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STUDIES ON THE INTERMEDIARY COMPLEXES OF SODIUM-POTASSIUM DEPENDENT ADENOSINETRIPHOSPHATASE FROM EEL ELECTRIC ORGAN

by

DAVID A. GEWIRTZ

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy. The City University of New York.

1977

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

STUDIES ON THE INTERMEDIARY COMPLEXES OF SODIUM-POTASSIUM DEPENDENT ADENOSINETRIPHOSPHATASE FROM EEL ELECTRIC ORGAN

by

David A. Gewirtz

Adviser: Dr. William A. Brodsky

A ^{14}C-labeled nucleotide-protein complex is observed after the steady state hydrolysis reaction at 0°C between eel electric organ (Na + K) ATPase and uniformly labeled [^{14}C] ATP ([U-^{14}C] ATP) is terminated by acid precipitation. Maximal levels of this complex are observed in the simultaneous presence of Mg, Na and K, and the formation of this complex is inhibited by ouabain.

An appreciable level of ^{32}P-phosphoprotein complex is observed after the steady state hydrolysis reaction at 0°C between eel electric organ (Na + K) ATPase and [γ-^{32}P] ATP in the presence of Mg, Na and K (as well as in the presence of Mg and Na) is terminated by acid precipitation. The formation of this complex is inhibited by ouabain.

The levels of the nucleotide-protein and phosphoprotein complexes observed in the simultaneous presence of Mg, Na and K show a dependency on the initial ATP

concentration similar to that of the overall hydrolysis. The rate of inorganic phosphate release is proportional to the steady state levels of the (Mg + Na + K)-dependent ^{14}C-nucleotide-protein complex and the (Mg + Na + K)-dependent ^{32}P-phosphoprotein complex; the breakdown of the (Mg + Na + K)-dependent ^{32}P-phosphoprotein is the rate-limiting step for the overall hydrolysis reaction.

When the hydrolytic reaction between eel electric organ (Na + K) ATPase and [γ-^{32}P] ATP is terminated at neutral pH by heat precipitation, a phosphoenzyme complex is formed which reaches maximal levels in the simultaneous presence of Mg, Na and K. After formation of a steady state level of phosphoenzyme in the presence of Mg and Na, a pulse of K increases the level of the heat-precipitated phosphoenzyme (while decreasing the level of the acid-precipitated phosphoenzyme).

The formation of the heat precipitated phosphoenzyme is clearly inhibited by ouabain only when the phosphoenzyme is formed in the presence of Mg, Na and K. Inorganic phosphate decreases the level of the heat-precipitated phosphoenzyme, but not that of the acid-precipitated phosphoenzyme (in the presence of Mg and Na or in that of Mg, Na and K). Moreover, a heat-precipitated, ouabain-sensitive phosphoenzyme forms in the reaction between the eel (Na + K) ATPase and $^{32}P_i$ with or without ATP.

The pH stability of the heat-precipitated phosphoenzyme complex is maximal at pH 6 to 8, and this complex shows little or no reactivity with neutral hydroxylamine, suggesting that the phosphate is not bound to an acyl residue of the protein.

These experiments indicate that both heat resistant and acid resistant phosphoenzymes are formed during the (Na + K) ATPase reaction at pH 7.4.

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Most of all, I am indebted to my wife, Fran, and to my parents and sister for their constant love and encouragement through the long and often discouraging days of my graduate career.

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INTRODUCTION

A. The Sodium Pump

The cell membrane separates the potassium-rich intracellular fluid from the sodium-rich extracellular fluid bathing the cells. The asymmetric distribution of cations between the two sides of the cell membrane is maintained by the sodium pump, which utilizes the energy derived from the hydrolysis of ATP to pump sodium ions out of the cell, and potassium ions into the cell (Glynn and Karlish, 1975).

The cell membrane is absolutely impermeable to the large polyvalent protein and organic phosphate anions contained within the cell, and relatively less permeable to sodium than to potassium. Potassium ions moving out of the cell along a concentration gradient are restrained by the increasing negative charge remaining within the cell (Pitts, 1968). In the resting or unstimulated state of the cell membrane, the cell interior remains approximately 70 to 80 milivolts negative with respect to the cell exterior, with most of the potential difference resulting from the separation of charge caused by the outward movement of potassium (there are also contributions to this potential from the asymmetric distribution across the cell membrane of the other less permeable ions).

As the equilibrium potential for potassium would be approximately -90 mV, there is a continuous potassium efflux along with a sodium influx. The sodium pump must pump potassium into and sodium out of the cell in order to maintain the potential gradients that would otherwise be dissipated by the passive ion movements (Sullivan, 1974).

The cell membrane is depolarized (actually, there is a reversal of membrane polarity) when an external or internal stimulus increases the membrane permeability to sodium, allowing a sudden influx of these positive ions from the extracellular fluid. The membrane returns to its original state of polarization and responsiveness to stimuli when an increase in the membrane permeability for potassium results in an efflux of potassium ions.

This series of events represents the cells' excitability mechanism, and allows for the transmission of impulses throughout the cells of the nervous system, for contraction of the muscle cells, and presumably for intracellular communication.

In excitatory tissues, such as the nerve, muscle, and brain, the function of the sodium pump, as described above, is to maintain a resting potential in the membrane which is to be the target of an excitatory stimulus. In the erythrocyte, and other cells as well, the expulsion of sodium prevents the swelling that would otherwise occur due to the high osmolarity of the cell interior. In the lens of the eye, the sodium pump prevents osmotic swelling which would result in opacity of the lens (Bonting, 1970).

The influx of sodium is sometimes coupled to the transport of glucose and amino acids, and the sodium pump works to maintain the gradient for continued sodium influx. For instance, in the intestine, the active transport of sodium from the epithelial cells to the blood sets up the gradient for the diffusion of sodium from the lumen into these cells. This carrier-mediated sodium diffusion provides for the co-transport of sugar and amino acids into the luminal epithelial cells.

In the kidney, the tubular reabsorption of sodium chloride is a primary homeostatic process for the prevention of salt and water loss. A sodium pump is located in the proximal tubule, distal tubule, and collecting duct. The pump responds to the mineralocorticoid, aldosterone, at the latter sites. In the loop of Henle, there is evidence that the sodium concentration gradients are maintained through the activity of a chloride pump (Sullivan, 1974).

The sodium pumps found in toad bladder as well as in the turtle urinary bladder are analagous to those in the human kidney tubules in that they are required for the reabsorption of salt and water from the filtered urine.

Of particular importance for the material presented below is the sodium pump found in the eel electric organ, as this is the tissue from which the (Na + K) ATPase used in our experiments was derived. This pump maintains a potential difference across each of thousands of electroplates set up in parallel in the organ (Keynes and Martins-Ferreira, 1953). In the resting state, at 24°C, each face of the electroplate (diagrammed in figure 1) maintains a resting potential of about 85 mV, with the inside of the cell membrane negative to the outside. On stimulation, the innervated face produces a large spike, at the peak of which the potential is reversed by approximately 65 mV (figure 2). The potential across the non-innervated face does not alter during this spike of about 2 msec.

As a result of these spikes, the voltage across the opposite faces of the electroplate are added together, each electroplate contributing approximately 150 mV to the total discharge.

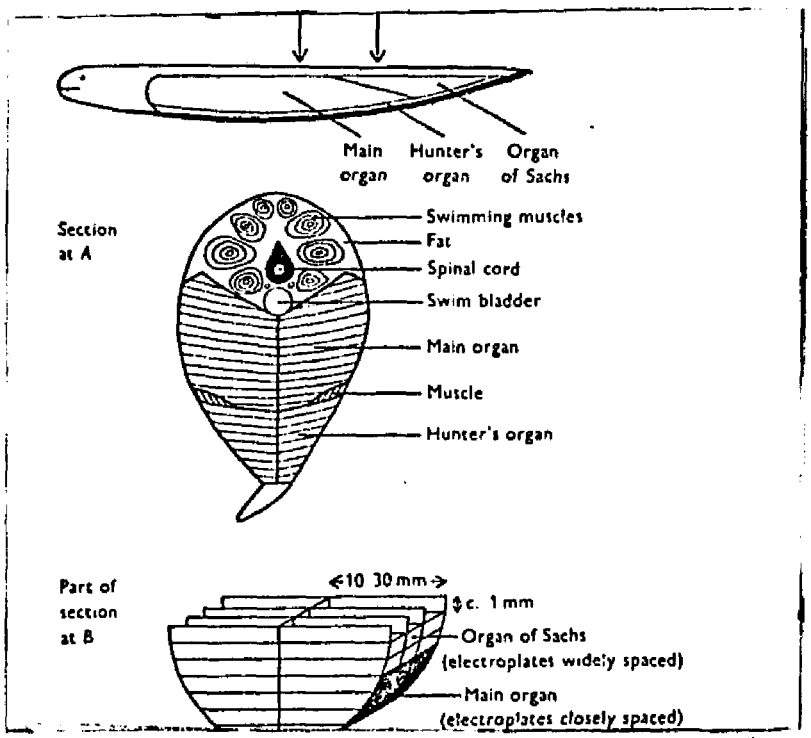

Figure 1. Diagrammatic representation of the anatomy of the electric organ in Electrophorus electricus. The lower section shows the arrangement of the electroplates in columns (from Keynes and Martins Ferreira, 1953).

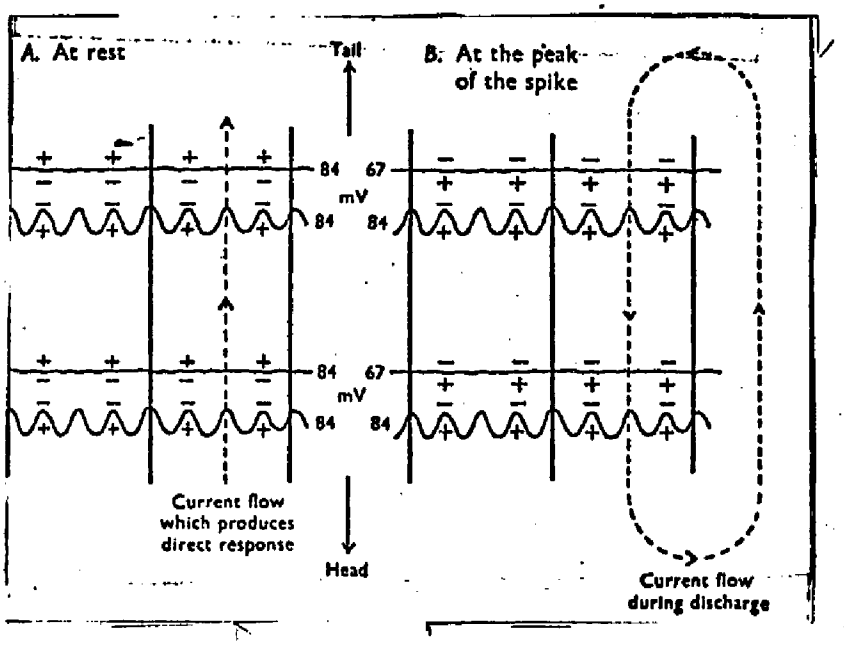

Figure 2. The mechanism of additive discharge in the electroplates of the electric organ. At rest (A), there is no net potential across the electroplates, but at the spike (B), all of the potentials are in series, and the head of the eel becomes positive with respect to the tail (Keynes, 1957).

During the resting state, the potentials across the opposite faces of the eel electroplate are opposed, and there is therefore no net voltage across the electroplate. In the active state, the electroplates act as a series of batteries discharging simultaneously. The reversal of potential across the nervous face of the electroplate is larger than that observed for any other excitable tissue.

The membrane of the nervous face of the electroplate is essentially a modified muscle membrane: it behaves in an approximately all-or-none fashion; the action potential is abolished in the absence of external sodium, suggesting that the reversal of potential results from a transient influx of sodium ions; and the resting potential is a function of the external potassium concentration.

The immediate source of the electrical energy dissipated in the discharge by the electroplate is the gradient of sodium and potassium maintained by the sodium pump.

B. (Na + K) ATPase as the Sodium Pump

The energy dependent transport of sodium by the sodium pump (usually accompanied by the transport of potassium in the opposite direction) is associated with the activity of a membrane bound enzyme, adenosine triphosphatase (ATPase), which catalyzes the hydrolysis of ATP. In 1957, Skou (J.C. Skou, 1957) discovered this enzyme in the microsomal fraction (cell membrane and endoplasmic reticulum) of the leg nerve of the shore crab, and it has since been established that this ATPase is part, if not all of the mechanism of the sodium pump.

Some of the basic properties shared by the sodium pump and by the enzyme extracted from the microsomal fraction of various tissues are the following:

a) both systems are located in the cell membrane;

b) both systems require the presence of both Mg and ATP to exhibit full activity;

c) in addition, both sodium and potassium ions are required, either ion alone being ineffective;

d) both systems are inhibited by the cardiac glycoside, ouabain (Glynn and Karlish, 1975).

Recently, it has been possible to purify the enzyme system, (Na + K) ATPase (Kyte, 1971), and to reconstitute an active sodium pump in artificial lipid vesicles (Goldin and Tong, 1974). This reconstituted system was capable of transporting sodium against its concentration gradient in an ATP dependent process. The activity of the reconstituted pump was inhibited by ouabain.

The sodium pump, (Na + K) ATPase system is oriented within the cell membrane in order to transport sodium to the extracellular fluid and potassium to the intracellular fluids. It has a molecular weight of approximately 250,000 (Kepner and Macy, 1968). The active form of the ATPase is thought to be composed of two different subunits (one of molecular weight between 40,000 and 50,000, and the other of molecular weight between 90,000 and 100,000), with each subunit in dimeric form (Kyte, 1971,1975; Hokin et al, 1973). The membrane bound ATPase requires a lipid environment for full activity, as demonstrated in experiments utilizing microsomal homogenates (Roelofson and Van Deenen, 1973).

It has been estimated that a macromolecule with a molecular weight of 250,000 and an assumed density of 1.3 would correspond to a spherical particle of diameter of 85 A^0 (Schwartz et al.,1975). This diameter would allow the protein to span the membrane and to be exposed to both intracellular and extracellular fluids. Recent experimental work on the proteins of the red blood cell membrane has supported the concept of proteins that span the membrane and act as gated transport systems for specific molecules (Rothstein et al., 1976).

The sodium pump has a sodium activation site at its cytoplasmic surface with a high affinity for sodium, and a potassium activation site at its extracellular surface with a high affinity for potassium. Intracellular ATP is the energy source for the pump activity, and the inorganic phosphate resulting from the hydrolysis of ATP is released into the cytoplasmic fluid for recycling to produce new ATP from ADP. The stoichiometry of the pump is that three sodium ions are pumped out of the cell for every two potassium ions pumped inward, and this unequal exchange of ions makes the pump an electrogenic system. One cycle of pumping requires the hydrolysis of one molecule of ATP (Glynn and Karlish, 1975).

Although the fragmented membrane preparation contains the complete sodium pump, and the membrane is in vesicular form, it is unclear whether ions per se are actually being transported across the vesicle membranes in these experimental preparations. It is certain that ions react with the (Na + K) ATPase in order to activate the ATP hydrolytic reaction and that there may be

competition between the sodium and potassium ions at their respective activation sites (Glynn and Karlish, 1975). However, if there is transport in these vesicles preparations, there may not be any detectable directionality since such vesicles are known to form in the inside-out as well as the right-side-out configuration after the extraction process.

It should be emphasized that both the sodium pump and the (Na + K) ATPase activity have a unique requirement for sodium ions. While the magnesium and potassium sites on the enzyme may accept other ions as analogs and maintain the activity of the pump at a reduced level, without sodium itself the pump will not function (Glynn, 1962; Post et al., 1960). This unique specificity for sodium is another of the characteristics shared by the sodium pump and the (Na + K) ATPase that has contributed to the concept that one is an expression of the other.

In order for transport of sodium to take place, the (Na + K) ATPase from the fragmented membrane preparations must interact with the ions as well as the substrate molecule. It has therefore proved important to examine the characteristics of the reactions leading to the hydrolysis of ATP (the intermediary reactions of ATP binding and phosphate binding), as well as the hydrolytic reaction itself, in terms of ionic requirements, kinetic characteristics, and response to varied stimulators and inhibitors in the hope of elucidating the steps of the reaction mechanism for the transport of sodium across the cell membrane.

The work to be discussed deals with the interactions among ATP, ADP, inorganic phosphate and the ATPase enzyme system in specific ionic environments; we can only infer that the mechanisms of these reactions are analogous to the activities of the pump in the cell membrane.

C. The Nucleotide-Protein Intermediate of the (Na + K) ATPase Reaction

The first step in the hydrolysis of ATP is, of necessity, the recognition of the substrate molecule by the enzyme, (Na + K) ATPase. This recognition step is highly specific, as is shown by the fact that the enzyme will hydrolyze the other nucleotide triphosphates at a much lower rate than ATP (Y.E. Shamoo and W.A. Brodsky, 1970).

The hydrolysis of ATP will not take place in the absence of magnesium ion, and it has been assumed that the undissociated Mg-ATP salt (or ion pair) is the true substrate for the hydrolytic reaction. A series of experiments by Robinson (1974) has provided quantitative evidence in support of this assumption. The K_m for magnesium was determined for the hydrolytic reaction at a constant level of ATP, and the K_m for ATP was determined for the hydrolytic reaction at a constant level of magnesium. Since the K_m for magnesium was found to be the same as the K_m for ATP, and since either free ATP or free magnesium was found to be inhibitory for the hydrolysis, it can be concluded that the undissociated Mg-ATP complex is the true substrate for the (Na + K) ATPase. A similar conclusion was drawn from the kinetic experiments of Hexum et al (1970).

However, Hegyvary and Post (1971) and Norby and Jensen (1971) have suggested that ATP can be bound to the (Na + K) ATPase in the absence of magnesium in a non-covalent linkage. At low temperature and in the absence of magnesium, [γ-^{32}P]ATP was allowed to equilibrate with the ATPase in an equilibrium dialysis chamber. The radiolabeled ATP molecule was subsequently chased by the addition of varying amounts of non-radiolabeled ATP, and the maximal ATP binding was estimated from a Scatchard analysis of data on the radiolabeled ATP released by the chase. The maximal ATP binding was consistent with the maximal formation of another intermediate in the reaction sequence, the phosphoenzyme complex.

These experiments also demonstrated that: the preference of this noncovalent enzymatic binding was greater for ATP than for other nucleotides; that the ATP binding could be inhibited by ouabain or by potassium; that the potassium induced decrease in the enzyme affinity for ATP was accompanied by an increase in the overall capacity for ATP binding; and that sodium counteracted the effects of potassium on the affinity of the enzyme for the ATP molecule. On the basis of these observations, it was concluded that the non-covalent binding of ATP is a physiologically significant part of the (Na + K) ATPase reaction.

In contrast to the magnesium-free conditions of the Hegyvary-Post and Norby-Jensen experiments, the binding of ATP to the (Na + K) ATPase has been determined under magnesium-rich conditions in turtle bladder microsomes (A.E. Shamoo and W.A. Brodsky, 1971, 1972, 1973; W.A. Brodsky and A.E. Shamoo, 1973). In these experiments, binding of ATP was taken as the amount of ^{14}C that remained attached to the

precipitated microsomes after terminating the reaction between [U-^{14}] ATP and microsomal (Na + K) ATPase by the addition of acid and repeated washings of the acid-precipitaed microsomes with non-radiolabeled nucleotides.

These experiments demonstrated that ATP could be bound to the (Na + K) ATPase in the presence of magnesium in an acid-stable linkage. Treatment of the enzyme preparation with ouabain reduced the ATP binding only in the simultaneous presence of Mg, Na and K. The conclusion drawn from these latter findings was that the formation of a complex between the (Na + K) ATPase and the substrate molecule is physiologically significant because this complex formation, like active sodium transport and maximal hydrolytic activity of the enzyme preparation, required the simultaneous presence of Mg, Na and K.

Evidence that the complex formed between the enzyme and the ^{14}C was, in part, an enzyme-ATP complex came from the following experiments: the ^{14}C-labeled, acid-precipitated microsomal enzyme was treated with various chemical compounds designed to break the bonds between the enzyme and the ^{14}C-containing compounds. The nucleotides released into the supernatant fluid by these procedures were chromatographed on thin layer plates, and it was found that at least 50% of the nucleotide content recovered by these treatments corresponded to intact ^{14}C-labeled ATP.

Another experiment which gave support to the physiological significance of the acid-precipitated nucleotide binding was an examination of the pH dependency of the reaction between the (Na + K) ATPase and the ATP. The pH curve for the binding of

substrate showed maximal binding of the ATP at about pH 7.4, with a decreased binding at either side of the neutral pH. This pH dependency was similar to that for the overall reaction of hydrolysis. The authors stated that: " This parallelism, together with the fact that both functions require the simultaneous presence of magnesium, sodium and potassium to be ouabain inhibitable, suggests that the reaction forming enzyme-ATP is in a series sequence with the overall (Na + K) ATPase catalyzed hydrolysis of ATP" (W.A. Brodsky and A.E. Shamoo, 1973).

D. The Phosphorylated Intermediate of the (Na + K) ATPase Reaction

After the formation of the nucleotide-protein complex, the next step in the reaction sequence of (Na + K) ATPase is the formation of a phosphate-protein complex utilizing the γ-phosphate of the ATP molecule (Glynn and Karlish, 1975). The presence of this second intermediate is demonstrated by incubating the membrane fragments with [γ-^{32}P] ATP, terminating the incubation by the addition of acid, repeatedly washing the labeled microsomal pellets with unlabeled nucleotide and inorganic phosphate, and finally measuring the radioactivity (^{32}P counts) of the precipitate. Maximal levels of ^{32}P labeling are found in the presence of Mg and Na; these levels are reduced by the addition of K to the (Mg + Na)-containing mixture. The concomitant increase in the overall hydrolytic rate suggests that one of the actions of potassium is to accelerate the dephosphorylation of the enzyme.

It is generally agreed that the formation and breakdown of the phosphoenzyme is a required step in the overall hydrolytic sequence of the (Na + K) ATPase because of the following evidence:

a) the maximal level of phosphoenzyme formed utilizing a particular enzyme preparation is directly proportional to the (Mg + Na + K)-dependent hydrolytic activity of the enzyme preparation (Bader et al., 1968);

b) sodium ion is an absolute requirement for achieving the maximal levels of phosphoenzyme, and no other ion can substitute for sodium in this reaction step as well as in the overall hydrolysis (Post et al., 1965);

c) under certain reaction conditions (described below), the phosphoenzyme can be used to phosphorylate ADP (Schwartz et al.,1975);

d) the addition of potassium to the (Mg + Na)-dependent phosphoenzyme will, under most conditions, produce a stripping of the phosphate from the enzyme and concomitantly increase the amount of inorganic phosphate (P_i) released (Post et al, 1965; Kanazawa et al, 1967; Fahn et al, 1968).

e) ouabain will inhibit the formation of the (Mg + Na)-dependent phosphoenzyme as well as inhibit the (Mg + Na)- and the (Mg + Na + K)-dependent hydrolysis (Post et al., 1965).

The most direct evidence for the concept that the hydrolysis reaction proceeds via the formation of a phosphoenzyme intermediate comes from the experiments of Kanazawa et al (1967), which attempt to relate the appearance of inorganic phosphate in the reaction supernatant with the fall in the level of phosphoenzyme. In the presence of 3 mM magnesium, 140 mM sodium, and 0.6 mM potassium, and with an

initial substrate concentration of 1 mM ATP, the observed rate of inorganic phosphate release could be calculated from the momentary level of the phosphoenzyme in the steady state. In other words, the rates of formation and breakdown of the phosphoenzyme were consistent with the catalytic activity of the enzyme. It is important to emphasize, with respect to the work to be described below, that the phosphoenzyme formed in the above reaction was initiated in the presence of potassium, and therefore does not coincide with the traditional "sodium phosphoenzyme". Rather, these experiments of Kanazawa support the concept that the phosphoenzyme formed in the presence of magnesium, sodium and potassium is a true intermediate in the hydrolysis reaction sequence, a finding consistent with the experiments to be presented below.

The bond between the enzyme protein and the phosphate moiety has the characteristics of an acyl-phosphate linkage (Bader et al, 1966). This conclusion is based on the sensitivity of the enzyme-phosphate linkage to treatment with hydroxylamine, acidified ethanol or methanol, and acyl phosphatase, as well as the acid stability and alkali lability of the bond.

Chromatography of the solubilized phosphoenzyme on SDS polyacrylamide gels reveals that the phosphate is bound to the larger subunit of the (Na + K) ATPase molecule, i.e., to the subunit of molecular weight of approximately 100,000 (Kyte, 1971; Collins and Albers, 1972). It should also be mentioned that there is evidence indicating that the large protein subunit of the ATPase

also interacts with the ATP molecule in the first recognition step of the hydrolytic reaction sequence (Hart and Titus, 1973).

Acid hydrolysis of the purified phosphoenzyme, with subsequent high voltage electrophoresis of the peptides formed has revealed that the phosphate is linked to the beta carboxyl group of an aspartyl residue, with a serine or threonine on one side and a lysine residue on the other side (Post et al, 1975).

The phosphoenzyme formed in the reaction between the (Na + K) ATPase and ATP is capable of phosphorylating ADP to produce ATP, under certain specified conditions:

a) in the presence of sodium and a low concentration of magnesium;

b) in the presence of sodium, magnesium, and the sulfhydryl reagent, N-ethylmaleimide (NEM);

c) in the presence of sodium, magnesium, and oligomycin; and

d) in the presence of sodium and calcium (Schwartz et al, 1975; Tobin et al, 1972).

To fit in with these observations, the following reaction pathway has been proposed and generally accepted (Siegel and Albers, 1967); reaction 1 has been added as a modification of the original reaction scheme of Siegel and Albers.

