration of ouabain varied as indicated. At 2 min either 30 mM KCl (△) or an equal volume of deionized water (●) was added, and the incubation was continued for an additional 3 min. The ^{32}P labeling, in picomoles of ^{32}P per milligram of protein, refers to the ouabain-dependent increment determined by subtracting the level in the absence of ouabain from the total incorporation in the presence of ouabain.

incubation of ouabain with microsomes at 37° and washed free of unbound ouabain as described in METHODS. This preparation, when cooled to 0°, incorporated ^{32}P to a steady-state level about 55% of that at 37°.

The extent of phosphorylation of the microsomal preparation is presented as a function of ouabain concentration in Fig. 1. Maximal labeling was reached at about 0.1 mM. The concentration for half-maximal labeling calculated from these data was 1.95×10^{-6} M, in good agreement with that for half-maximal inhibition of the over-all ($Na^+ + K^+$)-ATPase activity (14, 22).

In some of the experiments of Fig. 1, 30 mM K^+ was added in order to determine whether any competitive displacement of ouabain might be demonstrable. Were this the case, the addition of K^+, especially to those samples containing low concentrations of ouabain, might reduce the steady-state level of intermediate. However, the addition of K^+ 2 min after the initiation of the reaction did not significantly decrease the labeling in Fig. 1. Potassium ion was also without effect when added initially with the ouabain. The effects of this steroid on the phosphorylation from *p*-nitrophenyl phosphate, like those on the ($Na^+ + K^+$)-ATPase, are thus essentially irreversible.

Effects of various compounds on ouabain-dependent phosphorylation. Table 3 shows the effect of adding a number of unlabeled phosphate derivatives on the levels of phosphoprotein labeled by *p*-nitrophenyl phosphate-[^{32}P]. Phosphate compounds that are not substrates for K^+-dependent *p*-nitrophenyl phosphatase, such as glucose 6-phosphate, β-glycerophosphate, and phenyl phosphate (13), had insignificant effects on labeling. Partial reduction was produced by several adenine nucleotides which also are not substrates for the enzyme. ATP (22) and ADP (13) are, however, inhibitors of K^+-dependent phosphatases.

The effect of Tris-phosphate was of interest, since ouabain-dependent labeling by P_i had been demonstrated earlier (20), and it seemed likely that the effect noted in Table 3 could result from substitution of nonradioactive P_i into the same site labeled by *p*-nitrophenyl phosphate. The effect was therefore examined further. Figure 2 shows the extent of ouabain-dependent

TABLE 3

Effect of various compounds on ouabain-dependent incorporation of ^{32}P into beef brain microsomes

Each tube contained 0.5 mM ouabain, and conditions were as described in Table 1. Added compounds were at 2 mM concentration except for ATP, which was added at 10 mM.

Addition	^{32}P incorporation	Decrease in ^{32}P labeling
	μμmoles/mg protein	%
None	445	
Glucose 6-phosphate	411	8
p-Nitrophenol	410	8
β-Glycerophosphate	403	9
Phenyl phosphate	357	20
3′,5′-Cyclic AMP	352	21
5′-AMP	294	35
ATP	265	41
ADP	161	64
p-Nitrophenyl phosphate	161	64
Tris-phosphate	83	81

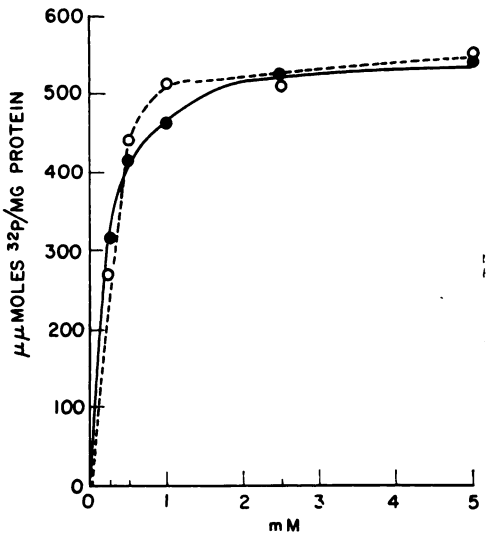

FIG. 2. *Effect of concentration of p-nitrophenyl phosphate-[^{32}P] or $^{32}P_i$ on ouabain-dependent incorporation of ^{32}P into beef brain microsomes*

The conditions were as described in Table 1. The concentration of disodium *p*-nitrophenyl phosphate-[^{32}P] (●) or Tris-phosphate-[^{32}P] (○) was varied as indicated. The data are for the ouabain-dependent increment, as in Fig. 1.

phosphorylation in the steady state as a function of $^{32}P_i$ or *p*-nitrophenyl phosphate-[^{32}P] concentration in the medium. The background radioactivity which was associated with protein and which persisted after washing the trichloracetic acid precipitate was consistently somewhat higher with P_i than with *p*-nitrophenyl phosphate-[^{32}P], but the ouabain-dependent labeling by the two sources was very similar.

Since the nature of these experiments required the use of unusually large concentrations of enzyme, there was always some slow hydrolysis of *p*-nitrophenyl phosphate by the Mg^{++}-dependent baseline activity that is not responsive to K^+. However, the phosphorylation of protein by P_i which was first released by this mechanism could not account for the labeling in the presence of *p*-nitrophenyl phosphate-[^{32}P]. Thus, with 0.4 mM *p*-nitrophenylphosphate-[^{32}P], a steady-state level of ouabain-dependent phosphorylation of 424 μμmoles of ^{32}P per milligram of protein was reached. At the time the steady state was attained, the level of $^{32}P_i$ in solution had reached 0.030 mM. With 4.0 mM *p*-nitrophenyl phosphate-[^{32}P], the steady-state level of phosphoprotein achieved in 2.0 min was 569 μμmoles of ^{32}P per milligram of protein, and $^{32}P_i$ had reached 0.088 mM. This amount could not account for more than 10–15% of the ^{32}P labeling (see Fig. 2).

From Fig. 2, it is apparent that 4.0 mM *p*-nitrophenyl phosphate-[^{32}P] is sufficient to saturate the phosphoprotein. This conconcentration was therefore used to establish the steady-state level of phosphoprotein in the exchange experiments of Fig. 3. The subsequent addition of an equimolar quantity of either unlabeled *p*-nitrophenyl phosphate or unlabeled Tris-phosphate resulted in a rapid loss of label and the establishment of a new steady state.

If, as suggested by Table 3 and Fig. 3, *p*-nitrophenyl phosphate and P_i label the same site on the enzyme, the addition of Tris-phosphate-[^{32}P] of the same specific activity as the *p*-nitrophenyl phosphate-[^{32}P] should not significantly change the ouabain-dependent steady-state level of phosphoprotein labeled by *p*-nitrophenyl phosphate. This was confirmed by the experiments of Table 4. The addition of Tris-

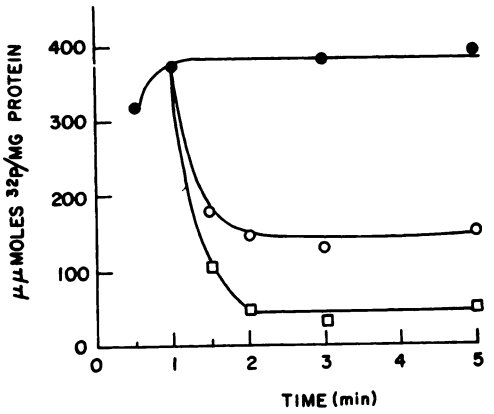

FIG. 3. *Effect of the addition of unlabeled p-nitrophenyl phosphate or phosphate on the steady-state level of ^{32}P microsomes labeled from p-nitrophenyl phosphate-[^{32}P]*

The microsomes were incubated with ouabain as described in METHODS and then added, unwashed, to tubes containing 4.0 mM *p*-nitrophenyl phosphate-[^{32}P]. After 1 min at 37°, the following additions were made: 4.0 mM disodium *p*-nitrophenyl phosphate (○), 4.0 mM Tris-phosphate (□), or deionized water (●). The incubation was then terminated as indicated. The data are for the ouabain-dependent increment, as in Fig. 1.

phosphate-[^{32}P] did not significantly change the level.

Properties of phosphoprotein intermediate. Trichloracetic acid precipitates from microsomal preparations labeled in the presence of ouabain by *p*-nitrophenyl phosphate-[^{32}P] and by $^{32}P_i$ were prepared. Since only small amounts, never exceeding 15%, of the total radioactivity could be removed by the lipid extraction procedure of Kennedy (23), the results could not be due to ouabain-dependent labeling of phospholipid.