(1) $E_1 + ATP \rightleftharpoons E_1 - ATP$

(2) $E_1 - ATP \overset{Na}{\rightleftharpoons} E_1 \sim P + ADP$

(3) $E_1 \sim P \overset{Mg}{\rightleftharpoons} E_2 - P$

(4) $E_2 - P \overset{K}{\rightleftharpoons} E_2 + P_i$

(5) $E_2 \overset{Na}{\rightleftharpoons} E_1$

The first phosphorylated intermediate, E_1P , is thought to be formed via a high energy linkage, which explains its capability of phosphorylating ADP in a reverse of reaction 2. Under standard reaction conditions, which include a high concentration of magnesium, the level of this high energy phosphorylated intermediate is low; presumably this is because there is rapid conversion of the phosphoenzyme to E_2P , a low energy phosphoenzyme which cannot re-phosphorylate the ADP molecule. The high levels of E_1P that are achieved under the conditions described above arise by the following mechanisms:

a) a low magnesium level prevents the conversion of E_1P to E_2P in reaction 3;

b) the substitution of calcium for magnesium prevents the magnesium induced transformation of E_1P to E_2P and likewise prevents the subsequent hydrolysis;

c) either NEM or oligomycin acts to prevent the energy loss associated with the change from E_1P to E_2P; these inhibitors also interfere with the potassium-induced breakdown of the phosphoprotein, suggesting that it remains in the E_1P conformation (Fahn et al, 1968).

Thus, E_1P can be distinguished from E_2P on the basis of the sensitivity of the phosphoenzyme to additions of ADP or potassium. The level of the E_1P form is decreased by ADP, but not by K; but that of the E_2P form is decreased by K (reaction 4), but not by ADP. However, the phosphopeptide composition of E_1P is indistinguishable

from that of E_2P insofar as enzymatic proteolysis of of (Na + K) ATPase after its reaction with [γ-^{32}P] ATP gives rise to phosphopeptides with similar electrophoretic mobilities (Post et al, 1969; Siegel et al, 1969). This suggests that the difference between E_1P and E_2P reflected in the reactivity to ADP and K must be attributed to differences in the conformation of the protein moieties of these two phosphoenzymes.

The phosphoenzyme intermediates described above are usually observed in the presence of Mg and Na, as these are the conditions which provide for maximal levels of the phosphoenzyme complex (after acid precipitation). However, most of the work to be described below deals with the relationship between the two intermediary complexes (the nucleotide protein and the phosphoprotein) and the overall hydrolysis in the simultaneous presence of Mg, Na and K. These, of course, are the conditions required for maximal hydrolytic activity.

We found it desirable and pertinent to determine the relationships between the enzymatic functions when the enzyme was in its "physiological" conformational state - that required for the coupling of ATP hydrolysis to the transport of sodium, rather than that required for determining the effect of a missing ion or ligand in an incomplete reaction sequence.

E. The Possibility of Another Phosphorylated Intermediate

As described above, the phosphoenzymes are traditionally isolated by acid precipitation of the protein after it has reacted with [γ-^{32}P] ATP. Such phosphoenzymes might be the result of an acid-induced (rather than an ATPase-induced) transfer of the phosphate to a particular site on the protein molecule. Moreover, no acid-labile phosphoenzyme can be detected after the acid-induced termination of the ATPase reaction.

Alexander and Rodnight (1974) dealt with this problem by terminating the (Na + K) ATPase reaction with sodium dodecyl sulfate (SDS), which procedure solubilizes the enzyme at a neutral pH. Gel electrophoresis of this solubilized enzyme preparation demonstrated that the neutral phosphoenzyme complex migrated similarly to the acid phosphoenzyme complex. The ion dependent levels of the neutral phosphoenzyme were similar to those of the acid phosphoenzyme (high in the presence of Mg and Na, and lower in the presence of Mg, Na and K), and the enzyme-to-phosphate bond was an acyl phosphate linkage in both cases.

We also dealt with this problem utilizing microsomal ATPase of the eel electric organ by terminating the (Na + K) ATPase reaction with rapid heating of the incubation mixture at neutral pH. It will be shown that several properties of the neutral, heat-precipitated phosphoenzyme were distinctly different from those of the conventional acid-precipitated phosphoenzyme.

MATERIALS AND METHODS

A. Preparation of Microsomal (Na + K) ATPase

An electric eel (Electrophorus electricus), from 2 to 4 feet long, and weighing from 2 to 4 kilograms, is made quiescent by packing in ice for one half hour (in a cold room). The eel is swiftly decapitated, and a cut is made running the dorsal length of the animal. The skin is then peeled off by alternate pulling of the skin along either side of the cut, and the electric organ-which runs along both sides of the spinal column, and makes up the major portion of the eel body mass-is exposed.

It is usually possible to remove the electric organ using the blunt end of the scalpel to peel the tissue away along the natural cleavage lines between the muscle-like organ and the spinal column. The tissue is rinsed in cold distilled water, and cut up into chunks of approximately 50 grams each for storage in liquid nitrogen until the extraction of the microsomal (Na + K) ATPase is carried out.

The extraction procedure to be described is a modification of the method of Albers et al (1963).

Approximately 50 grams of eel electric organ tissue is disintegrated in a Waring blender in 300 ml of "eel blending solution" containing 0.1 mM EDTA (ethylenediaminetetraacetic acid) and 0.4 mM Trizma Base (final pH = 7.4). The homogenate is strained through a cheesecloth (double layer) and then centrifuged at 10,000 x g for 30 minutes in a Sorvall RC-2B Centrifuge at $0^{o}C$

to remove the larger cellular debris such as the nuclei and mitochondria.

The precipitate from the above centrifugation is discarded, and the supernatant is centrifuged at 50,000 x g for 1 hour at 0°C; the precipitate resulting from this 50,000 x g spin contains the microsomal fraction (i.e. cell membranes and endoplasmic reticulum) which is the source of the (Na + K) ATPase activity. This 50,000 x g precipitate is resuspended by homogenization with a glass homogenizer in "eel homogenization solution" consisting of 5 mM Trizma Base adjusted to pH 7.4 with hydrochloric acid and 0.1 mM EDTA, and is re-centrifuged at 50,000 x g for one hour at 0°C. This re-suspension and re-centrifugation of the 50,000 x g precipitate is repeated twice more so that it is thoroughly washed, and the final pellet is suspended by homogenization with a glass homogenizer in a volume of from 5 to 10 ml of 1.0 mM EDTA (adjusted to pH 7.4 with Trizma Base); this results in a suspension with a protein concentration of from 2-4 mg/ml.

The microsomal suspension is divided into aliquots of 250 and 500 µl, and rapidly frozen in micro test tubes in a mixture of dry ice and acetone. The quickly frozen preparation remains viable at -20°C for several months with little loss of the ATPase activity. A flow sheet of the extraction procedure appears on page 22.

B. Assay of Hydrolytic Activity of the Microsomal ATPase

In micro test tubes with a total volume of 600 µl (6 mm x 50 mm),

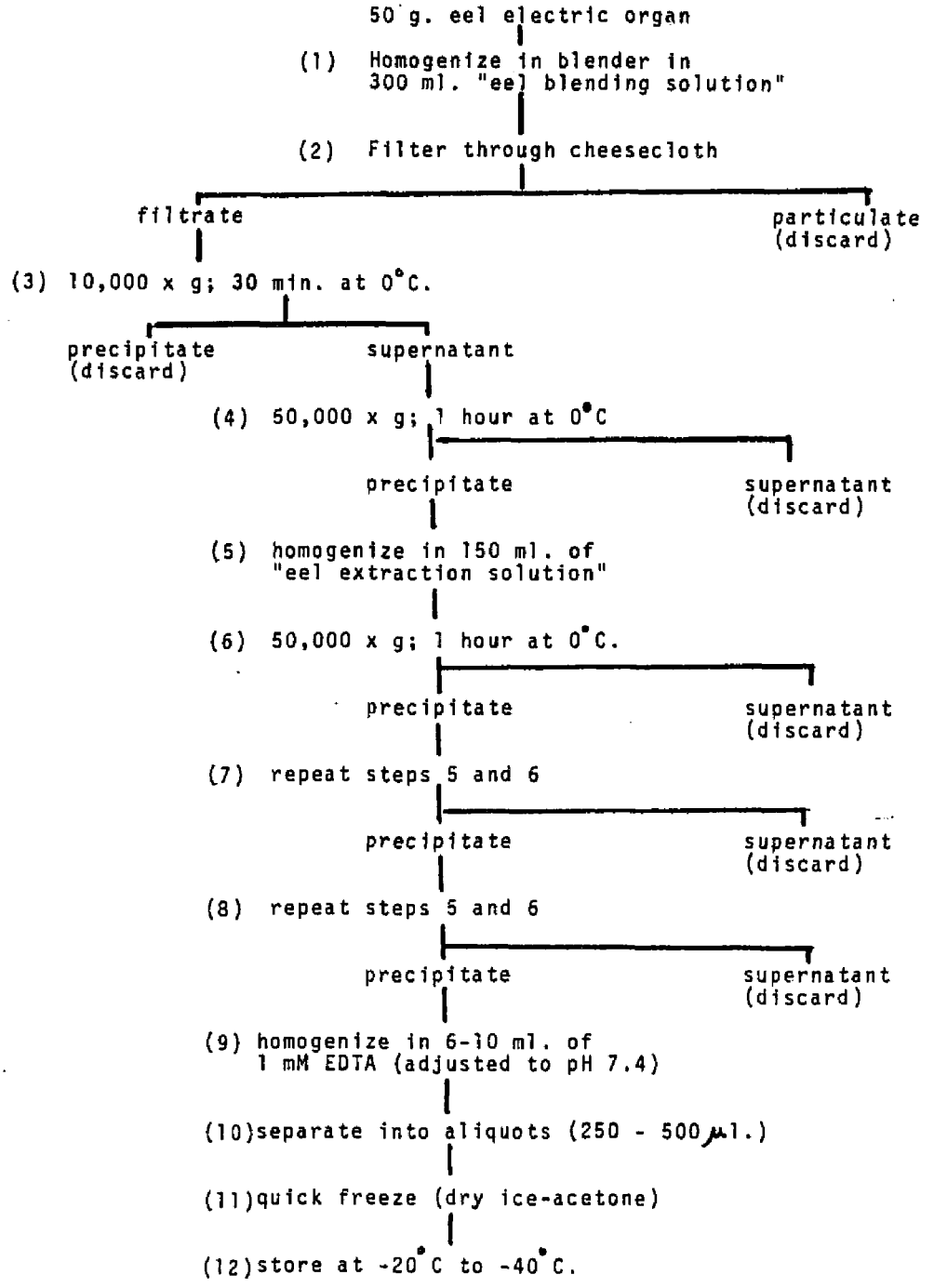

Figure 3. Outline of the extraction procedure for eel electric organ microsomes

50 to 100 μg of microsomal protein (in a final volume of 100 μl) were incubated for 10 minutes at 26°C in the presence of 3 mM $MgCl_2$, 60 mM NaCl when indicated, 25 mM KCl when indicated, 0.1 mM ouabain when indicated, and "eel reaction buffer" consisting of 40 mM Trizma Base (buffered at pH 7.4 with HCl) and 0.1 mM EDTA; the final pH of the reaction buffer was maintained at pH 7.4. After this "pre-incubation" step, the tubes were re-equilibrated in an ice bath at 0°C.

The hydrolysis reaction was triggered by the addition (at 0°C) of 2 to 500 μM [U-^{14}C] ATP or [γ-^{32}P] ATP (specific activity = 100 to 1,000 counts per minute/pmole of ATP), and the tubes were incubated at 0°C or 26°C for 5 to 180 seconds. The reaction was terminated by either:

(1) the addition of 25 to 100 μl of ice cold perchloric acid (2.5% to 6.5% final concentration (v/v); or

(2) immersion of the test tubes into a boiling water bath for 3 minutes, followed by the addition of 100 μl of ice cold distilled water.

Either of the above termination procedures resulted in the formation of a microsomal precipitate. The precipitate was then centrifuged at 20,000 x g for 20 minutes at 0°C, and the supernatant analyzed for the radiolabeled P_i content (in those tubes where [γ-^{32}P] ATP was the substrate) according to the method of Berenblum and Chain (1938).

An aliquot of the supernatant fluid was added to 35 μl of a solution of 5% ammonium molybdate in 1N H_2SO_4 so that the inorganic phosphate would react to form a phosphomolybdate complex, which

remains soluble. An aliquot of this solution was then extracted into 200 μl of isobutanol by mixing with a "Vortex-Genie" for approximately 30 seconds. The phosphomolybdate complex is taken up into the upper isobutanol layer while unreacted ATP (both radiolabeled and non-radiolabeled) remains in the lower aqueous phase. The two-phase system was centrifuged at 10,000 x g for 5 minutes at 0°C to assure full separation of the two phases. An aliquot of the isobutanol phase was removed for addition to a toluene-ethanol counting solution (toluene-ethanol, 1:1 (v/v); add 4 g Omnifluor per liter counting solution), and the radiolabeled P_i determined in a Beckman LS 230 scintillation counter.

C. Binding of Radiolabeled Substrate

The experimental protocol utilized was exactly the same as that for the hydrolysis reaction described above. Once the aliquot of supernatant had been removed for assay of the P_i release, the remaining supernatant was drawn off and discarded. The precipitated microsomes were washed free of non-specifically bound radioactivity by suspension in a solution containing the non-radiolabeled compounds Na_2ATP (20 mM) and NaH_2PO_4 (20 mM); and in those experiments utilizing [U-^{14}C] ATP as substrate, Na_2ADP (20 mM) and NaAMP (20 mM) in addition. In those reactions terminated by acid precipitation, the wash solution contained 0.3 N HCl so that the pH was approximately 1.0, and in those reactions terminated by heat precipitation, the wash solution was adjusted to pH 7.2 using 3N NaOH.

The suspended precipitate was centrifuged at 20,000 x g for 20 minutes at 0°C, and the supernatant removed. The procedure of suspension in wash solution and re-centrifugation was repeated 3 to 5 times, or until the supernatant wash solution contained a low, constant level of detectable radioactivity. The washed microsomal precipitate was then suspended in 200 µl of 90% formic acid, and quantitatively transferred (with three 200 µl washings of distilled water) to a vial containing 10 ml of toluene-ethanol counting solution for counting in the Beckman liquid scintillation counter.

In the "back-label" experiments, the experimental procedure was essentially that described above, with the radiolabeled compound being $H_3{}^{32}PO_4$, and Trizma-PO_4 the carrier compound.

The relationship between the rate of P_i release and the level of ^{32}P-phosphoprotein was based upon paired determinations made in a single set of incubation mixtures; i.e. the amount of P_i found in the supernatant was always compared with the amount of ^{32}P-phosphoprotein on the precipitated microsomes in the same test tube. However, the relationship between the level of ^{14}C-nucleotide-protein and the ^{32}P-phosphoprotein or P_i release was based upon concomitant determinations on aliquots of the same microsomes in two separate sets of tubes. Although determined separately, these functions were reproducible and well behaved when the original incubation of microsomes was carried out in the simultaneous presence of Mg, Na and K. Therefore, the

concomitant data on ^{14}C and ^{32}P labeling, taken at face value, could be interpreted as if both parameters had been measured in the same incubation flask.

D. "Complete" Ouabain Treatment of Microsomes

One aliquot of eel microsomes was incubated for 5 minutes at 26°C with 1 mM ouabain in the presence of 3 mM $MgCl_2$, 60 mM NaCl, 3 mM Na_2ATP, and eel reaction buffer (see page 23). Following this incubation, the temperature was reduced to 0°C, and the microsomal mixture was centrifuged twice at 65,000 x g for 1 hour. After each centrifugation, the supernatants were discarded, the pellets were suspended in eel homogenization solution (see page 21) containing 1 mM ouabain, and the suspension was similarly re-centrifuged. The final pellet, free of Mg, Na and ATP, was re-suspended in a solution of 1 mM EDTA containing 1 mM ouabain to a final protein concentration of 2 to 4 mg/ml.

A paired aliquot of the same microsomal preparation was carried through the same procedure with no exposure to ouabain in order to provide a paired control (or native) enzyme along with ouabain-treated preparation.

In those experiments where a "partial" or "incomplete" treatment with ouabain is mentioned, 1 mM ouabain was added to the enzyme preparation along with the indicated ions during the "pre-incubation" step of the reaction (see page 23). Although this procedure does allow for ouabain binding, as demonstrated by a ouabain-induced inhibition of enzyme hydrolytic activity,

the presence of potassium and/or the absence of ATP during the ouabain-binding reaction causes this reaction to be incomplete (Albers et al, 1968).

E. Chloroform-Methanol Extraction

The chloroform-methanol extraction procedure was a modification of the method described by Folch and Lees (1957). The precipitated, washed microsomes were suspended in 200 μl of a solution of chloroform-methanol, 2:1 (v/v), by repeated mixing at room temperature. (In some cases, either 50 μl of distilled water or 50 μl of 0.3 N HCl was also added to the chloroform-methanol solution). The suspension was then centrifuged at 15,000 x g for 15 minutes at 0°C, and the upper aqueous phase removed. The system was then washed with pure upper phase solution (resulting from a mixture of four parts chloroform-methanol, 2:1 (v/v),with one part 0.3 N HCl or distilled water), and this was added to the first aqueous phase removed. Then, the lower phase solution was removed.

The aqueous phase and the non-aqueous phase solutions were evaporated to dryness; and the sediments remaining from these evaporations, as well as the sediment remaining in the original tube (which had formed as an interphase between the two layers) were removed for scintillation counting with formic acid and distilled water (as described above for the binding of radiolabeled substrates).

F. pH Sensitivity of Precipitated Intermediary Complexes

In order to determine the pH sensitivity of the intermediary labeled complexes, the washed precipitates were suspended in 100 µl of a series of buffers. The buffers were all approximately 50 mM in concentration, and were taken from Methods in Enzymology (G.Gomori, 1955) as follows:

(a) buffer for pH 1-2 was KCl-HCl;

(b) buffer for pH 3-6 was citric acid-sodium citrate;

(c) buffer for pH 7-9 was Trizma Base-HCl;

(d) buffer for pH 9-10.7 was $NaHCO_3$-Na_2CO_3.

The suspended precipitates were incubated at 26°C for 30 minutes, centrifuged at 20,000 x g for 20 minutes at 0°C, and both the precipitate and supernatant were removed into scintillation vials for quantification of the associated radiolabel.

These treatments were carried out on successive days, utilizing buffers of pH 1, 3, 5, 7, 9 and 11 one day, and pH 2, 4, 6, 7, 8 and 10 the next day, so that the pH range from about 2 to 10 was covered each day.

G. Treatment of Microsomal Precipitates with Hydroxylamine

Hydroxylamine hydrochloride ($NH_2OH \cdot HCl$) was freshly made up as a 4N solution. Then 3 parts of 5N NaOH were mixed with 5 parts of 4N $NH_2OH \cdot HCl$ to provide a neutralized hydroxylamine solution (pH 7).

The washed precipitates were suspended in 150 µl of solution made up of either:

(a) 100 μl of Trizma Base-HCl buffer at pH 7 + 50 μl of 2.5 N NaCl as a control treatment, or

(b) 100 μl of Trizma Base-HCl buffer at pH 7 + 50 μl of 2.5 N $NH_2OH \cdot HCl$ (pH 7).

The suspended precipitate was incubated at 26°C for 10 minutes, centrifuged at 20,000 x g for 20 minutes at 0°C, and both the supernatant and precipitate were removed into scintillation vials (with 200 μl of 90% formic acid and 600 μl of distilled water, as described above) containing tolune-ethanol counting solution for quantification of the associated radiolabel.

H. Addendum to Methods

(1) Blank Values

Blank values in each experiment were determined by denaturing the microsomes with either acid or boiling water bath and then carrying these "pre-killed" microsomes through the reaction with substrate, and through the subsequent washing and centrifugation steps in the same manner as the "native" microsomes.

In some experiments on the heat precipitated ^{32}P-phosphoenzyme formation, blank values were determined by boiling the microsomal protein and the [γ-^{32}P] ATP separately, then mixing the protein and substrate together, and carrying this mixture through the steps of washing and centrifugation as described above.

(2) Basic Assay of Hydrolytic Activity

In order to determine that the extraction procedure for eel electric organ microsomes had been successful in maintaining the microsomal-associated (Na + K) ATPase activity, the following

assay was routinely performed on each batch of microsomes. Approximately 20 μg of microsomal protein was allowed to react with 3 mM [γ-^{32}P] ATP at 26°C for 2-10 minutes in the presence of (1) Mg, (2) Mg, Na and K or (3) Mg, Na, K and ouabain. This reaction was terminated by acid precipitation, and the amount of P_i released was measured in the supernatant as described above. We expected to find a ratio of ATPase activity in the microsomes incubated with Mg, Na and K that was 5 to 15 times greater than that observed in microsomes incubated with Mg or with Mg, Na, K and ouabain

(3) Cerenkov Counting

In some experiments, the ^{32}P-labeling of the protein and the $^{32}P_i$ release were measured by virtue of the Cerenkov radiation emitted by the isotope, i.e. in the absence of counting solution. For ^{32}P binding, the final microsomal precipitate was suspended in 200 μl of 90% formic acid, and the test tube placed in a counting vial. For measurement of $^{32}P_i$ release into the isobutanol layer (see above), an aliquot of the isobutanol phase was placed into a counting vial with no further additions. In both cases, the efficiency of counting by Cerenkov radiation was about 50% of that using toluene-ethanol counting solution.

(4) pH of the Heat-Terminated Reaction

The pH of the "eel reaction buffer" was measured at a series of temperatures in order to determine the pH at which the "heat-termination" step was taking place. The "eel reaction buffer" is a Trizma Base-HCl solution which has a high temperature dependency for dissociation, and one would expect that at 100°C, the pH of this buffer would be significantly different from that at 0°C or 26°C.

Figure 4 shows the straight line that can be drawn through the pH values observed for the "eel reaction buffer" at temperatures between 25°C and 75°C. When this line is extrapolated through a temperature of 100°C, the pH of the reaction buffer can be approximated as being about 6. Coincidentally, the pH for maximal stability of the heat precipitated phosphoenzyme falls between pH 6 and 8 (see figure 32).

(5) Materials

Electric eels were obtained from Paramount Research Supply, Ardsley, N.Y. Tris and disodium salts of ATP, ADP and AMP were obtained from Sigma Chemical Co., St. Louis, Mo. Ouabain and Hydroxylamine hydrochloride were likewise obtained from Sigma Chemical Co. Isotopically labeled forms of ATP included the ammonium salt of [U-^{14}C] ATP, (580 mCi/mMole), obtained from Amersham-Searle, Arlington Heights, Ill., and the ammonium salt of [γ-^{32}P] ATP (5 Ci/mMole), obtained from New England Nuclear Co., Boston, Mass. Labeled inorganic phosphate was obtained from the New England Nuclear Co. as $H_3{}^{32}PO_4$ in 0.02 M HCl (2 mCi/ml). Omnifluor for scintillation fluid was also obtained from the New England Nuclear Co.

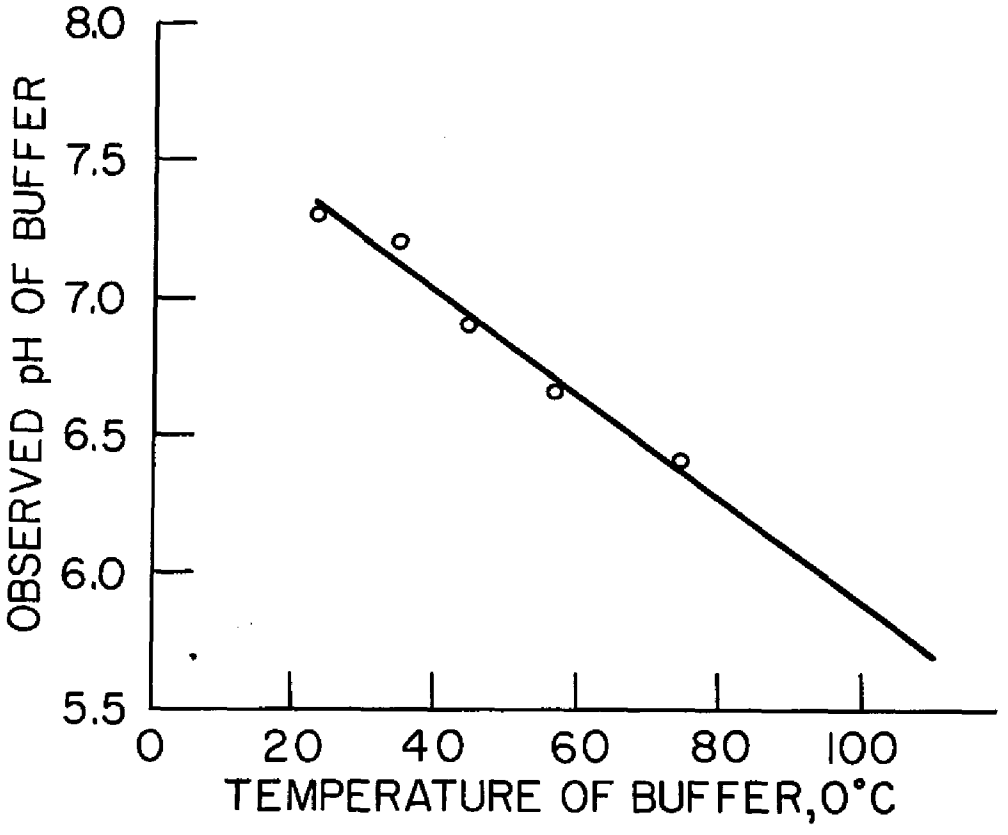

Figure 4. Temperature dependency of the pH of the buffer mixture used in assaying hydrolytic activity, phosphoenzyme formation, and nucleotide-protein formation. The pH of the Trizma Base buffer system (40 mM Trizma Base buffered at pH 7.4 at room temperature with HCl + 0.1 mM EDTA) was measured at a series of temperatures, and the pH of the buffer system at 100°C was extrapolated from the line drawn through these points.

RESULTS

The kinetic parameters for the hydrolysis of ATP by the (Na + K) ATPase and for the formation of the phosphoprotein and nucleotide-protein intermediates were measured at 0°C. At this temperature, the hydrolytic activity of the enzyme was relatively low, and steady state levels of the intermediates could be observed after 5 seconds of incubation.

A. Hydrolytic Activity

Figure 5 shows the amount of inorganic phosphate (P_i) released as a function of time during the (Na + K) ATPase reaction in eel electric organ microsomes under the specified ionic conditions.

The amount of P_i released, minimal in the presence of magnesium alone, was increased upon the addition of sodium, as has been observed before in microsomes from other tissues incubated at 0°C and at higher temperatures (Skou and Hilberg, 1969; Kanazawa et al, 1967). The amount of P_i released in the presence of Mg and Na was increased two or three fold by the addition of potassium. A stimulating effect of K (or of Na and K) on the ATPase activity at 0°C has previously been observed in eel microsomes (Siegel and Albers, 1967) and in turtle microsomes when the initial ATP concentration was low (A.E.Shamoo and W.A. Brodsky, 1972); however, in the case of ox brain microsomes (Skou and Hilberg, 1969) and rabbit brain microsomes (Kanazawa et al, 1967), a potassium-induced increment

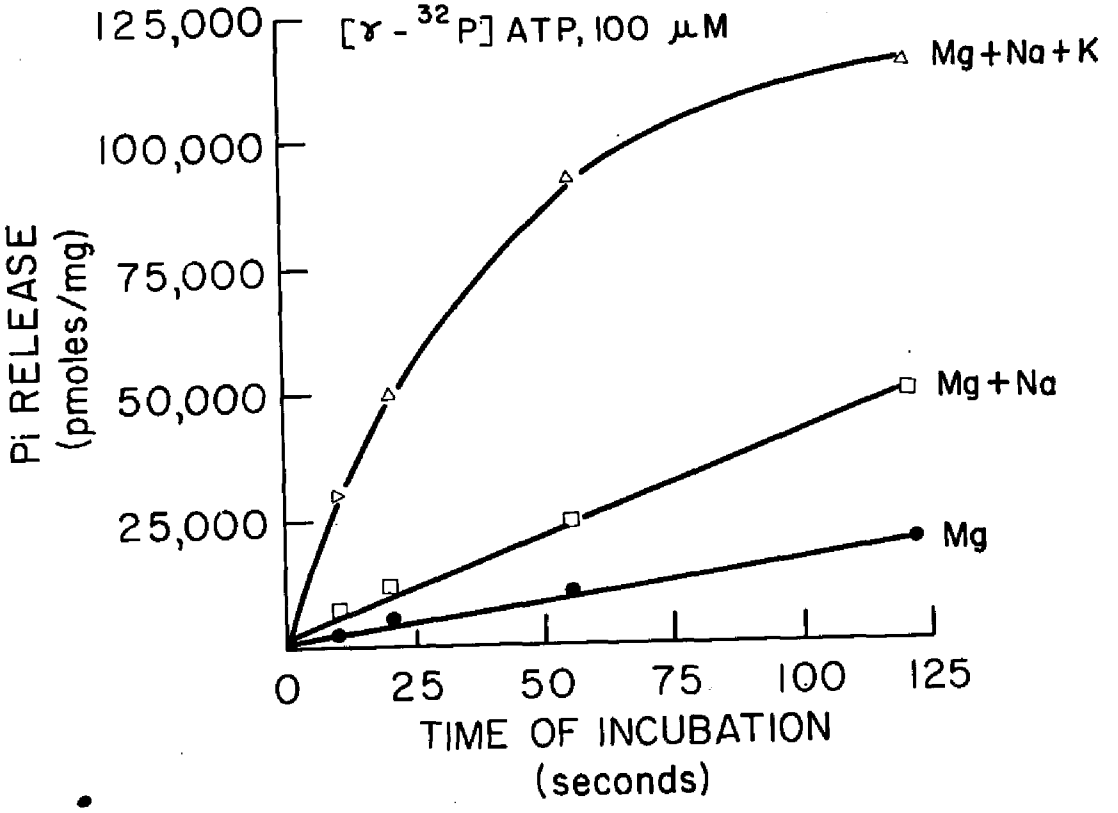

Figure 5. Time course for the release of the terminal phosphate (as inorganic phosphate) from [γ-^{32}P] ATP by eel electric organ (Na + K) ATPase under varied ionic conditions. Assays were performed at 0°C with an initial ATP concentration of 100 μM, and utilizing 55 μg of microsomal protein. The reaction was acid terminated. Blank values were subtracted, and each point represents the mean of 3-4 replicate determinations.

of hydrolytic activity was observed only at temperatures greater than $0^{o}C$.

It should be noted at the outset that: (i) the overall hydrolytic activity of the ATPase (figures 5 and 7) together with the concomitant level of ATP binding (Table I) reached maximal values in the simultaneous presence of Mg, Na and K; that (ii) functionally meaningful relationships between the intermediates and the final reaction products were found in the presence of Mg, Na and K, but not in the presence of Mg and Na; and that consequently (iii) most of the data to be shown below are from enzymatic reactions carried out in the simultaneous presence of Mg, Na and K.

Figure 6 presents data on the time-dependent release of P_i in the presence of Mg, Na and K at three different ATP concentrations. At initial ATP concentrations of 100 μM and 200 μM, the quantity of P_i released was a near-linear function of time between the 5th and 25th seconds of the reaction, which means that a steady state in the hydrolytic rate was being maintained during this interval. The maximal rate of substrate depletion was less than one percent between the 5th and 10th seconds of incubation-the time period used to determine concomitant levels of the intermediates and hydrolytic rates in the subsequent experiments in this report.

Figure 7 shows the effect of pretreatment of the enzyme with ouabain. Ouabain reduced the amount of P_i released in the presence of Mg and Na and of Mg, Na and K to that released in the presence of Mg alone. Similar inhibitory effects of ouabain have been found

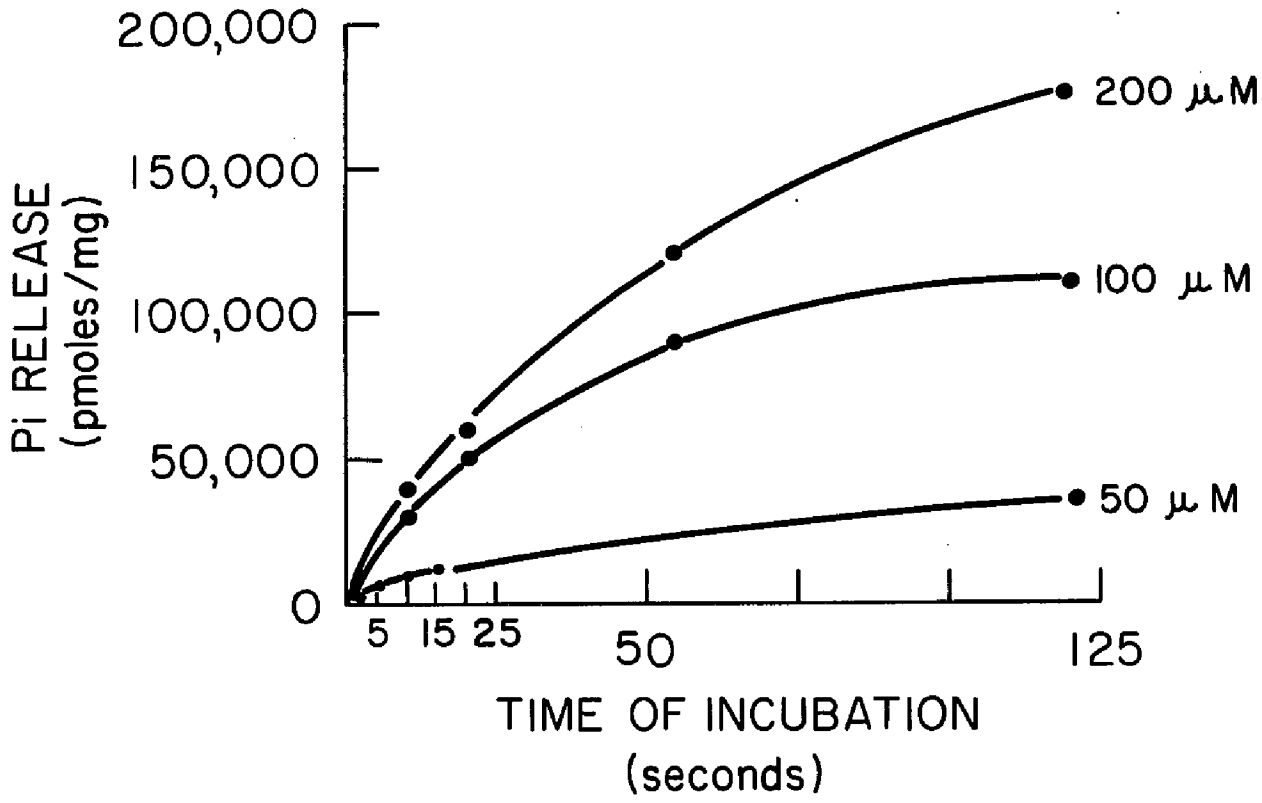

Figure 6. Time course for the release of inorganic phosphate from [γ-^{32}P] ATP by electric organ (Na + K) ATPase at varied concentrations of ATP and in the presence of Mg, Na and K. Assays were performed at 0°C, utilizing 58 μg of microsomal protein, and the reaction was acid terminated. Blank values were subtracted, and each point represents the mean of 3-4 replicate determinations.

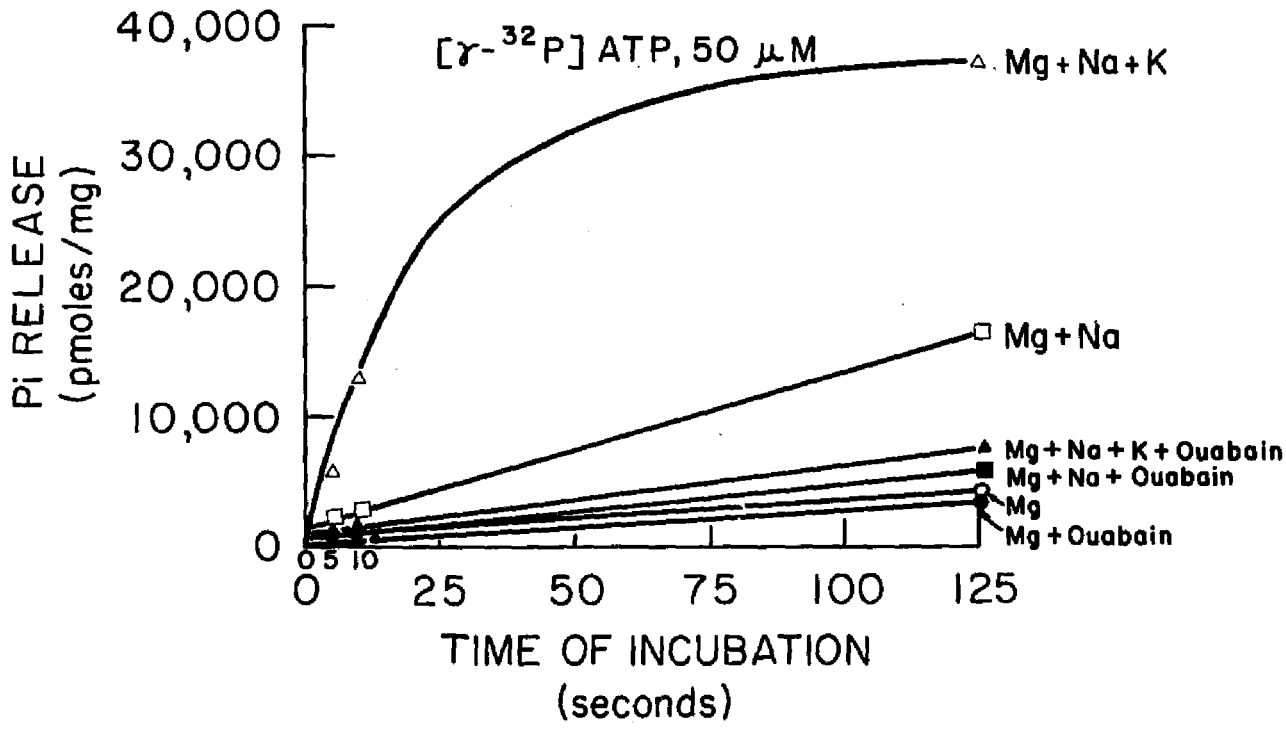

Figure 7. Effect of ouabain pretreatment of the microsomal (Na + K) ATPase on the time course of inorganic phosphate release from [γ-32P] ATP under varied ionic conditions. Assays were performed at 0°C utilizing from 60 to 80 μg of microsomal protein, and the reaction was acid terminated. The initial ATP concentration used was 50 μM. Blank values were subtracted, and each point represents the mean of 3 to 4 replicate determinations.

elsewhere with (Na + K) ATPase preparations from other tissues (Post et al, 1965; Albers et al, 1968; Skou et al, 1969; Blostein, 1970).

Figure 8 shows that the hydrolytic reaction velocity (rate of P_i release) in the presence of Mg, Na and K at 0^oC was a Michaelis-like function of the initial concentration of ATP. A Lineweaver- Burke plot of these data (figure 9) showed that the maximal reaction velocity (V_{max}) was 3100 pmoles P_i/mg protein/hr, and the apparent K_m of the reaction was about 40μ M.

This value of the apparent K_m at 0^oC in the electric organ microsomes was essentially the same as that observed in rabbit brain microsomes under similar reaction conditions at 10^oC (Kanazawa et al, 1967). Of particular importance is the fact that the K_m for the hydrolytic activity of the (Na + K) ATPase is approximately equal to the K_m for phosphoprotein formation and nucleotide formation under the identical reaction conditions (see figures 13 and 21).

Because of the importance usually attached to the (Mg + Na)-dependent formation of the phosphoenzyme intermediate (Glynn and Karlish, 1975), it was pertinent to determine the substrate concentration dependency of the hydrolytic reaction occurring in the presence of Mg + Na. Figure 10 presents the results of three separate experiments to obtain such a functional relationship in the eel electric organ microsomes. There was no clearly reproducible pattern for the concentration dependency under these reaction conditions, except for inhibition of the

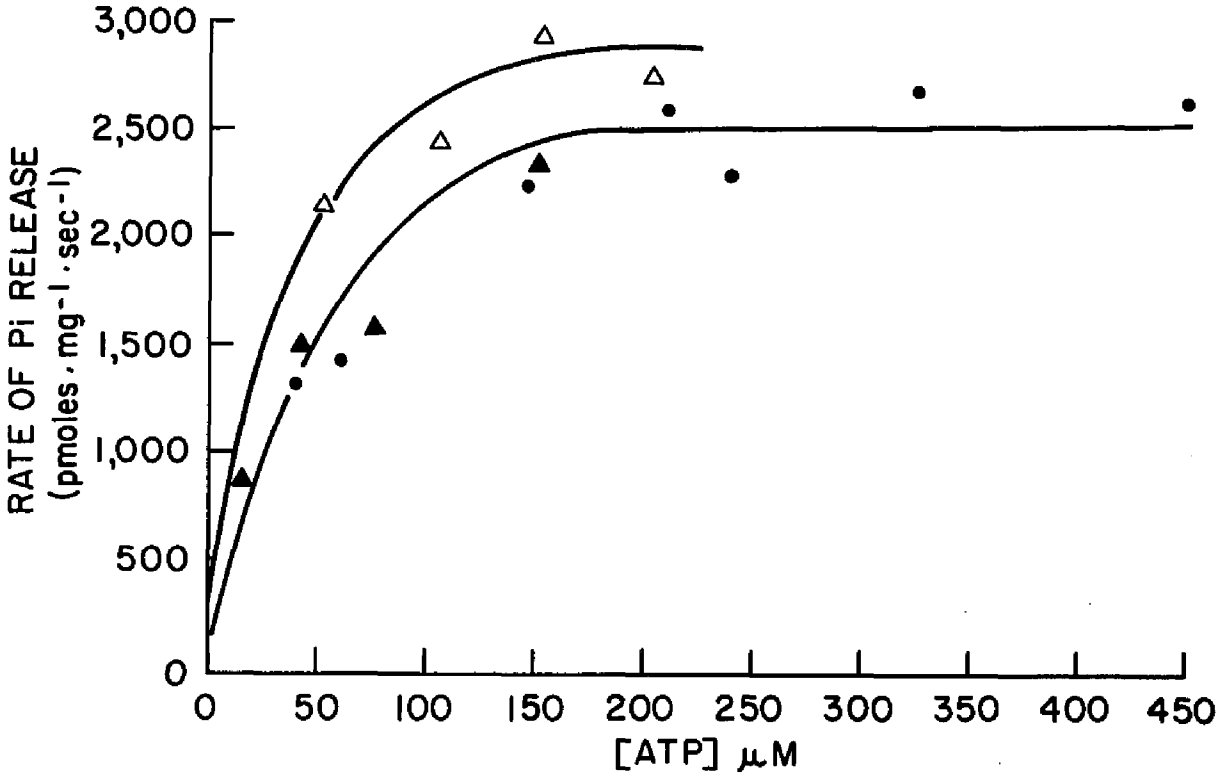

Figure 8. Hydrolytic reaction velocity in the presence of Mg, Na and K as a function of the initial concentration of ATP. The hydrolytic reaction, at 0°C, was terminated after 7.5 seconds by acid precipitation. The reaction took place with 44 to 52μg of microsomal protein. Blank values were subtracted, and each point represents the mean of four replicate determinations. Each symbol represents a separate experiment, utilizing a different batch of microsomes.

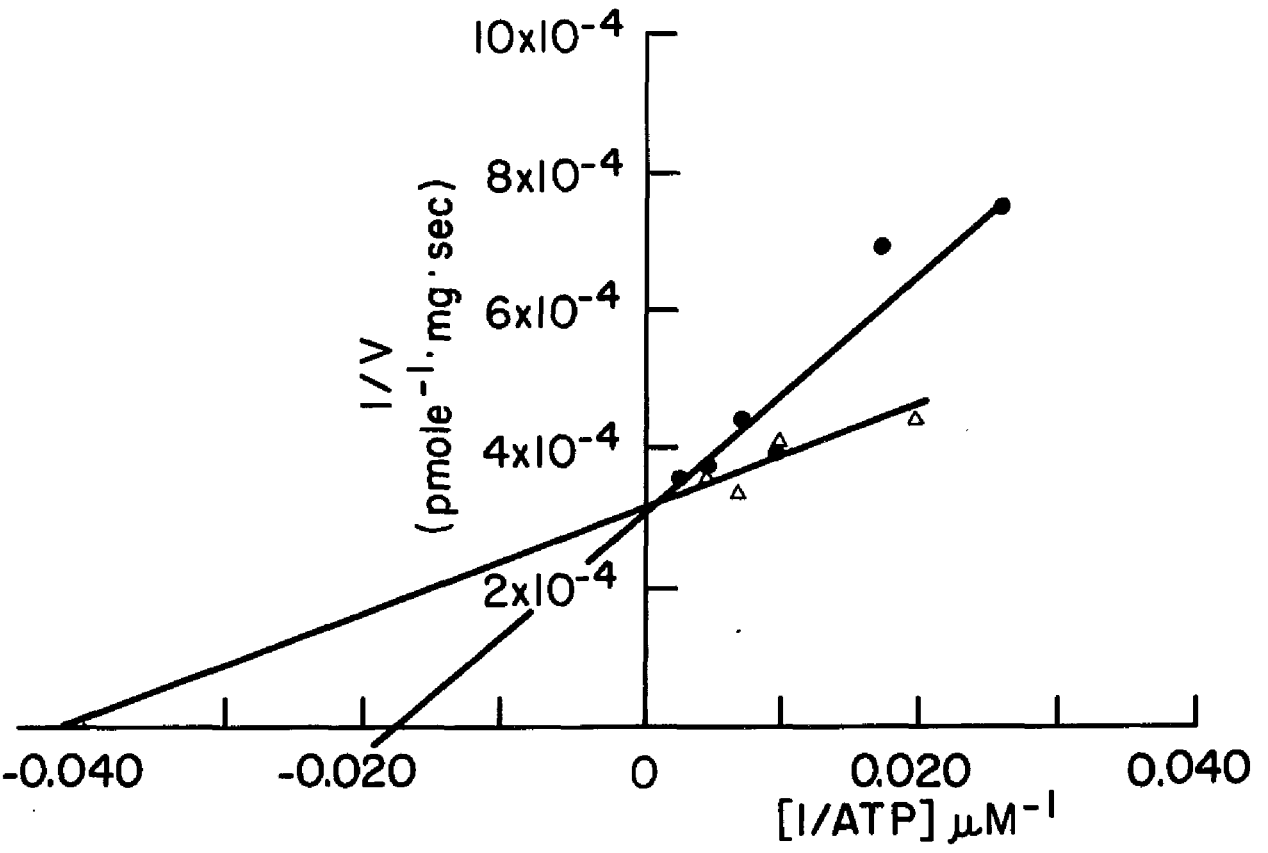

Figure 9. Double reciprocal plot of hydrolytic reaction velocity versus initial ATP concentration. Reaction conditions were those of figure 8. Best straight lines were drawn from a least squares regression analysis.

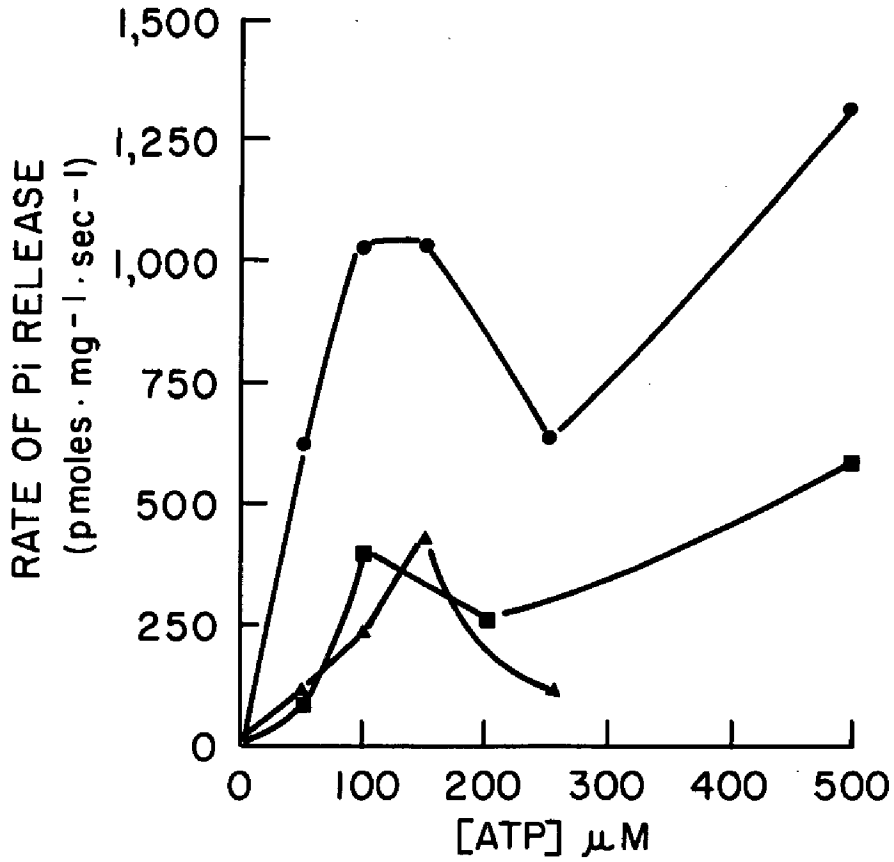

Figure 10. Hydrolytic reaction velocity in the presence of Mg and Na as a function of the initial concentration of ATP. The hydrolytic reaction, at 0°C, was terminated after 7.5 seconds by acid precipitation. The concentration of microsomal protein used was: ● 52.5 g; ■ 78.5 g; ▲ 44.8 g. Blank values were subtracted at each concentration, and each point represents the mean of four replicate determinations. Each set of symbols represents a separate experiment with a different batch of microsomes.

hydrolytic reaction rate at high substrate concentrations. However, the incubation time utilized to obtain these curves was that designed to observe steady state levels of P_i release in the (Mg + Na + K) - dependent reaction, i.e. 7.5 seconds. Reference to figure 5 suggests that a more reliable estimate of the hydrolytic activity in the presence of Mg and Na might have been obtained if the reaction had been allowed to proceed for longer periods of time.

B. Phosphoenzyme Formation (Acid-Stable)

The formation of phosphoenzyme was assayed simultaneously with and in the same incubation-reaction vessel as was the rate of P_i release. Thus, each and every level of phosphoenzyme has a corresponding hydrolytic rate (e.g. the data in figures 11 through 15 came from the same incubation flask as the data in figures 5 through 9).