Partial hydrolysis of 1 mg of the trichloracetic acid precipitate with pepsin according to the procedure of Albers *et al.* (24) resulted in solubilization of 84% of the precipitate labeled by $^{32}P_i$ and 78% of that introduced from *p*-nitrophenyl phosphate-[^{32}P]. The rates of hydrolysis were not significantly different. Treatment of the trichloracetic acid precipitate with 2 M NH_2OH at pH 6.5 for 30 min at 22° released 80% of the bound ^{32}P, as would be expected if the phosphoprotein were an acyl phosphate (25).

TABLE 4

Effect of addition of $^{32}P_i$ to a steady-state level of ^{32}P microsomes labeled from p-nitrophenyl phosphate-[^{32}P]

Each tube contained 4.0 mM *p*-nitrophenyl phosphate-[^{32}P] (2.7×10^6 cpm/tube), 1.25 mM $MgCl_2$, 25 mM Tris-HCl (pH 7.4), and 1.7 mg of beef brain microsomes in a final volume of 1 ml. Ouabain, where indicated, was added at 0.5 mM. After 2 min at 37°, 4.0 mM Tris-phosphate-[^{32}P] (2.7×10^6 cpm/tube) was added, and the incubation was continued for an additional 3 min.

Time	$^{32}P_i$	^{32}P incorporation		
		With ouabain (A)	No ouabain (B)	A − B[a]
min		*μμmoles/mg protein*		
2	−	647	79	568
5	−	693	129	564
	+	934	333	601

[a] Ouabain-dependent incorporation.

DISCUSSION

An attempt to explain the relationship of the K^+-dependent phosphatase to the $(Na^+ + K^+)$-ATPase must take into account the known sequence of intermediate reactions of the latter. In the reaction scheme first proposed by Albers (3) and now widely accepted, Na^+ stimulates a reversible phosphorylation of enzyme protein by the terminal phosphate of ATP (26, 27). In the presence of sufficient Mg^{++}, the phosphoprotein is converted by reaction 2 to a form which can now be dephosphorylated by the K^+-dependent reaction 3.

$$E + \text{ATP} \underset{\cdot}{\overset{Na^+ + Mg^{++}}{\longleftrightarrow}} E \sim \text{P} + \text{ADP} \quad (1)$$

$$E \sim \text{P} \xrightarrow{Mg^{++}} E\text{—P} \quad (2)$$

$$E\text{—P} + H_2O \xrightarrow{K^+} E + P_i \quad (3)$$

Addition of oligomycin or prior treatment with *N*-ethylmaleimide apparently blocks reaction 2, since the reversibility of reaction 1 becomes demonstrable (26, 27) under these conditions and the over-all $(Na^+ + K^+)$-ATPase reaction is blocked, but the K^+-

dependent phosphatase activity with acetyl phosphate or *p*-nitrophenyl phosphate as substrate is either unaffected (oligomycin) or only slightly inhibited (*N*-ethylmaleimide) (4). Ouabain inhibits both the ($Na^+ + K^+$)-ATPase and the K^+-dependent phosphatase and does not enhance the Na^+-dependent ADP-ATP transphosphorylation (22, 26, 27). The substrate, *p*-nitrophenyl phosphate, must therefore enter the reaction sequence after step 2, and the effect of ouabain is to inhibit step 3. The rate of formation of the irreversible enzyme-ouabain complex, however, is strongly dependent on the events in steps 1 and 2. Post *et al.* (28) have recently reviewed the evidence that the transformation of $E \sim P$ to E—P represents a change in conformation of the enzyme system, and that the latter of these phosphorylated forms preferentially reacts with ouabain. If the dephospho-enzyme is stabilized in the reactive conformation with magnesium, the binding of ouabain is enhanced by the presence of P_i, and cooperative effects of P_i and ouabain may account for the fact that $^{32}P_i$ is rapidly incorporated into phosphoprotein by several ATPase preparations (20, 28, 29) when Mg^{++} and the steroid are present. Lindemayer *et al.* (29) reported that incorporation into a preparation from calf brain was further enhanced by K^+, but no K^+ requirement was demonstrable in the present work with either $^{32}P_i$ or *p*-nitrophenyl phosphate-[^{32}P] at K^+ concentrations up to 30 mM. In the ($Na^+ + K^+$)-ATPase preparation from eel electroplax, ouabain can be bound to about the same extent in samples treated with *N*-ethylmaleimide as in controls, but the incorporation of $^{32}P_i$ does not occur with the treated enzyme.