Figure 11 shows the time-dependent levels of phosphoenzyme formed during the (Na + K) ATPase reaction of electric organ microsomes in the presence of the specified combinations of ions. The level of the Mg-dependent phosphoenzyme, along with the hydrolytic rate, was minimal. The level of the (Mg + Na)-dependent phosphoenzyme was maximal, as has been observed elsewhere at 0^oC and at higher temperatures (Post et al, 1965; Kanazawa et al, 1967; Fahn et al, 1968; Skou et al, 1969). The addition of K to the incubation mixture containing Mg and Na decreased the level of phosphoenzyme to about one-third that observed in the presence of Mg and Na. This

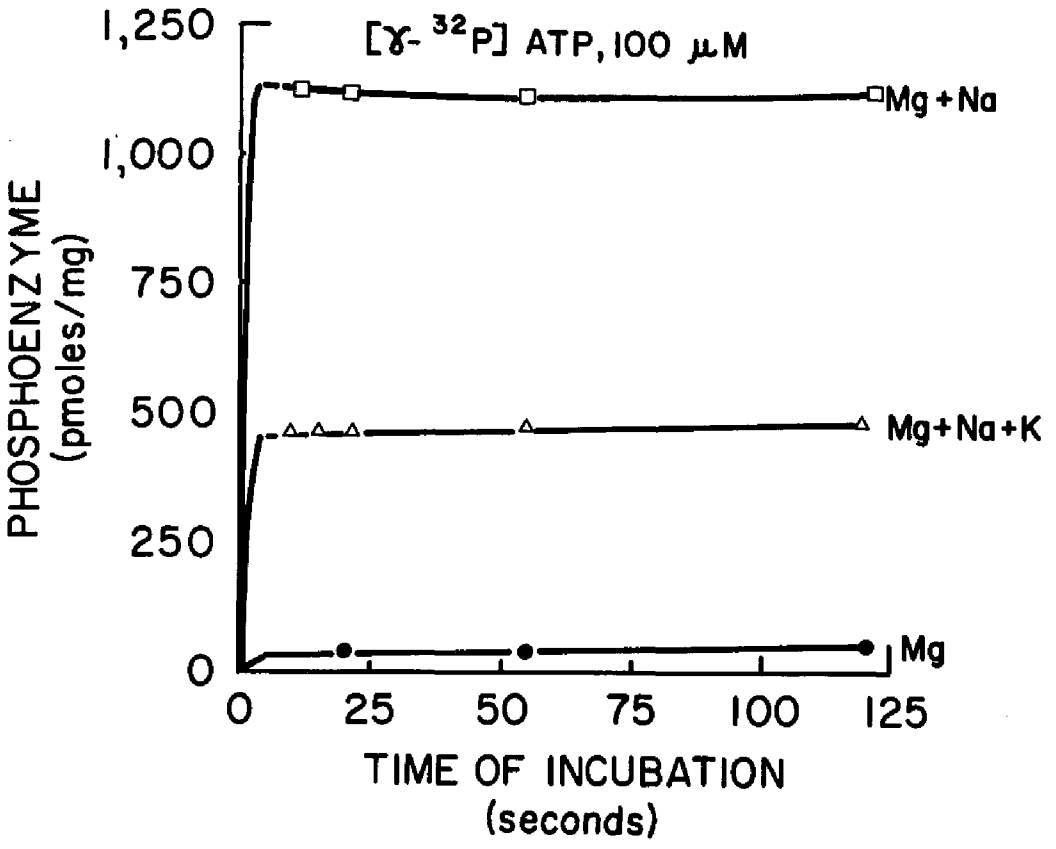

Figure 11. Time-dependent level of ^{32}P-phosphoenzyme under varied ionic conditions. Phosphorylation assays were performed as described in Methods section, at 0°C; the amount of microsomal protein used was 54.6 μg, the initial ATP concentration was 100 μM, and the reaction was acid terminated. Blank values were subtracted, and each point represents the mean of four replicate determinations.

effect of potassium, presumably due to a stripping of phosphate from the phosphoenzyme, was accompanied by an increased release of P_i into the supernatant (see figure 5).

The level of phosphoenzyme under the three ionic conditions reached a steady state level within less than ten seconds (data points before 10 seconds are not shown in figure 11), and maintained this steady state level throughout the time that the reaction was observed. This time-dependent pattern of the level of phosphoenzyme is similar to that reported by Fahn for phosphoenzyme levels of eel electric organ ATPase at $23^{o}C$ in the presence of Mg and Na (Fahn et al, 1968) and to that reported for phosphoenzyme levels of rabbit brain ATPase at $0^{o}C$ in the presence of Mg, Na and K (Kanazawa et al, 1967).

Figure 12 presents the time-dependent level of phosphoenzyme in the presence of magnesium, sodium and potassium at three different ATP concentrations. For concentrations around 100 μM ATP, the steady state level of phosphoenzyme was reached within 5 seconds, and maintained for at least 10 seconds more-which justifies a quantitative comparison of the phosphoenzyme level with the concomitant rate of Pi release (see below).

Figure 13 shows that the level of the phosphoenzyme formed in the simultaneous presence of magnesium, sodium and potassium was a Michaelis-like function of the initial ATP concentration. From figure 13, and from the double reciprocal transformation of these data in figure 14, it can be estimated that the maximal level of phosphoenzyme formed in the presence of magnesium, sodium

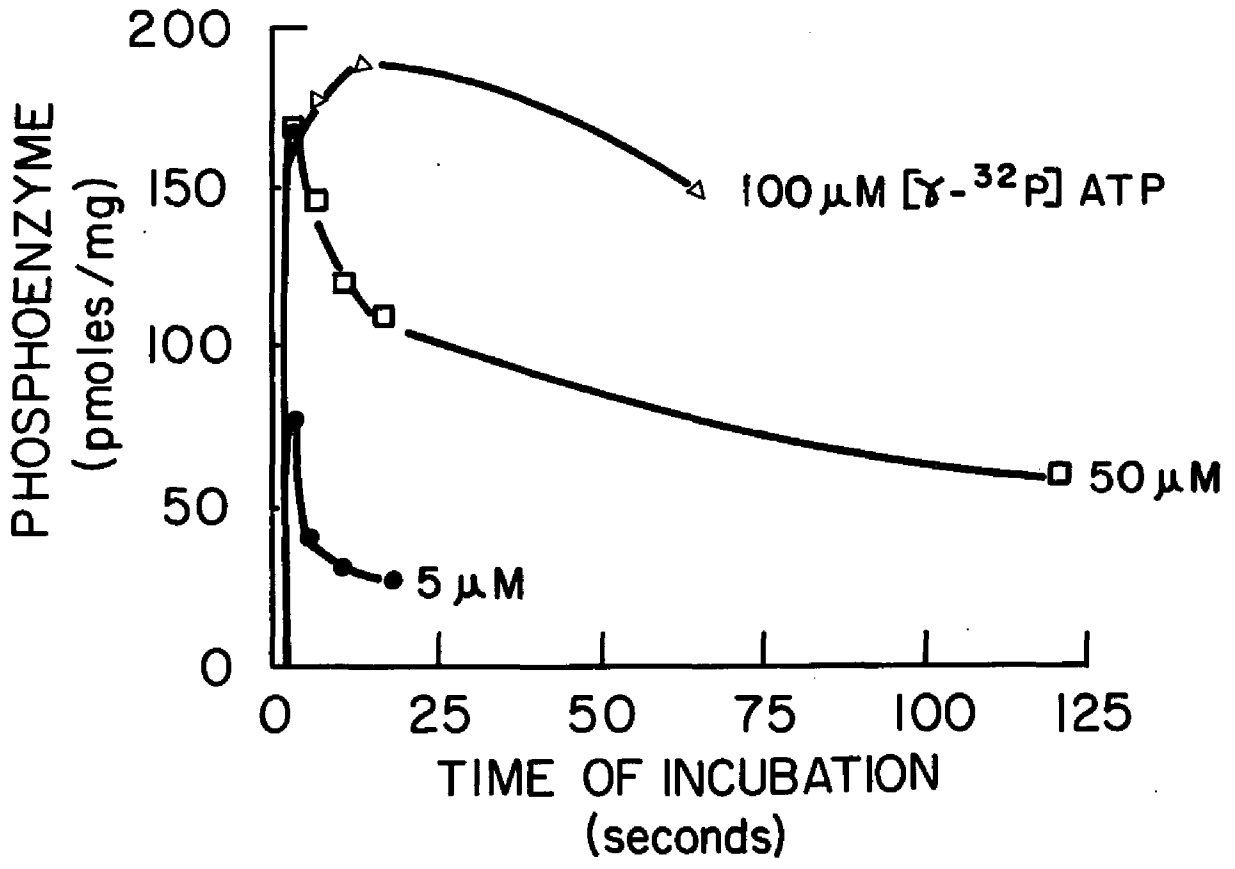

Figure 12. Time-dependent level of ^{32}P-phosphoenzyme with varied initial concentrations of ATP in the presence of Mg, Na and K. Phosphorylation assays, at 0°C, were terminated at the given times by acid precipitation. The amount of microsomal protein utilized was either 58μg (▲,■), or 90μg (●). Blank values were subtracted, and each point represents the mean of three replicate determinations.

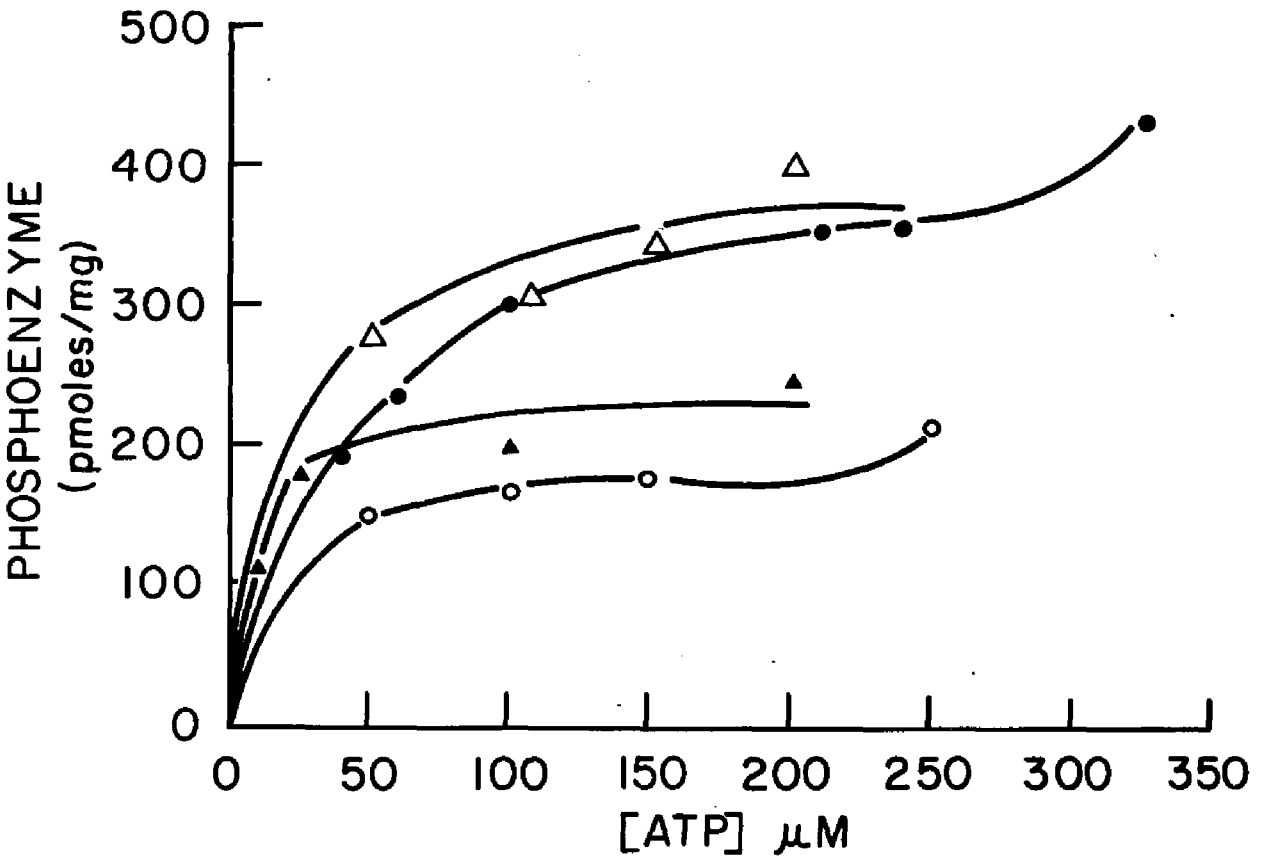

Figure 13. Level of ^{32}P-phosphoenzyme as a function of the initial concentration of ATP. The phosphorylation and hydrolysis assay, at 0°C, and in the presence of Mg, Na and K was terminated after 7.5 seconds by acid precipitation. The amount of microsomal protein used for these assays was either 52.5 μg (●, △, ○), or 43.5 μg(▲). Blank values were subtracted at each concentration, and each point represents the mean of four replicate determinations. Each set of symbols represents an independent experiment.

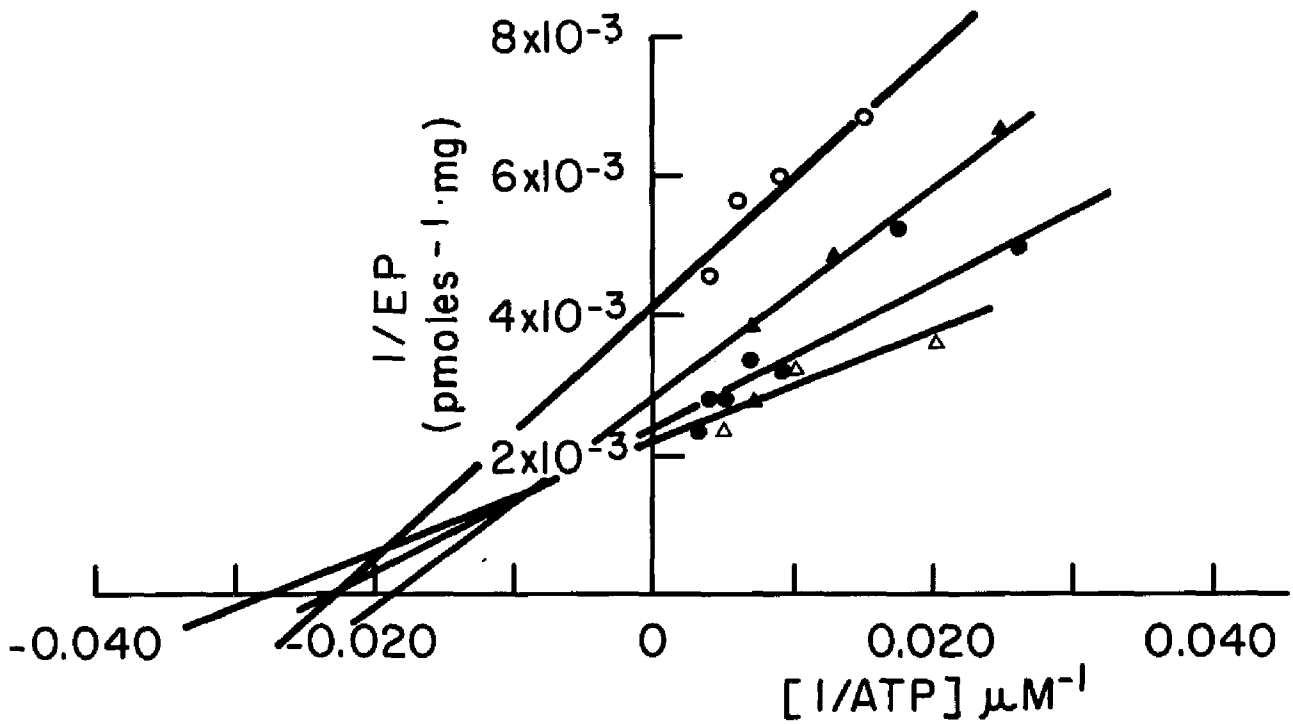

Figure 14. Double reciprocal plot of ^{32}P-phosphoenzyme level as a function of the initial concentration of ATP. Reaction conditions were those of figure 13. Best straight lines were drawn from a least squares regression analysis of the data.

and potassium is approximately 400 pmoles/mg protein, while the K_m for phosphoenzyme formation falls between 30 and 50 μM ATP. This K_m for phosphorylation is approximately equal to the K_m for the hydrolysis reaction under the same reaction conditions determined in figure 9; this suggests that the kinetics of phosphoenzyme formation are consistent with the concept that the phosphoenzyme is an intermediate in the hydrolytic reaction sequence.

Figure 15 shows that the effect of pretreating the enzyme with ouabain was to reduce the level of the (Mg + Na)- as well as the (Mg + Na + K)-dependent phosphoenzyme to that observed in the presence of magnesium alone. This effect of ouabain on the phosphoenzyme level matches that on the rate of P_i release, and corresponds to the effect of ouabain on sodium transport in the intact cell system. The significance of these observed ouabain effects will be explored in the Discussion section.

It was next important to determine whether or not the level of the (Mg + Na + K)-dependent phosphoenzyme could be directly related to the rate of inorganic phosphate release in these enyme preparations.

Figure 16 is a plot of values of the rate of P_i release (into the reaction supernatant) versus those of the concomitant level of phosphoenzyme (on the subsequently acid-precipitated microsomes in the same flask) in the presence of Mg, Na and K.

The straight line relationship suggests that the phosphoenzyme could be a rate limiting intermediate for the production of P_i

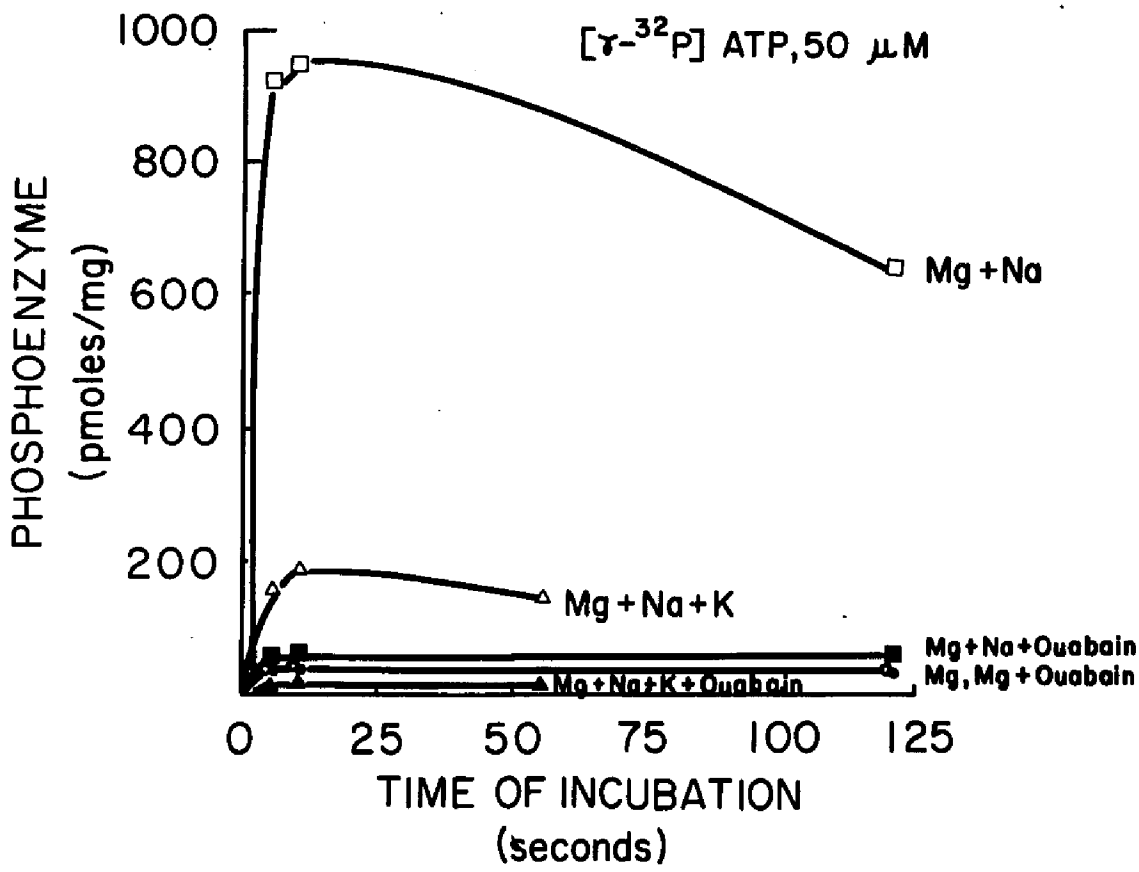

Figure 15. Effect of ouabain pretreatment of the microsomal (Na + K) ATPase on the time-dependent level of ^{32}P-phosphoenzyme under varied ionic conditions. Phosphorylation assays, at 0°C, with an initial ATP concentration of 50μM, and amounts of microsomal protein varying between 60 and 80μg, were acid terminated. Blank values were subtracted, and each point rep resents the mean of three replicate determinations.

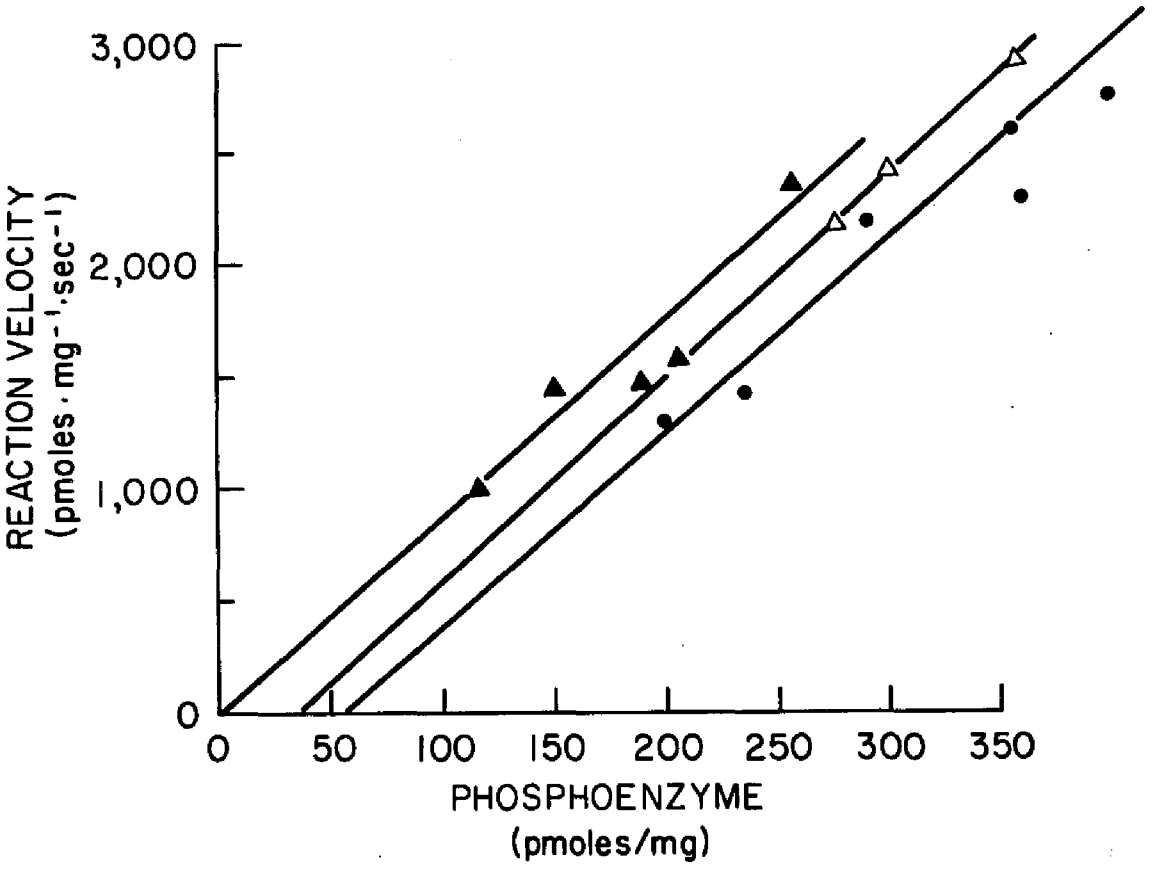

Figure 16. Linear relation between the hydrolytic reaction velocity in the steady state, and the concomitant level of ^{32}P-phosphoenzyme formed in the presence of Mg, Na and K. Data for this graph were taken from experiments which measured the reaction velocity and the level of phosphoenzyme (in the same tube) as a function of the initial concentration of ATP (see figures 8 and 13). Best straight lines were drawn from a least squares regression analysis.

under these ionic conditions. This linear relationship is similar to those found at 23°C with guinea pig cortex ATPase (Post et al, 1965, fig.5) and rabbit brain microsomal ATPase at 15°C (Kanazawa et al, 1970, fig.21). However, this relationship has not previously been observed at 0°C because most tissues (other than the electric organ and the turtle bladder) show little or no K-induced increment of the (Na + K) ATPase-dependent hydrolysis at 0°C (Kanazawa et al, 1967; Skou and Hilberg, 1969).

The linear relationship generated in figure 16 was lost at ATP concentrations above 200 μM or at phosphoenzyme (EP) levels in excess of 350 pmoles/mg, raising the possibility that some of the ATP-induced phosphorylation is due to a relatively low affinity phosphate binding to enzymes other than the (Na + K) ATPase. Although we did not pursue this question, if the binding of phosphate at ATP concentrations above 200 μM could have been shown to be insensitive to ouabain, this latter possibility would have been validated. In this regard, we have observed the presence of 15-20 other protein species in the microsomal extract of the eel electric organ by SDS-polyacrylamide gel electrophoresis.

The phosphoprotein function obtained in the presence of Mg and Na (figure 17) differed markedly from that obtained in the presence of Mg, Na and K (figure 13). The (Mg + Na)-dependent phosphoenzyme reached maximal levels at low substrate concentrations (< 10 μM ATP), whereas the (Mg + Na + K)-dependent phosphoenzyme reached maximal levels at concentrations of ATP greater than 100 μM.

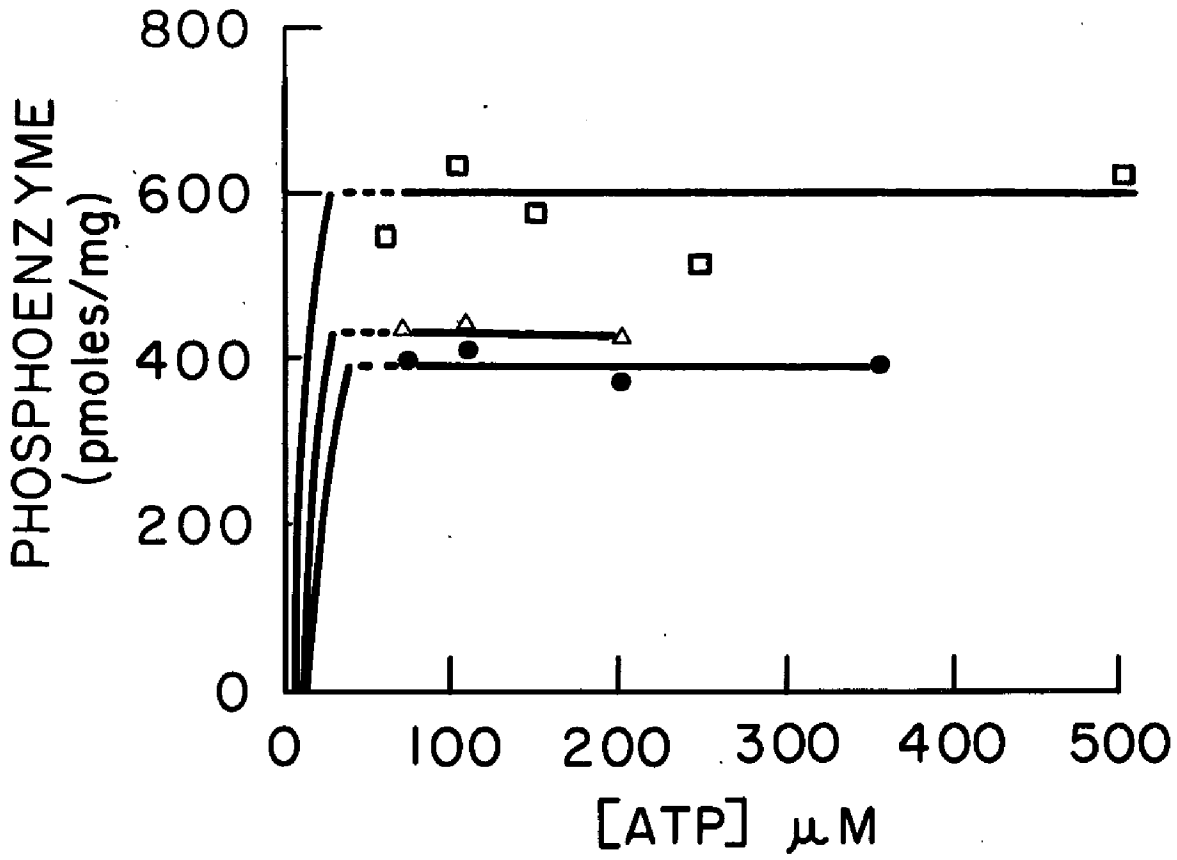

Figure 17. Level of ^{32}P-phosphoenzyme as a function of the initial concentration of ATP. The phosphorylation-hydrolysis assay, in the presence of Mg and Na (at 0°C) was terminated after 7.5 seconds by acid precipitation. Blank values were subtracted at each concentration, and each point represents the mean of four replicate determinations. Each set of symbols represents a separate experiment, utilizing a different batch of microsomes. The amount of microsomal protein used was 44.8 μg (△), 52.5 μg (□), or 78.5 μg (●).

The (Mg + Na)-dependent phosphoenzyme also bore no functional relationship to the concomitant rate of P_i release.

The above observations are consistent with the findings of Kanazawa et al (1967), who showed that: (i) the apparent K_m for the formation of the (Mg + Na)-dependent phosphoenzyme was approximately 1 μM and (ii) it was not possible to show a functional relationship between the (Mg + Na)-dependent phosphoenzyme level and the (Mg + Na)-dependent hydrolysis at 0^oC.

The finding of a very low K_m for phosphorylation suggests that the ATP concentrations utilized to generate the curves of hydrolytic activity vs. ATP concentration (figure 10) were too high by a factor of 100. It is therefore possible to explain the observed decline in hydrolytic activity at higher substrate concentrations on the basis of substrate inhibition of the reaction.

C. Nucleotide-Protein Formation

It is generally agreed that the formation of an enzyme-ATP (E-ATP) complex must precede the formation of the EP complexes (E_1P and E_2P) in the (Na + K) ATPase reaction sequence. Therefore, part of the measured phosphoenzyme may actually be an E-ATP complex.

The acid-precipitated binding of ^{14}C (from [^{14}C] ATP) to the (Na + K) ATPase has been measured by others

(Post et al, 1965; Fahn et al, 1968) in order to determine the quantity of E-ATP that forms and how much of the phosphoprotein can be attributed to the binding of the complete ATP molecule. But the level of such binding was too low (e.g. 3 to 10 pmoles/mg) to be considered seriously when compared to that of phosphate bound (400-800 pmoles/mg).

Therefore, before attempting to estimate the level of E-ATP from that of [U-^{14}C] ATP binding, it was necessary to determine that: (i) the ^{14}C-binding level of an enzymatically active preparation was significantly greater than that of an enzymatically dead preparation and that (ii) the ^{14}C-labeling levels of acid precipitated microsomes were dependent on the ionic environment in which the microsomes were incubated with [U-^{14}C] ATP prior to the acid precipitation procedure.

We found significant, albeit small increments of [^{14}C] ATP binding to live (native) over that to dead (pre-acid-denatured) microsomes, and this enzymatically dependent increment of binding was related to the ionic composition of the incubation mixture as well as showing sensitivity to ouabain.

Table I presents mean values of ^{14}C-labeling levels in paired aliquots of live and dead enzyme preparations that had been incubated with 100 μM [U-^{14}C] ATP. The amount of ^{14}C counts recovered on the precipitated

Ionic Conditions	Binding (mean value) Live - Dead / Dead	S.D.*	S.E.**	t	p
Mg	0.199	0.122	0.055	3.6	0.02
Mg + Na	0.149	0.112	0.045	3.3	0.02
Mg + Na + K	0.285	0.122	0.026	10.96	0.001

Table I. Fractional increase in binding of ^{14}C to native microsomes over that to pre-denatured microsomes under varied ionic conditions. Binding of ^{14}C (nucleotide-protein levels) was measured after the reaction between the microsomal (Na + K) ATPase and [U-^{14}C] ATP at 0°C, as described in the Methods section. The initial substrate concentration was 100 or 200 μM ATP. "Dead" microsomes were denatured with acid before exposure to substrate, while live microsomes were denatured with acid 7.5 seconds after reaction with substrate. Labeling of dead microsomes represents the blank subtracted in the experiments to be described.

S.D.* = standard deviation S.E.** = standard error

microsomal enzyme was between 0.01% and 0.1% of that initially added as [U-^{14}C] ATP. The mean percentage increment in the ^{14}C-labeling of live over dead enzyme was maximal (28.5%) in the presence of Mg, Na and K, minimal in the presence of Mg and Na (14.9%), and intermediate (19.9%) in the presence of Mg alone. What follows is concerned with the characteristics of the ^{14}C-labeling function in the simultaneous presence of Mg, Na and K, unless otherwise specified.

It was necessary to determine that the observed binding of the ^{14}C was to a protein rather than a lipid moiety of the membrane preparation , as the ATPase is known to be composed of a protein and a glycoprotein moiety (Kyte, 1971). It should be noted that some lipid involvement in the binding reaction would not be surprising, as a lipid environment is required for full enzymatic activity of the ATPase (Goldman and Albers, 1973).

The reaction between microsomal (Na + K) ATPase and 100 μM [U-^{14}C] ATP was allowed to progress for 15 seconds before termination by acid precipitation. The ratio of ^{14}C binding to the native microsomes versus that to the pre-denatured microsomes was of the order of 1.3 to 1, which is a fairly common ratio for the reaction taking place in the presence of Mg, Na and K.

Table II presents the results of a chloroform-methanol extraction of the above ^{14}C-complexed precipitates, according to a modification (see Methods) of the method of Folch and Lees (1957). This procedure is designed to determine what proportion of the bound ^{14}C counts could be extracted into a lipid solvent. In both cases, the percentage of lipid-extractable counts amounted to about 10% of the total bound ^{14}C. These data suggest that up to 90% of the ^{14}C is bound to non-lipid moieties, presumably the protein of the (Na + K) ATPase molecule.

Preliminary experiments were also performed to determine the chemical identity of the bound ^{14}C compound. After terminating the reaction between microsomal (Na + K) ATPase and 100-200 μM [U-^{14}C] ATP with acid, and washing the precipitates (see Methods), the resulting ^{14}C-labeled precipitates (formed in the presence of Mg, Na and K) were exposed to a carbonate-bicarbonate buffer system at pH 10.3 (for 30 minutes at 26^{o}C). This treatment released 55-75% of the ^{14}C label from the microsomal precipitate into the supernatant.

After subjecting the ^{14}C-containing supernatants to thin layer chromatography (using either 0.3 M LiCl or acidified 0.25 M KH_2PO_4 as the solvent), it was found that 50-80% of the ^{14}C comigrated with ATP and ADP; the remainder was associated with either AMP or cyclic-AMP. This finding constitutes a partial confirmation of

Chloroform-methanol	Aqueous	Precipitate
8.1 ± 0.5 %	46.2 ± 3.4 % (acidified)	46%
12.5 ± 2.0 %	6.9 ± 0.9 %	81%

Table II. Chloroform-methanol treatment of the ^{14}C-nucleotide-protein precipitate. After formation of the ^{14}C-nucleotide-protein complex at $0^{o}C$ (reaction for 7.5 seconds with 200 μM ATP, using 44 or 52 μg of microsomal protein), the reaction was terminated with acid, and the precipitate exposed to chloroform-methanol, as described in the Methods section. The amount of radiolabeled ^{14}C in the chloroform-methanol fraction, the aqueous fraction, and in the remaining precipitate were measured. There was an 80 to 100% recovery of the ^{14}C counts after this procedure, and this table shows the percentage of ^{14}C counts in each fraction.

previously reported data on ^{14}C-labeled microsomes formed in the acid terminated reaction between turtle bladder epithelium microsomal ATPase and [U-^{14}C] ATP (A.E. Shamoo and W.A. Brodsky, 1971).

We also attempted to isolate the ^{14}C-nucleotide-protein complex by electrophoresis on an SDS-polyacrylamide gel. However, solubilization of the acid-precipitated complex with SDS resulted in nearly complete dissociation of the ^{14}C-nucleotide-protein, as shown by the observation of free ATP (and/or ADP) after electrophoresis in this gel system. Parenthetically, about 12 protein or polypeptide bands were found by gel electrophoresis, with most of the material in the region between 50,000 and 200,000 Daltons.

Figure 18 presents the level of the ^{14}C-labeled (nucleotide) protein complex as a function of time at three different initial concentrations of ATP. The level of nucleotide-protein is maintained at a steady state between 5 and 10 seconds of reaction time under the initial ATP concentrations tested. This justifies a comparison of this parameter with the formation of the phosphoprotein and the overall hydrolysis at 7.5 seconds. At an initial ATP concentration of 100 μM, the steady state level of nucleotide protein is maintained for the full 60 seconds that the reaction was observed, and this coincides with the time dependency of the phosphoprotein (figure 11).

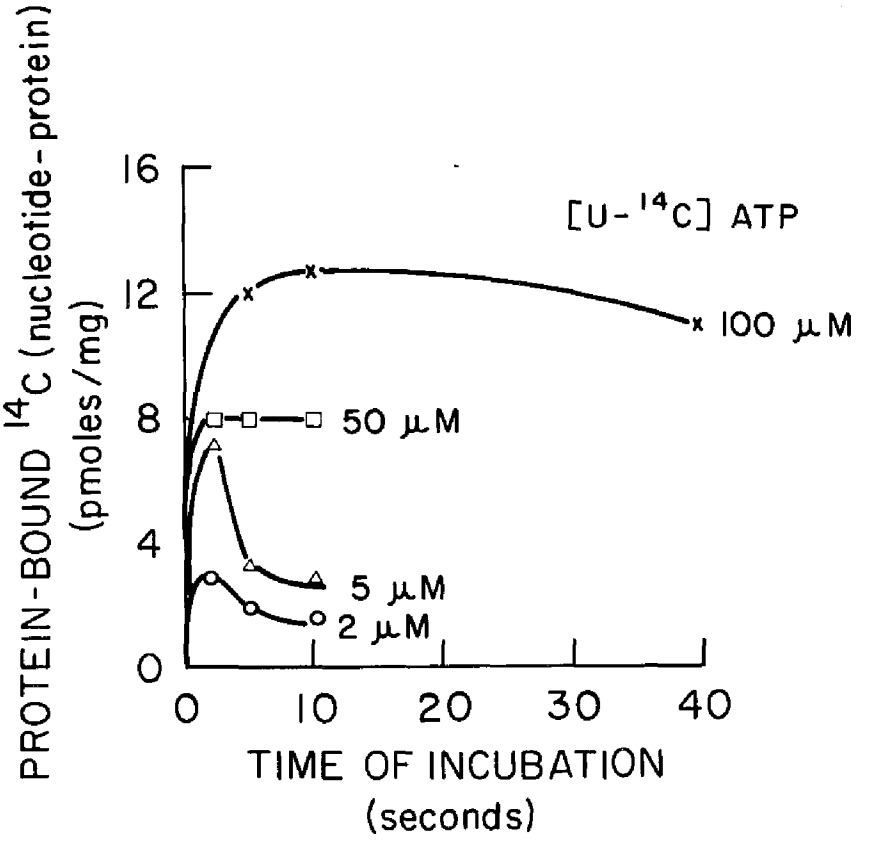

Figure 18. Time-dependent level of ^{14}C-nucleotide-protein complex. Nucleotide-protein assays were performed, at 0°C, as described in the Methods section, at varied initial concentrations of ATP. The amount of microsomal protein used was either 59 μg (X), 85 μg (□), or 87 μg (o,Δ). The reaction was acid terminated. Blank values were subtracted, and each point represents the mean of four replicate determinations.

Figures 19, 20 and 21 present data showing the effect of the substrate concentration on the radiolabeling of live and dead enzyme preparations by [U-^{14}C] ATP. The level of ^{14}C-labeling of the live enzyme was significantly greater than that of the dead enzyme over a wide range of initial ATP concentrations (figure 19). The estimated level of the ^{14}C-nucleotide-protein (figure 20) increased with increasing substrate concentrations in both the live and the dead enzyme preparations. However, the binding to the native (live) microsomes was consistently higher at each concentration of ATP, reinforcing the presumption that some of the binding is related to the enzymatic activity of the microsomal preparation.

The enzymatically dependent increment of ^{14}C-nucleotide-protein formation (that formed by live enzyme minus that formed by dead enzyme at the same ATP concentration) was apparently a double saturation function of the initial substrate concentration (figure 21). For ATP concentrations of less than 200 μM, the first saturation level of ATP binding (30 pmoles/mg for curve A) was associated with an apparent K_m of 35 μM ATP. A similar value for the K_m can be determined from curve B, which represents an enzyme preparation with lower ATP binding activity (maximal binding of approximately 19 pmoles/mg). For ATP concentrations in excess of 200 μM, the second saturation level (70 pmoles/mg) was associated with a second apparent K_m of 230 μM ATP.

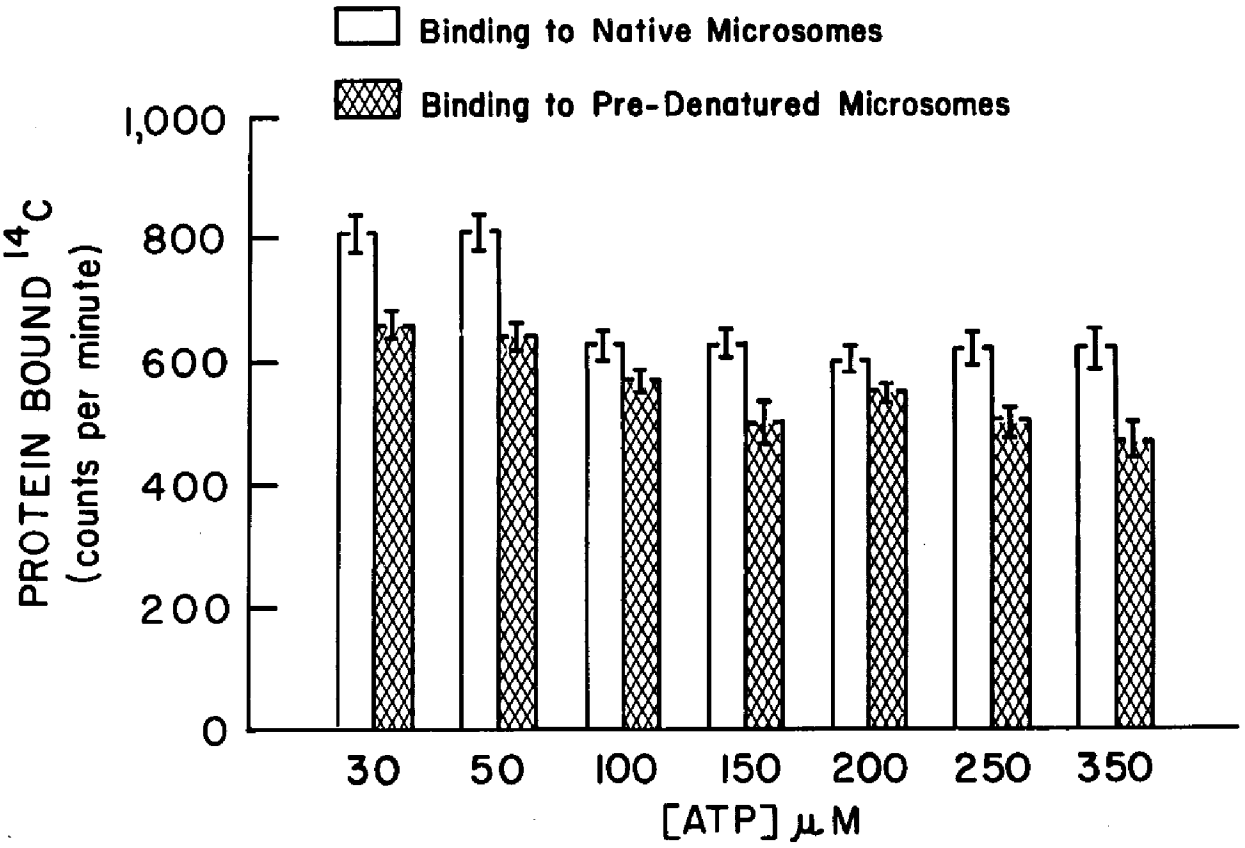

Figure 19. Binding in raw counts of ^{14}C to native and pre-denatured microsomes as a function of the initial concentration of ATP. Reaction of the microsomal (Na + K) ATPase with [U-^{14}C] ATP was carried out at 0°C, for 7.5 seconds, in the presence of Mg, Na and K, with varied initial concentrations of ATP. The amount of microsomal protein used was 52.2 μg. The standard errors were calculated for 4 to 5 replicate determinations at each concentration of ATP.

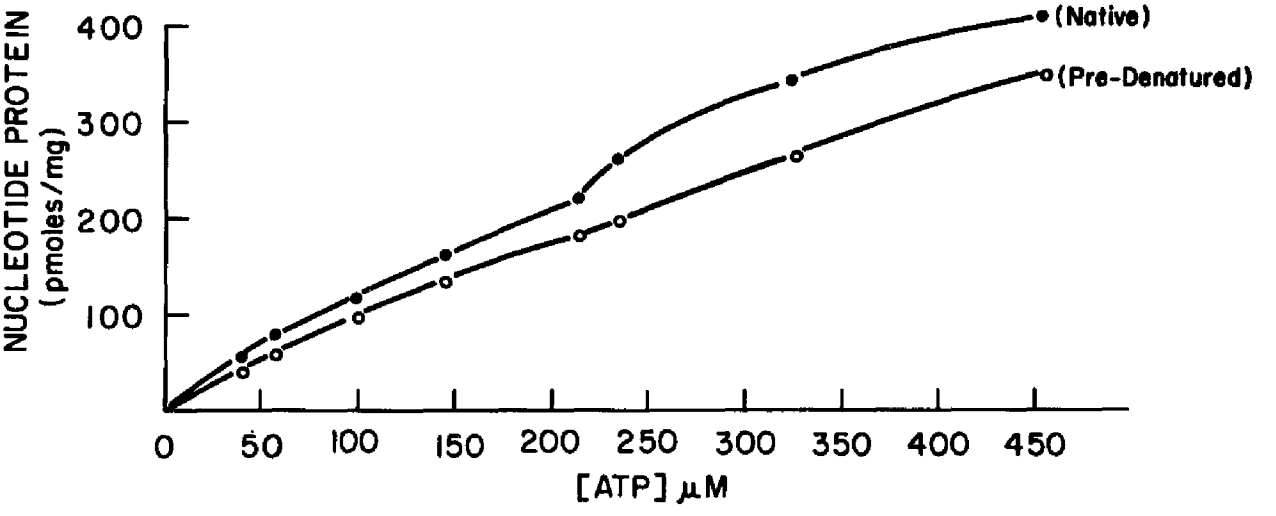

Figure 20. Binding (in pmoles/mg) of ^{14}C to native and pre-denatured microsomes. The raw counts obtained in figure 19 were transformed into binding as pmoles/mg protein.

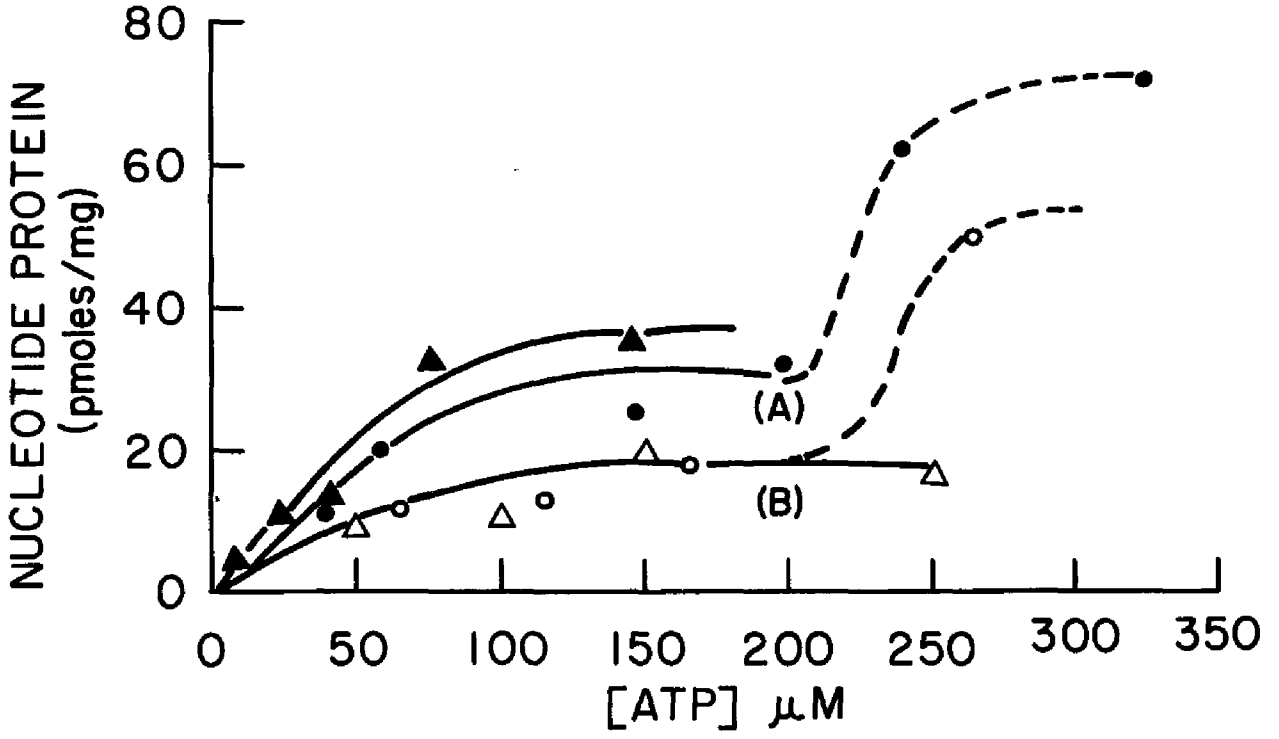

Figure 21. Enzymatically dependent binding of ^{14}C to microsomal (Na + K) ATPase as a function of the initial concentration of ATP. Reaction conditions are those of figure 19. The amount of microsomal protein used was either 52.5 μg or 43.5 μg. Blank values subtracted in all cases from those shown are taken as the amount of ^{14}C bound by pre-acid denatured microsomes (paired aliquots for each concentration of ATP). Each set of symbols represents a separate experiment.

Since the first apparent K_m of ATP binding was the same as that of the concomitant hydrolysis and phosphoenzyme formation (compare with figures 8 and 13), it can be inferred that part of the enzymatically dependent ATP binding (that which occurs with substrate concentrations of less than 200 μ M ATP) reflects the level(s) of the E-ATP (and or P-E-ADP) complexes that form during the (Na + K) ATPase reaction sequence. The second apparent K_m of ATP binding could not be related to the concomitant hydrolysis and phosphoenzyme formation-which remained constant at ATP concentrations in excess of 200 μ M. The secondary or low affinity ATP-binding function could be ascribed in part to the presence in these microsomes of other ATP-binding proteins (e.g. adenylate cyclase, protein kinase and pyrophosphatase activities have been found in these microsomes), in part to the binding of ATP to the non-hydrolytic subunit of the native (Na + K) ATPase (Glynn and Karlish, 1975), and in part to the relatively low specific activity of the [U-^{14}C] ATP at concentrations of 200 μ M and greater.

It has been shown that ouabain inhibits both the hydrolysis and the formation of phosphoenzyme (figures 7 and 15). Since the first apparent K_m of the ^{14}C-nucleotide-protein binding reaction suggests that the nucleotide-protein may be an intermediate in the

(Na + K) ATPase reaction pathway, it was decided to determine the effect of ouabain on the formation of the ^{14}C-nucleotide-protein complex.

If the formation of the phosphoenzyme and the subsequent hydrolysis are inhibited because the formation of the nucleotide-protein is inhibited, we would expect this to be reflected in a decrease in the amount of ^{14}C bound to microsomes pretreated with ouabain.

Figure 22 shows the time dependency of the enzymatic increment of ^{14}C-nucleotide-protein formation by a mated pair of native (upper curve) and ouabain-treated (lower curve) enzyme preparations after their reaction with 100 μM [U-^{14}C] ATP in the presence of Mg, Na and K. The level of the ^{14}C-nucleotide-protein formed by the ouabain-treated enzyme preparation was less than 20% of that formed by the native enzyme preparation between 0 and 5 seconds of reaction time, and about 50% of that of the native preparation between 10 and 360 seconds of reaction time.

In three other experiments using a less stringent ouabain treatment, the degree of ouabain inhibition of nucleotide-protein formation (at 10 seconds of reaction time) amounted to 46% (using 100 µM [U-^{14}C] ATP) and between 29% and 51% (using 200 µM [U-^{14}C] ATP).