One of the most interesting problems in the ATPase mechanism is the chemical nature of the phosphoprotein intermediate. This is recovered from reaction mixtures by precipitation with trichloracetic acid (16). In the material thus isolated, the phosphate is linked as an anhydride of a carboxyl group (25), presumably from a γ-glutamyl moiety (30). Although the studies on the effects of inhibitors and magnesium can best be explained by the existence of two forms of phosphorylated intermediate, there is no evidence to indicate that more than one form is isolated by trichloracetic acid precipitation. Paper electrophoretic patterns of the peptides obtained after peptic digestion of trichloracetic acid precipitates reveal that the same site is labeled by AT^{32}P in the sodium-dependent mechanism, by $^{32}P_i$ in the ouabain-dependent mechanism (28, 31), and by acetyl phosphate (22) in an apparently sodium-dependent (32) mechanism. In the present studies we have been unable to demonstrate labeling of microsomal protein from *p*-nitrophenyl phosphate-[^{32}P] under conditions that support labeling by AT^{32}P or acetyl phosphate-[^{32}P]. The ouabain-dependent reaction with *p*-nitrophenyl phosphate, however, apparently labels the same site as all the other phosphate sources. It remains puzzling that P_i and a relatively low-energy phosphate ester such as *p*-nitrophenyl phosphate should equilibrate so readily with what appears to be a protein-phosphate bond of high energy, since the latter exhibits properties of an acyl phosphate in trichloracetic acid precipitates. Possible explanations of this have been discussed (20, 31). In any case, our observations suggest that *p*-nitrophenyl phosphate has access to the same site as other substrates and products of the ($Na^+ + K^+$)-ATPase complex. It is interesting in this connection that β-glycerophosphate and glucose 6-phosphate, classically used substrates for alkaline phosphatase, are neither inhibitors nor substrates of K^+-dependent phosphatase, and neither inhibit nor donate phosphorus in the ouabain-dependent reaction with *p*-nitrophenyl phosphate.

Although the site that is labeled by phosphate donors and is responsive to sodium in the reactions discussed above must be a component of the active center, the fact that it is labeled by *p*-nitrophenyl phosphate does not necessarily indicate that it is the catalytic center responsible for the K^+-dependent hydrolytic attack. Nucleophilic groups capable of initiating the latter process might be expected to react with diisopropyl fluorophosphate, and inhibition of ($Na^+ + K^+$)-ATPase from brain and kidney has indeed been observed after exposure to this agent (33, 34). These observations by Hokin and collaborators were confirmed

by our experiments with beef brain enzyme.[4] After 20 min of exposure to concentrations of 1, 2, and 10 mM diisopropyl fluorophosphate at 37° ($Na^+ + K^+$)-ATPase activities were reduced to 86, 53, and 10%, respectively, of control values. The K^+-dependent *p*-nitrophenyl phosphatase activities (87, 58, and 20%) were not significantly different. The reaction mixture contained 3 mM Mg^{++} and 10 mM K^+. Omission of the latter decreased the effect of diisopropyl fluorophosphate against K^+-dependent *p*-nitrophenyl phosphatase by 44%, and that against the ATPase, by 38%.

These observations, together with the protection against inactivation afforded by the substrate, ATP (33), offer presumptive evidence that a common nucleophilic group plays a catalytic role in both the K^+-dependent *p*-nitrophenyl phosphatase and ($Na^+ + K^+$)-ATPase reactions. Recent studies suggest that this group may lie on a different protein from that labeled by ATP and P_i. That component which is labeled by diisopropyl fluorophosphate-[^{32}P] and which can be protected against the introduction of the diisopropyl fluorophosphate label by ATP was not identical with the component labeled by ATP and P_i when microsomal proteins from a rat kidney ATPase preparation were disaggregated and examined by electrophoresis on polyacrylamide (35). The simplest explanation for these observations would be that in the enzymatically active complex the protein carrying the phosphorylated intermediate and that carrying the attacking group are closely associated in such a way that adsorption of ATP on the first would protect the second against diisopropyl fluorophosphate.

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