In short, 50 to 80% of the measured ^{14}C-nucleotide-protein formation is apparently ouabain sensitive, and

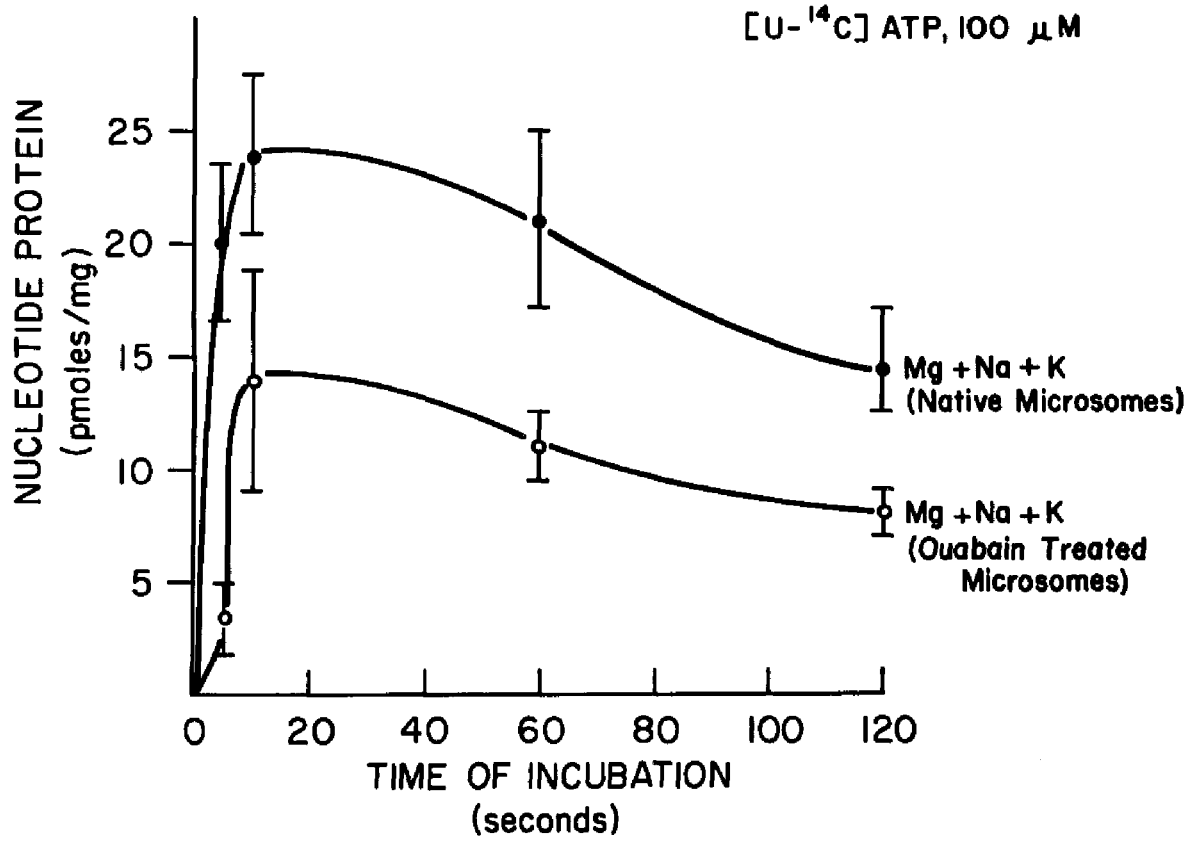

Figure 22. Effect of ouabain pretreatment of the microsomal (Na + K) ATPase on the time-dependent level of ^{14}C-nucleotide-protein formed in the presence of Mg, Na and K. Nucleotide-protein formation, at 0°C, utilizing an initial ATP concentration of 100 µ M and 52.5 µ g of microsomal protein, was terminated by acid precipitation. Blank values were subtracted, and each point represents the mean of four replicate determinations.

therefore provides a presumptive measure of the E-ATP (and P-E-ADP) complexes that form during the (Na + K) ATPase reaction. It follows that the measured level of the ^{14}C-nucleotide-protein should bear a functional relationship to the overall P_i release and phosphoenzyme level, as well as to the substrate concentration.

Figure 23 presents data showing that the rate of P_i release was a straight line function of the concomitant level of E-ATP formed in the presence of Mg, Na and K for ATP levels of less than 200 μM; the rate of P_i release was independent of the level of E-ATP for ATP levels in excess of 200 μM ATP.

Figure 24 presents data showing that the level of phosphoenzyme (EP) was linearly related to that of the nucleotide-protein (E-ATP) in the presence of Mg, Na and K (for E-ATP levels of less than 30 pmoles/mg and initial ATP concentrations of up to 200 μM).

The data of figures 19 through 24 are consistent with the hypothesis that over half of the ^{14}C-nucleotide-protein consists of the primary enzyme-substrate complex(es) that form between the (Na + K) ATPase and ATP and that the level of the nucleotide-protein, like that of the phosphoprotein, is directly related to the rate of hydrolysis of ATP when the reaction is carried out in the simultaneous presence of Mg, Na and K.

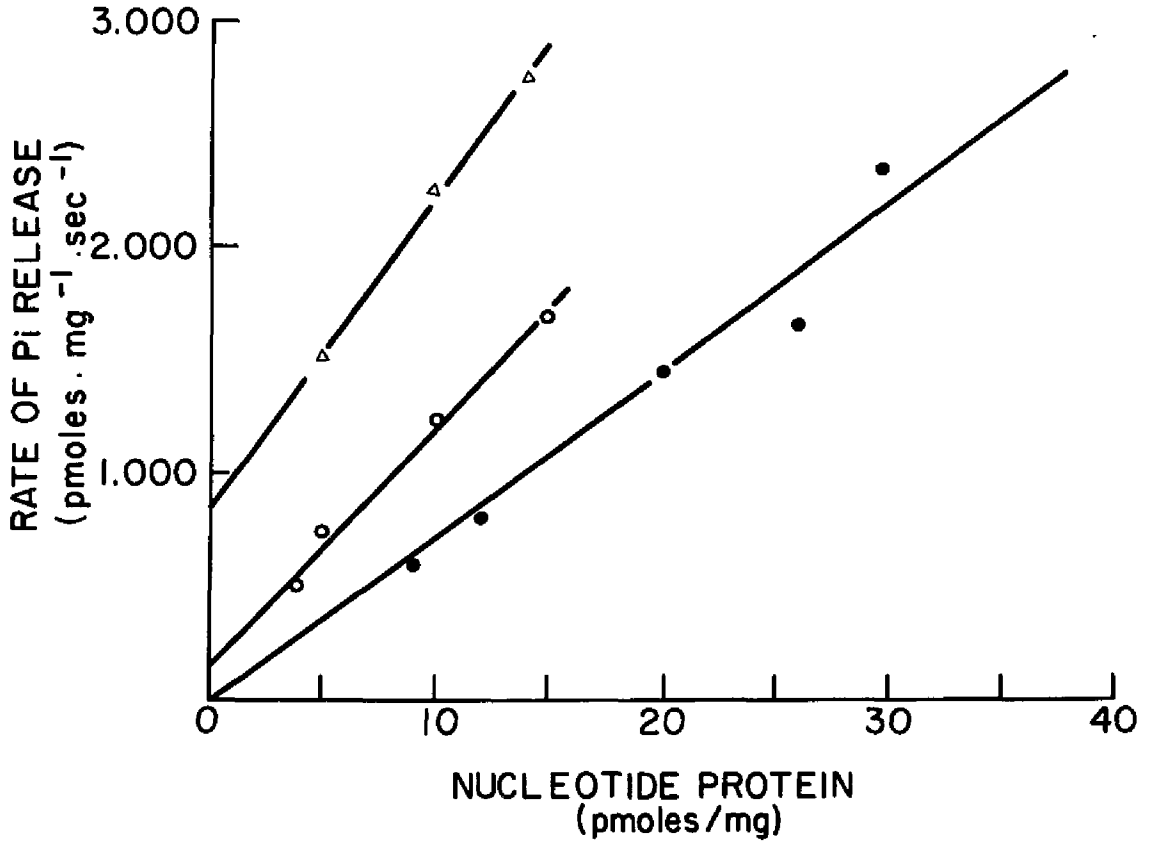

Figure 23. Straight line relationship between the rate of inorganic phosphate release and the level of nucleotide-protein in the steady state. Both parameters were determined after 7.5 seconds of reaction at 0°C in the presence of Mg, Na and K, using varied concentrations of ATP (see figures 8 and 21). Data points were taken from the "best" curves drawn for each parameter. Best straight lines were drawn froma a least squares regression analysis.

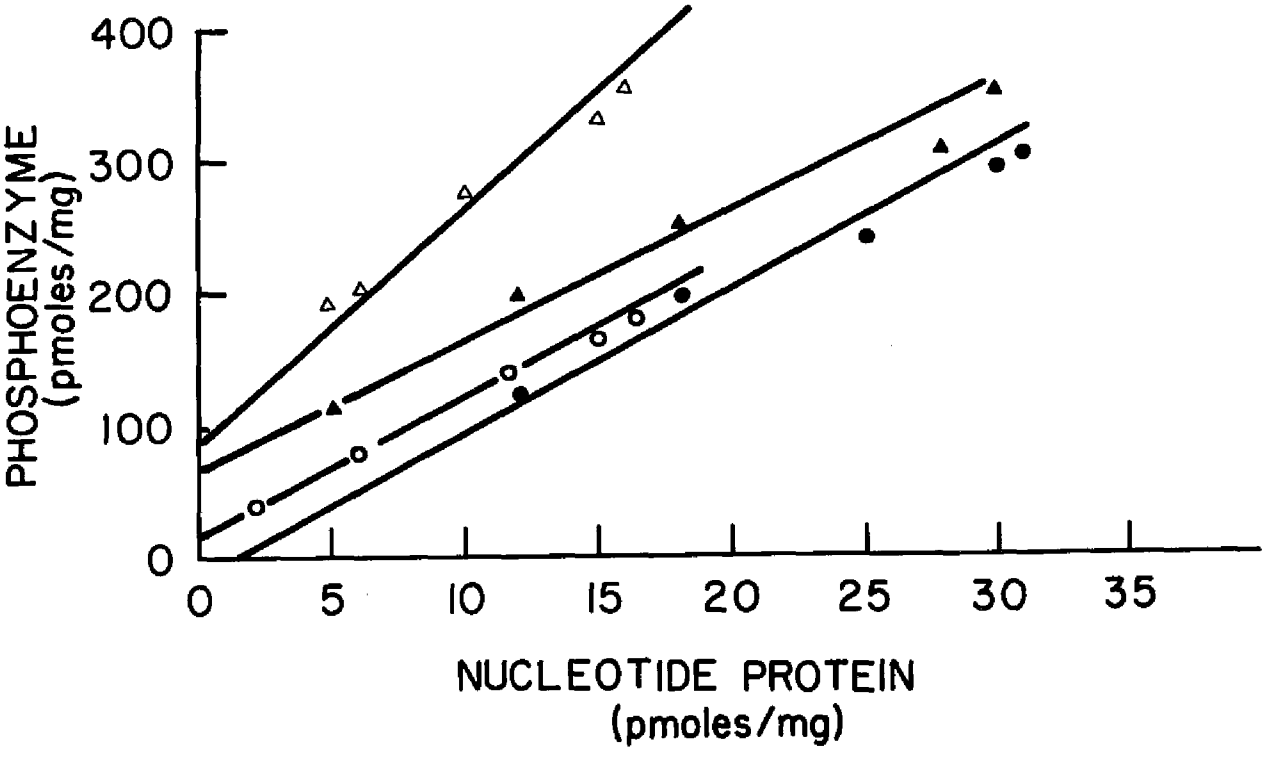

Figure 24. Straight line relationship between the level of the ^{32}P-phosphoprotein and the level of the ^{14}C-nucleotide-protein in the steady state. Both parameters were determined after 7.5 seconds of reaction at 0°C in the presence of Mg, Na and K under varied concentrations of ATP (see figures 13 and 21). Data points were taken from the "best" curve drawn for each parameter. Best straight lines were drawn from a least squares regression analysis.

D. Heat-Terminated Hydrolysis and Phosphoenzyme Formation

The formation of a heat-precipitated, ion-dependent phosphorylated enzyme complex during the reaction between eel electric organ (Na + K) ATPase and [γ-^{32}P] ATP was first described in 1974 (Brodsky and Sohn). The experiments that follow were designed to determine the kinetic parameters of the phosphorylation and hydrolysis reactions, as well as the chemical characteristics of the heat-precipitated phosphoenzyme complex.

Figure 25 is a plot of the time-dependent release of inorganic phosphate from ATP, catalyzed by eel electric organ microsomes under the designated ionic conditions, in a reaction terminated by heat precipitation. The amount of P_i released, minimal in the presence of magnesium, was not significantly changed by the addition of either sodium or potassium during the early part of the reaction; the simultaneous addition of sodium and potassium together increased the inorganic phosphate released by three to four fold.

Thus, the ionic sensitivity of the (Na + K) ATPase reaction terminated by sudden heating is qualitatively similar to the same (Na + K) ATPase reaction terminated by acid. The fact that the amount of inorganic phosphate released after the heat-induced termination is greater than that released after the acid-induced termination under each ionic condition can be ascribed to the short-

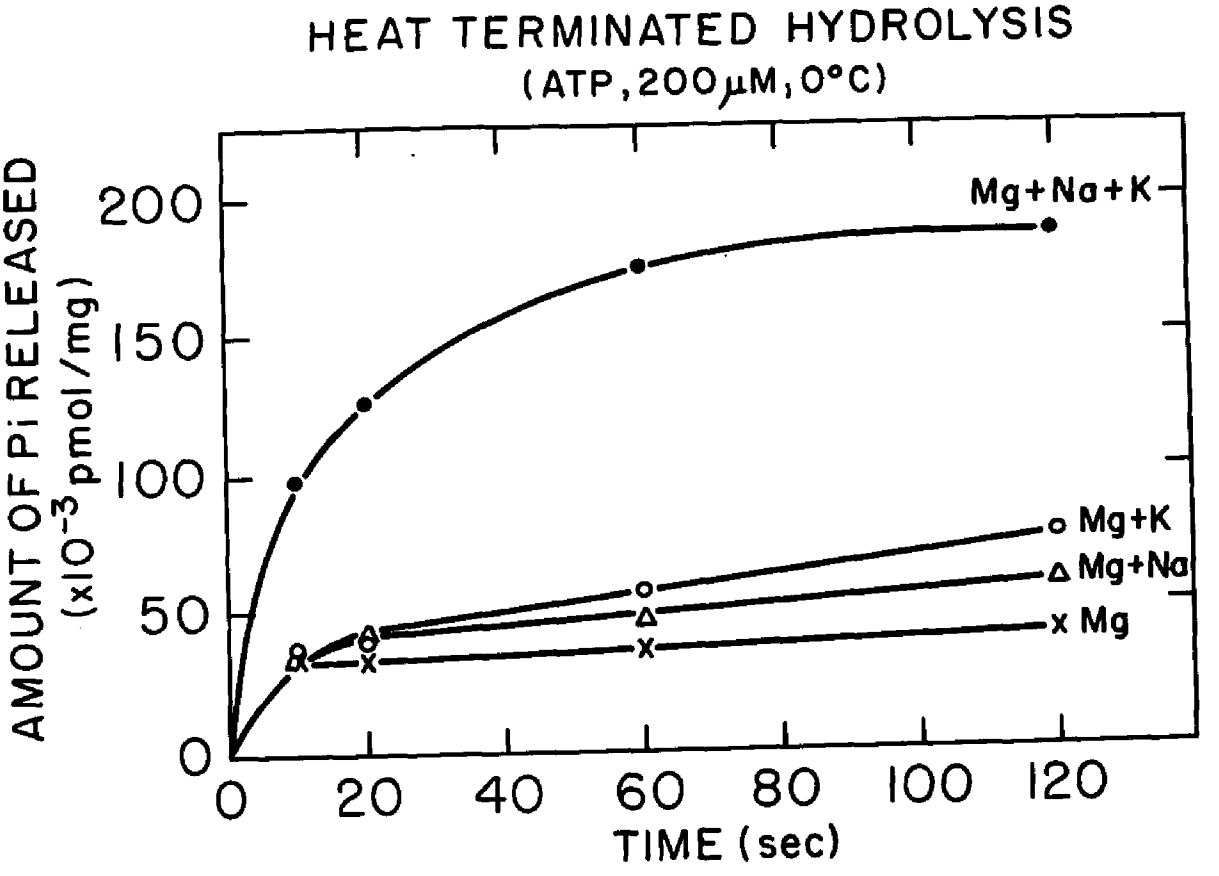

Figure 25. Time course for the release of inorganic phosphate from [γ-32P] ATP by electric organ (Na + K) ATPase under varied ionic conditions. Assays were performed at 0° C with an initial ATP concentration of 200 μM, and 58 μg of microsomal protein. The reaction was terminated by heat precipitation. Blank values were subtracted, and each point represents the mean of 3-4 replicate determinations.

lived period of continuing hydrolysis that occurs while the temperature of the reaction mixture is increased from $0^{o}C$ to $100^{o}C$ and before the enzyme has been fully denatured and thereby inactivated.

Figure 26 is a plot of the time-dependent formation of a heat-precipitated phosphoenzyme complex under the same ionic conditions (i.e. in the same reaction vessel) used to determine the time dependency for the hydrolytic reaction. The level of phosphoenzyme, low in the presence of magnesium, was increased by the addition of either sodium or potassium; however, this Na- or K-induced stimulation was not consistently observed. The simultaneous addition of sodium and potassium (to the magnesium reaction mixture) induced a maximal level of the heat-precipitated phosphoenzyme.

This pattern of ionic responses is in sharp contrast to the pattern of ionic dependency for formation of the acid-precipitated phosphoenzyme-in which case the addition of sodium induces a maximal level of phosphoenzyme-and the further addition of potassium reduces the level of the (Mg + Na)-dependent phosphoenzyme (see figure 11). In the case of the heat-terminated reaction, the conditions which provide for maximal hydrolytic activity (i.e. Mg, Na and K) also provide for the maximal level of phosphoenzyme observed.

The heat-precipitated phosphoenzyme complex reaches

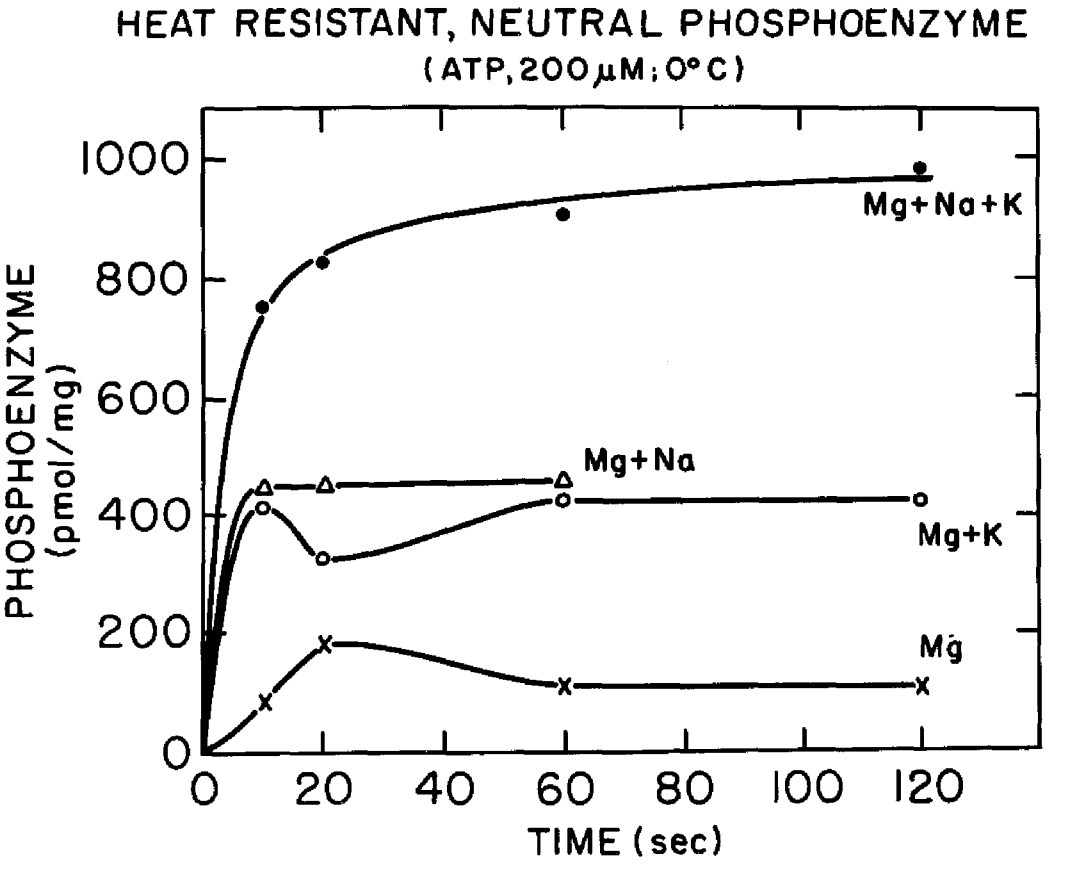

Figure 26. Time-dependent level of heat-precipitated ^{32}P-phosphoenzyme under varied ionic conditions. Phosphorylation assays were performed, as described under Methods, at 0°C; the amount of microsomal protein used was 58 μg, and the initial ATP concentration was 200 μM. The reaction was terminated by heat precipitation. Blank values were subtracted, and each point represents the mean of 3-4 replicate determinations.

a steady state level within the first 10 to 20 seconds of the reaction, while the acid-precipitated phosphoenzyme complex reaches a steady state level within about 5 seconds.

In order to confirm the effect of potassium on the level of the heat-precipitated phosphoenzyme presented in figure 26, the following experiment was performed. The phosphoenzyme formed after five seconds of incubation in the presence of Mg and Na was pulsed with potassium, and the reaction was allowed to proceed for five seconds longer before termination by heat precipitation. The pulse of potassium (figure 27) increased the level of the phosphoenzyme by about 50% at the same time as it increased the overall rate of hydrolysis. We decided to study the heat-precipitated phosphoenzyme formation and hydrolysis in the presence of Mg, Na and K, since these ionic conditions provided for the maximal level of phosphoenzyme, as well as for maximal hydrolytic activity.

Another reason for choosing the heat-precipitated (Mg + Na + K)-dependent phosphoenzyme for further study was the observation that a consistent effect of ouabain in reducing the level of the phosphoenzyme was obtained only under these ionic conditions. When the heat-precipitated phosphoenzyme was formed in a reaction between microsomal (Na + K) ATPase and 200_{μ} M [γ-^{32}P] ATP at 0^{o}C (reaction time, 15 seconds), the following effects of ouabain were observed. For the Mg-dependent

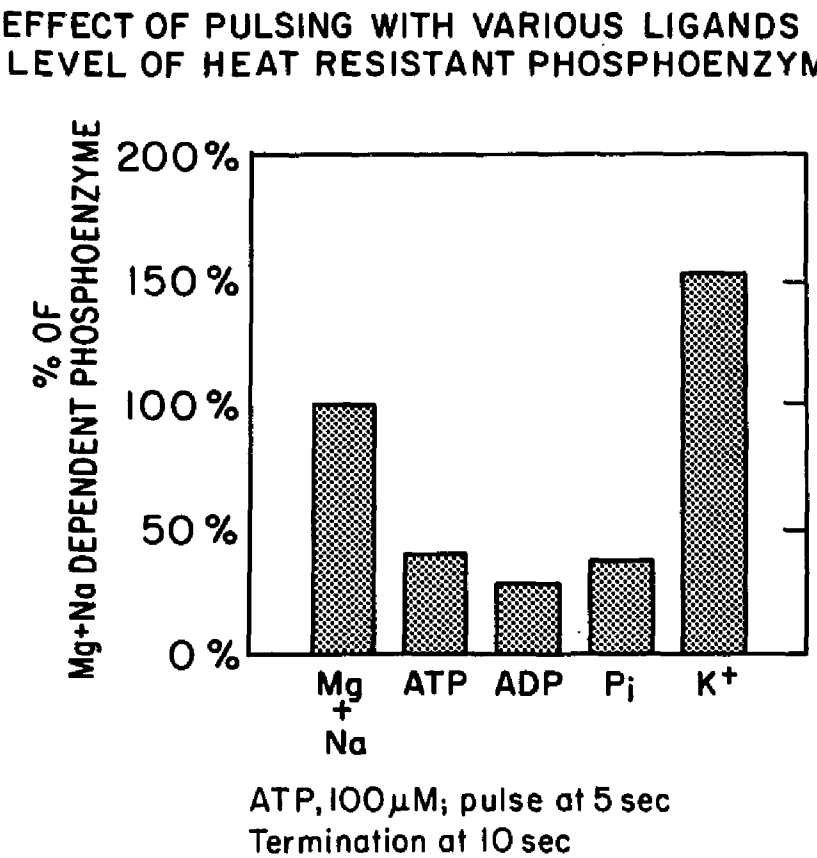

Figure 27. Effect of pulsing the reaction with various ligands on the level of the heat-precipitated phosphoenzyme formed in the presence of Mg and Na. The phosphorylation-hydrolysis reaction, at 0°C, and in the presence of Mg and Na, was initiated with 100 μM [γ-^{32}P] ATP, as described in the Methods section. After 5 seconds, the reaction was pulsed with 1 mM concentrations of the given ligands, and the reaction was terminated 5 seconds later by heat precipitation. Blank values were subtracted, and each level of phosphoenzyme represents the mean of three replicate determinations.

phosphoenzyme, ouabain decreased the level of phosphoenzyme by 9 to 24% or increased the level by 28%. For the (Mg + Na)-dependent phosphoenzyme, ouabain either decreased the level by 28% or increased the level by 28%. However, in the case of the (Mg + Na + K)-dependent phosphoenzyme, ouabain consistently caused a decrease in the level of phosphoenzyme of from 40 to 70%.

Figure 28 plots the rate of inorganic phosphate release from ATP, at $0^{o}C$, by the microsomal ATPase as a function of the initial concentration of ATP, in the presence of Mg, Na and K. In this reaction, terminated by heat precipitation after 5 or 10 seconds, the maximal rate of hydrolysis was approximately 35 μmoles/mg protein/hr, and the K_m was estimated as 65 μM. A similar K_m was estimated for the reaction terminated after 30 seconds. The K_m for the identical hydrolytic reaction terminated by acid precipitation was about 40 μ M (see figure 9).

As the method utilized for termination of the hydrolytic reaction should in no way alter the interaction between the enzyme and the substrate, the observed increase in the K_m of hydrolysis when the reaction is terminated by heat precipitation must be ascribed to the transient increase in the reaction temperature at the onset of heating. This increase in the K_m is consistent with the fact that the hydrolytic reaction for eel electric organ microsomes at $23^{o}C$ has a K_m

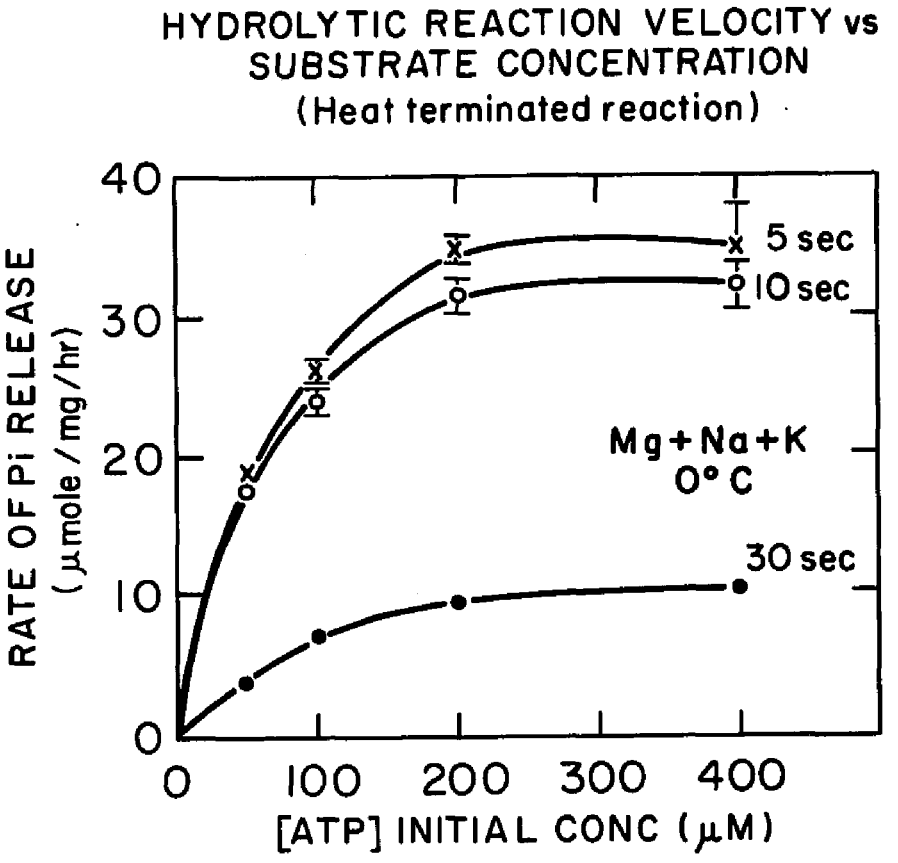

Figure 28. Hydrolytic reaction velocity in the presence of Mg, Na and K as a function of the initial concentration of ATP. The hydrolytic reaction, at 0°C, was terminated at various times by heat precipitation. The amount of microsomal protein utilized in these experiments was 25 μg (×), 45.9 μg (○), or 73.5 μg (●). Blank values were subtracted at each concentration, and each point represents the mean of four replicate determinations.

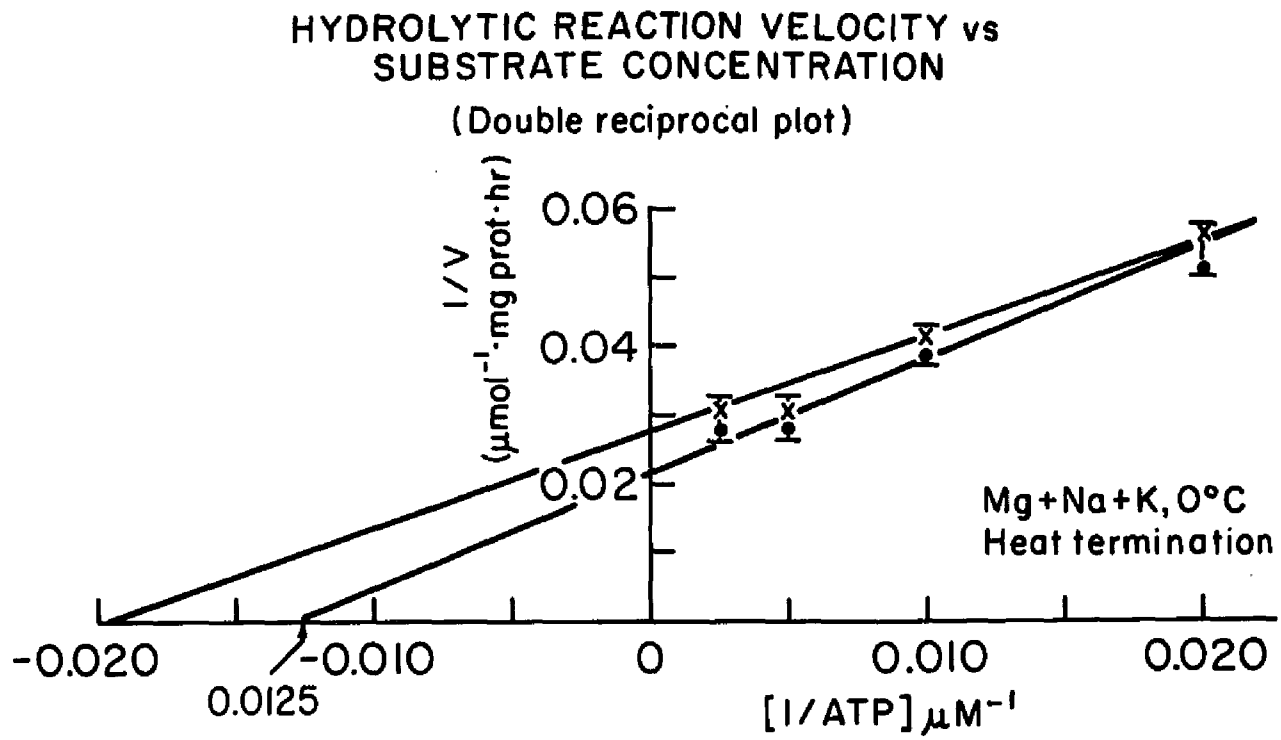

Figure 29. Double reciprocal plot of hydrolytic reaction velocity versus initial ATP concentration. Reaction conditions were the same as those in figure 28. Best straight lines were drawn from a least squares regression analysis of the data.

of 290 μM (Dixon and Hokin, 1974).

Figure 30 plots the level of the heat-precipitated phosphoenzyme (formed concomitantly with the above hydrolytic reaction) as a function of concentration of ATP (corrected for the degree of hydrolysis). From a double reciprocal plot of these data (figure 31), it can be estimated that the maximal level of the heat-precipitated, (Mg + Na + K)-dependent phosphoenzyme that could be observed with these enzyme preparations would be about 500 pmoles/mg; the estimated K_m for this phosphorylation reaction is approximately 100 μM ATP.

As the apparent K_m for phosphoenzyme formation is significantly greater than that for hydrolytic activity, only part of the phosphoenzyme complex could be related to the hydrolytic activity. This is understandable, as the microsomal preparation utilized in our experiments is not a purified enzyme, and it is to be expected that other, non-ATPase proteins would be capable of binding the phosphate in a heat-precipitated reaction.

In order to ascertain that the bound ^{32}P-phosphate measured after heat precipitation was, in actuality, bound to protein rather than lipid moieties of the microsomal preparation, a chloroform-methanol extraction was performed on the phosphoenzyme formed in the presence of Mg, Na and K. With about 87% of the bound ^{32}P-phosphate recovered overall, it was found that the chloroform-

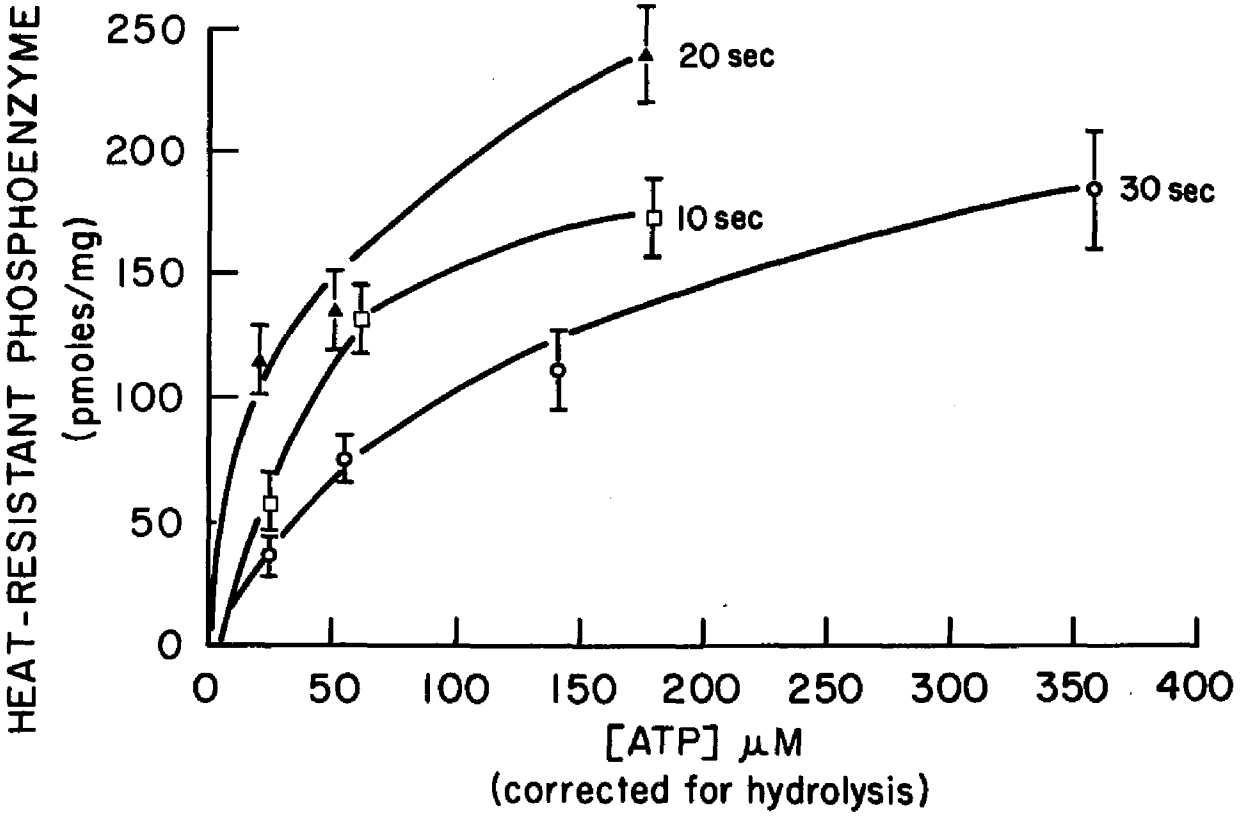

Figure 30. Level of heat-precipitated ^{32}P-phosphoenzyme as a function of the level of ATP (corrected for the degree of hydrolysis). The phosphorylation-hydrolysis assay, at 0°C, and in the presence of Mg, Na and K, was terminated at various times by heat precipitation. The amount of microsomal protein used in these experiments was 45.9 μg (□), 47 μg (▲), or 73.5 μg (○). Blank values were subtracted at each concentration, and each point represents the mean of four replicate determinations.

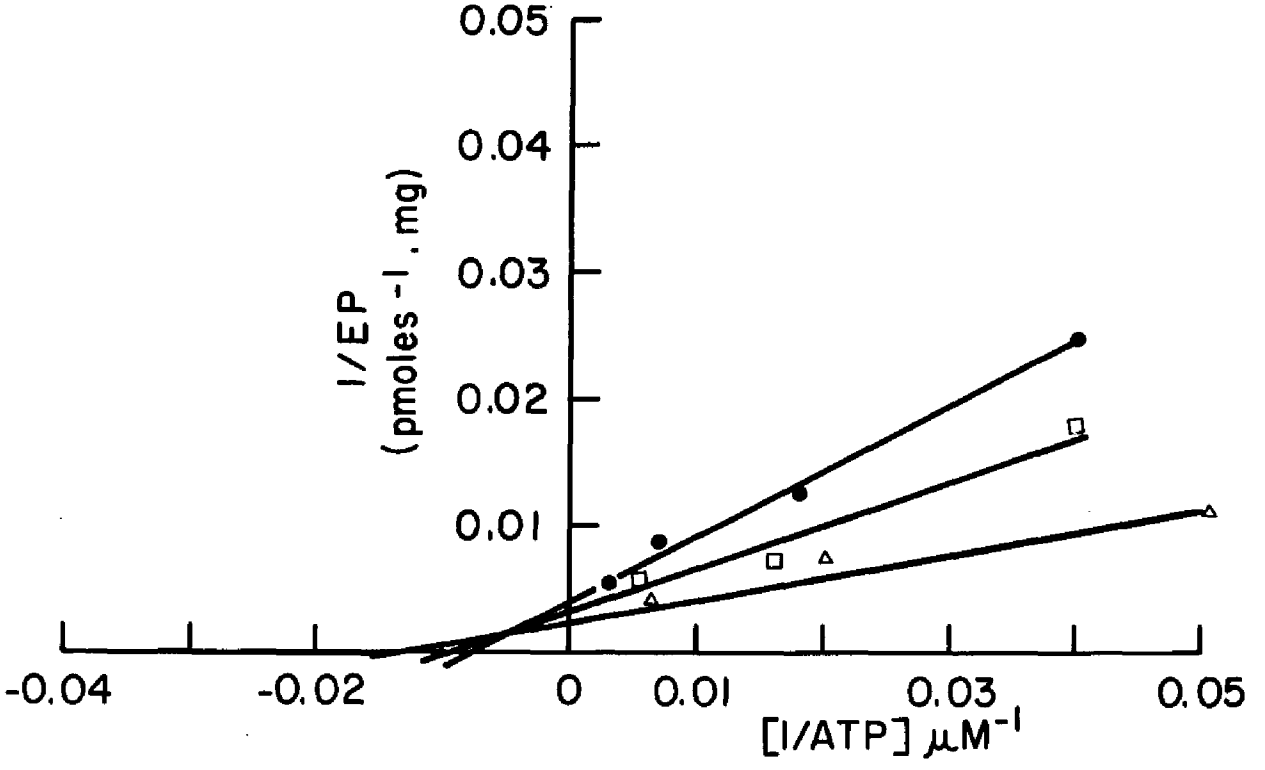

Figure 31. Double reciprocal plot of heat-precipitated ^{32}P-phosphoenzyme level as a function of the initial concentration of ATP. Reaction conditions were those of figure 30. Best straight lines were drawn from a least squares regression analysis.

methanol extraction procedure did not solubilize the phosphate binding moiety. This supports the contention that the substance binding the phosphate is protein and not lipid in nature.

It has been established that the acid-precipitated phosphoenzyme formed in the presence of Mg and Na is formed via an acyl-phosphate linkage (Bader et al,1966). In order to determine whether the (Mg + Na + K)-dependent phosphoenzyme observed after heat precipitation of the hydrolysis reaction is formed via a similar chemical linkage, its stability to incubation at various pHs was measured and compared to that of the acid-precipitated (Mg + Na)-dependent phosphoenzyme (figure 32).

The washed ^{32}P-phosphoprotein precipitates were suspended in 100 µl of a 50 mM solution of buffers at various pHs, and incubated for 30 minutes at 26°C. One set of ^{32}P-phosphoprotein precipitates was left untreated in each case to provide a measure of the baseline level of phosphorylation.

Figure 32 plots the percentage of phosphoenzyme remaining (compared to the total amount of phosphoenzyme found before treatment) as a function of the pH of treatment. The acid-precipitated phosphoenzyme showed a high degree of stability at acid pHs up through pH 8, with a sharp decline in stability at more alkaline pH,

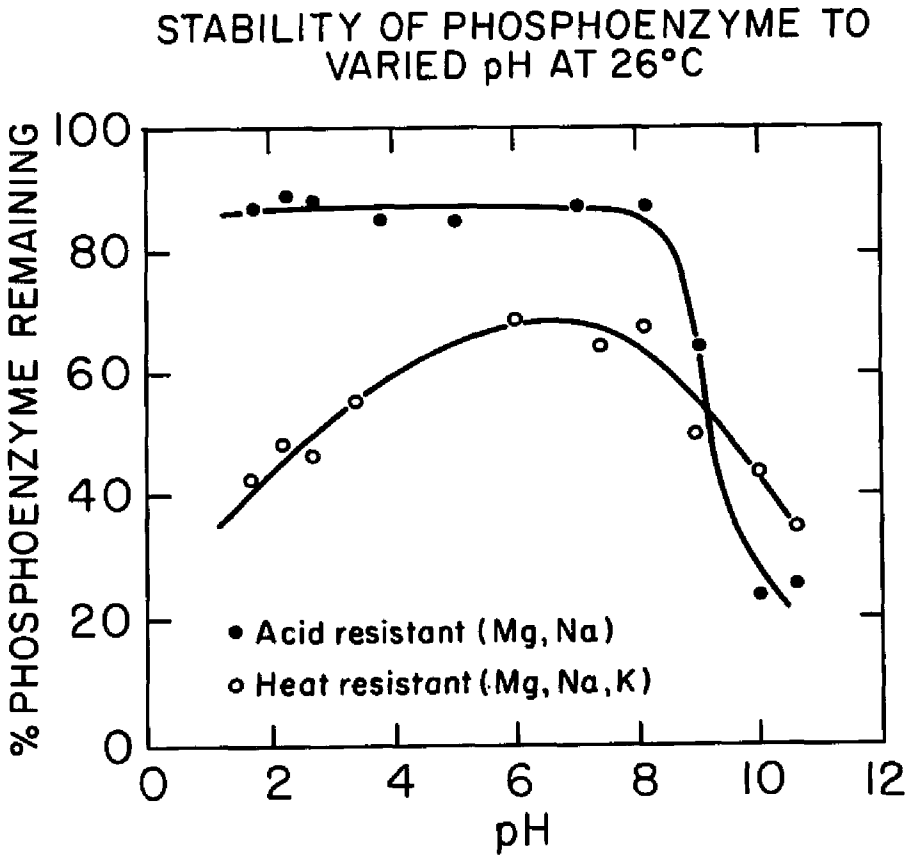

Figure 32. Stability of heat-precipitated and acid-precipitated phosphoenzymes to varied pH treatment at 26°C. The phosphoenzymes were formed at 0°C after reaction with [γ-^{32}P] ATP ([ATP] = 200 μM) for 10 seconds in the presence of Mg and Na (acid precipitation) or Mg + Na + K (heat precipitation). After treatment with buffers of varied pH, the amount of ^{32}P in the precipitate and the buffer supernatant was measured. The ordinate shows the amount of ^{32}P remaining bound to the precipitate as a percentage of that bound before the treatment with buffer.

a pattern of stability consistent with the findings of others on the ATPase phosphoenzyme (Hokin et al, 1965; Bader et al, 1966; Ratanabanangkoon and Hokin, 1973).

The heat-precipitated phosphoenzyme, in contrast, showed a broad peak of maximal stability between pH 6 and 8, suggesting that the heat-precipitated phosphoenzyme complex was not formed via an acyl-phosphate linkage. The validity of these observations is strengthened by the fact that: (i) the same batch of microsomes was utilized for both sets of experiments; (ii) the pH ranges were repeated with buffers covering the same pH range; and (iii) the hydroxylamine sensitivity studies in figure 33, which support the acyl-phosphate nature of the acid-precipitated phosphoenzyme, and the non-acyl-phosphate nature of the heat-precipitated phosphoenzyme.

The heat precipitated phosphoenzyme showed an overall higher degree of lability to mechanical manipulation than the acid-precipitated phosphoenzyme. In figure 32, incubation of the heat-precipitated phosphoenzyme at 26^{o}C for 30 minutes, even at the pH of maximal stability (6 to 8), reduced the level of the phosphoenzyme by about 30%. In contrast, these manipulations, which include a suspension and centrifugation step, reduced the level of the acid-precipitated phosphoenzyme by only about 10%.

The tendency of the heat-precipitated phosphoenzyme complex to dissociate under repeated mechanical manipulations was underscored during the normal washing procedure utilized to free the precipitate of non-specifically bound ^{32}P (see Methods). After two or three turns of re-suspension and re-centrifugation, the heat-precipitated microsomal pellet showed a tendency to migrate into the wash fluid; it became necessary to increase both the time of the centrifugation as well as the gravitational force exerted in order to keep the precipitate tightly packed. The acid-precipitated pellet tended to remain well packed through repeated rounds of suspension and centrifugation.

In order to further confirm that the heat-precipitated phosphoenzyme bond was not an acyl-phosphate linkage, hydroxylamine was used as a probe of the chemical reactivity of the bond. Hydroxylamine (at neutral pH) is known to break apart an acyl phosphate linkage by the formation of a hydroxamate bond (Lippman and Tuttle, 1945) according to the following reaction:

$$E\text{-}C(=O)\text{-}O\text{-}P + NH_2OH \longrightarrow E\text{-}C(=O)\text{-}NHOH + P_i$$

and other laboratories have previously shown that the acid-precipitated sodium phosphoenzyme bond is broken by treatment with neutral hydroxylamine (Hokin et al, 1965)

The heat-precipitated (Mg + Na)-dependent and (Mg + Na + K)-dependent phosphoenzymes as well as the acid-precipitated (Mg + Na)- and (Mg + Na + K)-dependent phosphoenzymes were treated with 0.8 M hydroxylamine (at neutral pH) for 10 minutes at 26°C, and the amount of radiolabeled ^{32}P-phosphate released into the supernatant was determined. The ^{32}P-phosphoenzymes formed after acid precipitation were, as expected, highly sensitive to hydroxylamine treatment (figure 33), and from 70 to 90% of the bound ^{32}P-phosphate was released into the supernatant. Treatment with a neutral buffer system (the same as that utilized with the hydroxylamine) removed little of the bound label. In contrast, the neutral hydroxylamine had little or no advantage over the neutral buffer treatment in removing the ^{32}P-phosphate bound in the heat-precipitated ^{32}P-phosphoenzyme complexes.

The similar sensitivity of the acid-precipitated phosphoenzymes formed in the presence of Mg and Na and in the presence of Mg, Na and K to hydroxylamine treatment is consistent with the idea that both linkages are of the acyl-phosphate type, and that different levels of the same phosphoenzyme are formed under the two ionic conditions. Similarly, the lack of effect of hydroxylamine on the heat-precipitated phosphoenzymes suggests that both

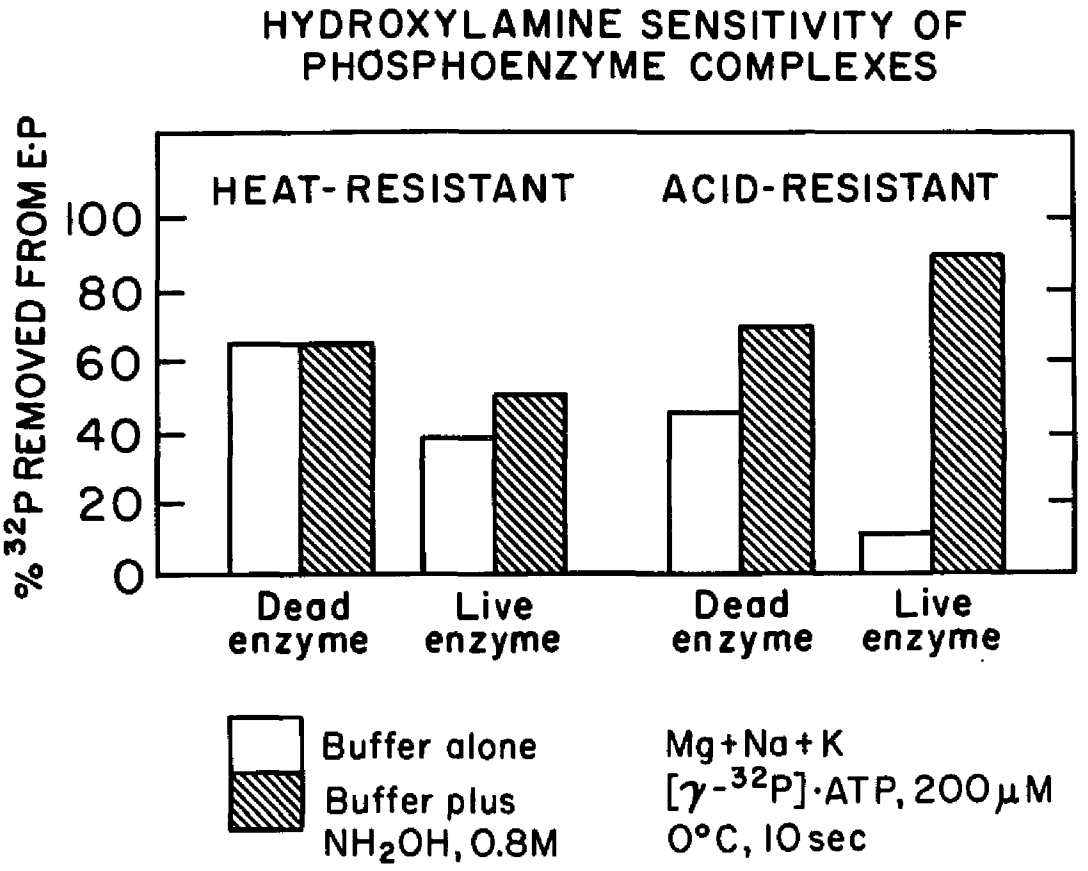

Figure 33. Hydroxylamine sensitivity of the heat- and acid-precipitated phosphoenzyme complexes. Heat- and acid-precipitated ^{32}P-phosphoenzymes were formed after the reaction of microsomal (Na + K) ATPase with [γ-^{32}P] ATP ([ATP] = 200 μM) for 10 seconds at 0°C in the presence of Mg and Na and in that of Mg, Na and K. Both sets of precipitated microsomes were treated with neutral buffer and hydroxylamine in neutral buffer, and the percentage of the bound ^{32}P that was removed by these treatments was measured.

the (Mg + Na)- and the (Mg + Na + K)-dependent phosphoenzymes observed after heat precipitation might be formed via the same chemical linkage. This hypothesis, however, requires a detailed study of the chemical nature of the two heat-precipitated phosphoenzymes.

The next series of experiments was designed to examine the effects of various ligands on the level of phosphorylation in the heat-terminated reaction. The formation (and breakdown) of the phosphoenzyme in the presence of Mg and Na or Mg, Na and K was allowed to progress for 5 seconds before the reaction was pulsed with either distilled water (for control level of phosphorylation), 1 mM ATP (final concentration), 1 mM ADP, or 1 mM inorganic phosphate. After the pulse (or chase), the phosphorylation reaction continued for 5, 10 or 15 seconds longer before termination by heat precipitation and determination of the level of phosphoenzyme.

Figure 34 shows that the addition of an excess of unlabeled ATP to the phosphorylation reaction taking place in the presence of Mg, Na and K caused an almost linear fall (with time) of the level of the phosphoenzyme. This suggests that the (Mg + Na + K)-dependent phosphoenzyme is turning over while the hydrolysis of ATP is taking place; that is, the addition of non-radiolabeled ATP to the reaction causes

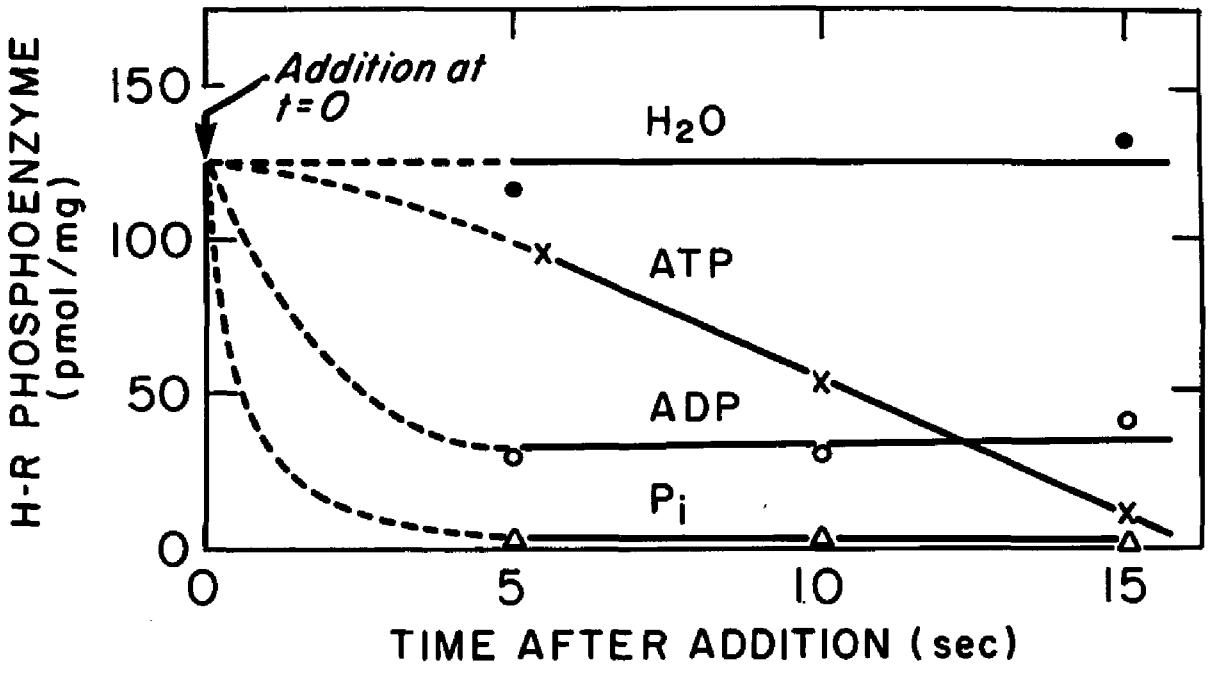

Figure 34. Effect of pulsing with various ligands on the time-dependent level of the heat-precipitated ^{32}P-phosphoenzyme formed in the presence of Mg, Na and K. The ^{32}P-phosphoenzyme was formed after reaction of the microsomal (Na + K) ATPase with [γ-^{32}P] ATP ([ATP] = 200 μM) for 5 seconds at 0°C; at this point, the reaction was pulsed with 1 mM concentrations of ATP, ADP or inorganic phosphate, and the level of ^{32}P-phosphoenzyme determined 5, 10 and 15 seconds after the pulse in a reaction terminated by heat precipitation. Blanks were subtracted, and each point is the mean of four replicate determinations.

the replacement of the radiolabeled phosphate bound to the enzyme with non-radiolabeled phosphate from the "chasing" ATP molecule.

The level of the (Mg + Na)-dependent phosphoenzyme was reduced by approximately 50% five seconds after a pulse of non-radiolabeled ATP (see figure 27). This suggests that the (Mg + Na)-dependent phosphoenzyme is also turning over during the hydrolytic reaction (perhaps at a slower rate than the (Mg + Na +K)-dependent phosphoenzyme).

The effect of an excess of inorganic phosphate as the chasing ligand (figure 34) was to bring the level of the (Mg + Na + K)-dependent phosphoenzyme down to zero within five seconds. The fact that the phosphoenzyme can rapidly and effectively exchange with inorganic phosphate while the hydrolysis reaction is taking place suggests that the heat-precipitated phosphoenzyme is formed via a low-energy linkage similar to that proposed for the E_2P complex of the (Na + K) ATPase reaction scheme described in the *Introduction*.

A similar (though apparently incomplete) exchange of the phosphoenzyme with inorganic phosphate is observed for the (Mg + Na)-dependent reaction (figure 27). This suggests that the heat-precipitated, (Mg + Na)-dependent phosphoenzyme also has some characteristics of a low-energy linkage.

An excess of ADP as the chasing ligand brings the level of the (Mg + Na + K)-dependent phosphoenzyme down to about one fifth that of the water-pulsed control (figure 34). As the reduction in the phosphoenzyme level is initially more effective than that induced by ATP as the chasing ligand, it must be assumed that the action of ADP is not to compete with the [γ-^{32}P] ATP for binding sites on the enzyme; if the action of the ADP was to compete with the radiolabeled ATP molecule, then ATP would have been a more effective competitor. Rather, the decreased phosphoenzyme level resulting from the ADP pulse suggests that the ADP is being phosphorylated by the phosphoenzyme in a reversal of reaction 2 (see Introduction). This phosphorylation of ADP suggests that a high energy bond exists between the enzyme and the phosphate. This conclusion is in apparent contradiction to the idea that the enzyme-phosphate bond is of the low energy type, which was suggested by the efficacy of the chase by inorganic phosphate.

The fact that the phosphoenzyme level is maintained at a constant level after the ADP pulse suggests that an equilibrium may have been reached between the phosphoenzyme and the ADP, at least in the time interval over which the reaction was observed.

The effect of an ADP pulse in reducing the level

of the (Mg + Na)-dependent phosphoenzyme (figure 27) suggests that this heat-precipitated phosphoenzyme also has some characteristics of a high energy linkage, allowing for the phosphorylation of the ADP molecule.

The experiments described in figures 27 and 34 again suggest a pattern of similarities between the (Mg + Na)- and the (Mg + Na + K)-dependent phosphoenzymes observed after heat precipitation (as do the hydroxylamine-sensitivity experiments described by figure 33), and it would be worthwhile to study both sets of phosphoenzymes in more detail.

Further support for a mixed ADP and P_i sensitivity of the heat-precipitated phosphoenzymes is obtained from experiments in which ADP and/or P_i were included in in the original incubation mixture along with the [γ-^{32}] ATP. Figure 35 shows that both ADP and inorganic phosphate were effective in reducing the level of phosphoenzyme ultimately observed, whether the reaction was carried out in the presence of Mg and Na or Mg, Na and K.

In light of the probable low energy nature of the phosphate-enzyme linkage ultimately observed as the heat-precipitated ^{32}P-phosphoenzyme complex, a series of experiments were performed to determine whether the enzyme could be phosphorylated from inorganic phosphate ($H_3{}^{32}PO_4$) in a reaction terminated by heat precipitation.

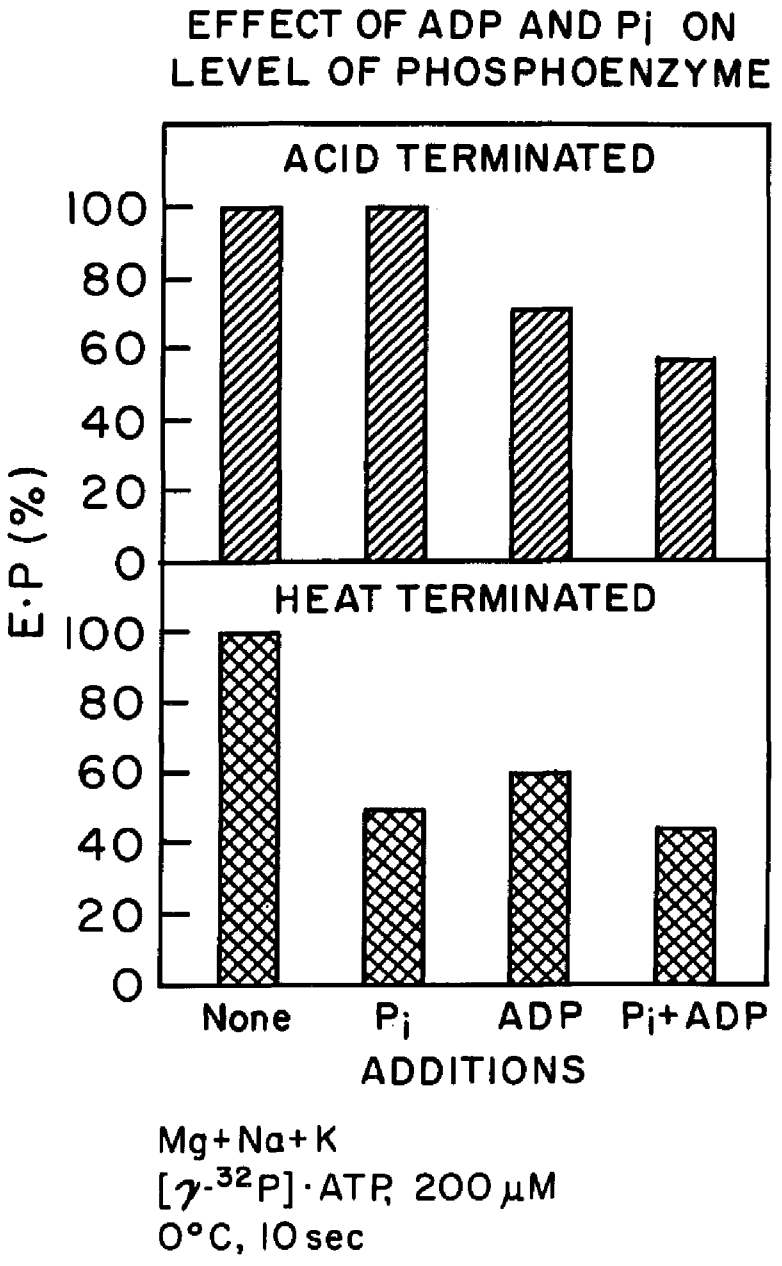

Figure 35. Effect of ADP and P_i in the reaction mixture on the level of the heat- and acid-precipitated 32P-phosphoenzymes. The ^{32}P-phosphoenzymes were formed in the presence of Mg and Na or in that of Mg, Na and K, and isolated after heat or acid precipitation. The phosphorylation reactions were carried out at 0°C for 10 seconds using [γ-^{32}P] ATP ([ATP] = 200 μM) with and without 200 μM ADP, 200 μM P_i, or 200 μM ADP and 200 μM P_i. The amount of microsomal protein used was 54 μg.

Figure 36 shows that this "back-labeling" reaction occurred equally well in the presence of Mg and Na and in that of Mg, Na and K, and was unaffected by the absence or presence of ATP. Furthermore, when the enzyme (microsomal) preparation was pre-treated with ouabain, the back-labeling reaction was inhibited by 35 to 50%; this suggests that the phosphorylation by inorganic phosphate requires an enzymatically active enzyme preparation.

When the back-labeling reaction was terminated by acid precipitation, the amount of of labeling by the radiolabeled inorganic phosphate was practically zero, and this is consistent with the findings of others (Albers et al, 1968; Lindenmayer et al, 1968). Binding of the inorganic phosphate after acid precipitation tends to reach significant levels in experiments where the enzyme preparation has been pre-treated with ouabain; this approach is thought to stabilize the E_2 form of the enzyme which should readily exchange with inorganic phosphate (Post et al, 1969; Siegel and Josephson, 1972).

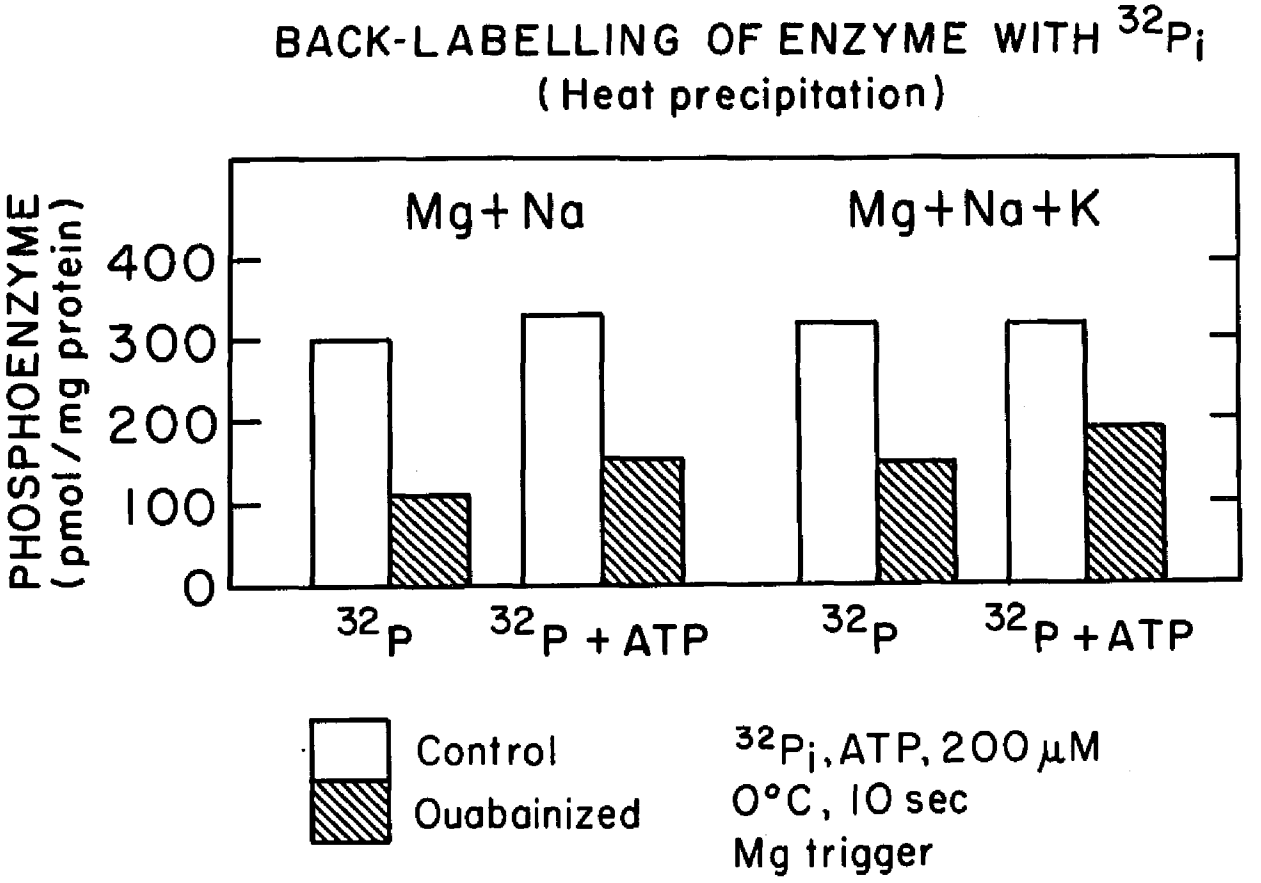

Figure 36. "Back-labeling" of microsomal (Na + K) ATPase with $^{32}P_i$. Native and ouabain-treated microsomes were incubated for 10 seconds at 0°C with $H_3{}^{32}PO_4$ (concentration of carrier Tris-PO_4 was 200 μM) in the presence of Mg and Na and in that of Mg, Na and K. In some experiments, non-radiolabeled ATP (200 μM) was also included. The reaction was terminated by heat precipitation, and the assay for bound ^{32}P was carried out as described in the Methods section. Blanks were subtracted, and each level of phosphoenzyme represents the mean of 3-4 replicate determinations.

DISCUSSION

The reaction sequence for the catalyzed hydrolysis of ATP by (Na + K) ATPase has usually been elucidated through characterization of the intermediary complexes formed by blocking the reaction in mid-sequence. In order to observe binding of ATP to the enzyme, in the first step of the reaction sequence, Hegyvary and Post (1971) and Norby and Jensen (1971) utilized a magnesium-free environment, which eliminated all hydrolytic activity. In order to observe maximal binding of the phosphate to the enzyme, experimental procedures have generally involved a potassium-free environment (magnesium + sodium alone); this ionic environment allows for the binding of ATP and subsequent phosphorylation but keeps hydrolysis at a minimum level by preventing the K-induced stripping of the bound phosphate.

The above experimental approaches assume that the enzyme is in its "natural" configuration even in the absence of the ionic environment surrounding the enzyme protein in the living cell. It is difficult to accept this point of view since the binding or removal of ligands might well induce conformational changes in the protein; some evidence for these effects of ion binding comes from the observation that the phosphoenzyme changes from an E_1P conformation, sensitive to ADP

to an E_2P conformation sensitive to K upon the addition of a high concentration of magnesium to the reaction vessel (Glynn and Karlish, 1975). Racker's group (Y. Kuriki et al, 1976) has recently presented data showing that conformational changes resulting from ion binding to the protein can provide the energy required to phosphorylate ADP. Hart and Titus (1973) have also demonstrated that binding of sodium, potassium, magnesium, ATP or ouabain, alone and in varied combinations, induces conformational changes in the enzyme protein-as measured by the number of sulfhydryl groups available for complexation by NEM.

The experiments described in this dissertation demonstrate that it is possible to isolate and characterize the intermediary complexes formed during the steady state hydrolysis of ATP by the (Na + K) ATPase in the simultaneous presence of magnesium, sodium and potassium; that is, under the optimal conditions for hydrolytic activity in vitro and sodium pump activity in vivo.

A. The Nucleotide-Protein Complex

The level of the nucleotide-protein complex observed (in the absence of magnesium) through the equilibrium dialysis experiments of Hegyvary and Post (1971) and Norby and Jensen (1971) is decreased by the addition of ouabain or potassium and increased by the addition of sodium. Although potassium reduces the affinity of the

(Na + K) ATPase for the ATP molecule, the authors are able to show that the maximal binding capacity is increased. Scatchard analysis of the bound [γ-^{32}P] ATP displaced from the enzyme by the non-radiolabeled ATP allows the authors to estimate that the maximum number of ATP binding sites (in the absence of any ions) is equal to the maximum number of phosphorylation sites in the presence of magnesium and sodium. The apparent K_m for the formation of this enzyme-ATP complex is approximately 0.1 μM in the absence of any ions and about 20 μM in the presence of potassium.

Maximal levels of the acid-precipitated nucleotide-protein complex are observed in the simultaneous presence of Mg, Na and K and the formation of this complex is inhibited by ouabain. The maximal level of ^{14}C-nucleotide-protein complex observed with eel electric organ microsomes is of the same order as that found with ox brain microsomes by Klodos and Skou (1975); and the apparent K_m for E·ATP formation, 35 μM, is close to that previously found by Shamoo and Brodsky with turtle bladder microsomes (Shamoo and Brodsky, 1971).

It is important to emphasize that Norby and Jensen (1971) and Hegyvary and Post (1971) had found that K increases the maximum level of E·ATP as well as the K_m(apparent) of its formation by kidney microsomes in a Mg-free (non-hydrolyzing) system. Thus, K increases the maximum level of E·ATP that can be observed in a

Mg-free as well as Mg-rich incubation mixture, suggesting that K binding to the free enzyme is required for the conformational change resulting in the maximum number of ATP binding sites as well as the optimal hydrolytic activity.

In this context, it is pertinent to note some of the current interpretations of K effects on various steps in the (Na + K) ATPase reaction sequence.

Robinson (1967) and Hexum (1970) have shown that potassium, even in the presence of magnesium, increases the K_m for ATP in the hydrolysis reaction. Skou and Hilberg (1969) and Siegel and Goodwin (1972) have shown that potassium can inhibit both phosphorylation and hydrolysis at ultra-low ATP concentrations. However, at the ATP concentrations utilized in our experiments, potassium unequivocally increases the V_{max} of the hydrolytic reaction (see figure 5), and this effect of potassium is consistent with the increased level of nucleotide-protein observed after the addition of potassium to the (magnesium + sodium)-reaction mixture.

Peter and Wolf (1972) have suggested that potassium is required for the binding of sodium and Mg-ATP to the free enzyme-on the basis of their kinetic analysis of the overall hydrolytic reaction. This is consistent with our present observations on the effects of potassium on the intermediary and overall steps of the (Na + K) ATPase reaction sequence.

Post et al (1972) have suggested that the potassium-enzyme complex does not react with ATP until it is dissociated by sodium, which then leads to formation of the phosphoenzyme. The high concentration of sodium used in our experiments (in conjunction with magnesium and potassium) allows for the incorporation of this observation into a reaction scheme in which sodium, as well as potassium, is involved in the formation of the E·ATP complex during the active hydrolytic sequence.

Banerjee and Wong (1972) have suggested that the potassium-induced acceleration of the ATP:ADP exchange reaction (in the presence of sodium) results from a potassium-induced blockade of the conversion of E_1P to E_2P (see reaction scheme in the Introduction); this, in turn, could result in an accumulation of E·ATP-as was found in the presence of magnesium, sodium and potassium.

B. Kinetics of the Intermediary and Overall (Na + K) ATPase Reaction Sequence

The level of the acid-precipitated nucleotide-protein complex observed in our experiments was approximately one-tenth the level of phosphoenzyme formed under the same reaction conditions (i.e. in the presence of Mg, Na and K). This finding is consistent with a reaction in which the nucleotide-protein turns over to produce phosphoprotein at a high rate during the steady state

reaction (see calculations of the rate constants for the turnover of nucleotide-protein to phosphoprotein and of phosphoprotein to inorganic phosphate, below).

The apparent K_m for nucleotide-protein formation, as well as that for phosphoprotein formation, is approximately the same as the Km of the hydrolytic reaction in the presence of Mg, Na and K. This finding is consistent with the idea that both the phosphoprotein and the nucleotide-protein are intermediates in the hydrolytic reaction sequence. However, it should be emphasized that this finding is not adequate proof for this hypothesis. In the case where the levels of the putative intermediates have reached a maximum (in the steady state) without a concomitant maximal hydrolysis, one would be forced to rule out these complexes as reaction intermediates. However, if the K_m for formation of the putative intermediate complexes was greater than the K_m for hydrolysis, the involvement of these complexes in the reaction sequence could not be ruled out. In this latter case, one could hypothesize regulatory binding sites for the ATP and the inorganic phosphate.

In order to clarify the steady state kinetics of the partial and overall reaction sequence of (Na + K) ATPase in the presence of Mg, Na and K, the reaction sequence shown in the Introduction can be re-expressed in the form below:

$$E + ATP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot ATP \xrightarrow{k_2} E\cdot P + ADP \xrightarrow{k_3} E + P_i \quad (1)$$

where E·ATP is estimated from the level of the ^{14}C-nucleotide-protein, and E·P from the level of the ^{32}P-phosphoprotein (presumably the sum of E_1P, E_2P and E·ATP). The kinetic constants, k_{-2} and k_{-3} are neglected based on the assumption that the reaction steps E·ATP to E·P and E·P to E + P_i are only slightly reversible. This assumption is justified by the fact that both of these reactions (in the forward reaction) are accompanied by the loss of a considerable amount of energy.

In this analysis, it is assumed that normal Michaelis-Menten kinetics are obeyed for an enzymatic reaction consisting of two reactive intermediates in accordance with the criteria set forth by Zerner and Bender (1964) as reviewed by Zeffren and Hall (1973). The designated rate constants can be evaluated from the present data which fit the above criteria insofar as:

(i) steady state conditions were shown to hold for the overall and intermediary reactions;

(ii) the concentration of total enzyme (E_t) was much less than that of the substrate, ATP. (If one mole of enzyme weighs 250,000 grams, then 50 μg of enzyme would be equal to 200 pmoles of enzyme; in our experiments, the final reaction volume is equal to 100 μl, and the concentration of enzyme would be 2 μM if the microsomal preparation were composed solely of (Na + K) ATPase).

(iii) the time-dependent changes in the concentrations of E·ATP and E·P were negligible compared to the quantity of substrate depletion or product accumulation; i.e. the reaction is in a steady state; and
(iiii) the binding of ATP by the enzyme is a rapid equilibrium, reversible reaction which is not rate-limiting for the overall P_i release.

Steady state conditions require that the rate of hydrolysis (v) equal that of E·ATP breakdown as well as that of E·P breakdown, or that

$$v = k_2 [E \cdot ATP] = k_3 [E \cdot P] \qquad (2)$$

Values of k_2 and k_3, 68 sec^{-1} and 10 sec^{-1} respectively, were obtained from the regression coefficients of the plots of the rate of P_i release versus [E·ATP] (figure 23), and the rate of P_i release versus [E·P] (figure 16). The low value of k_3 compared to that of k_2 suggests that the breakdown of E·P into P_i and E is the rate-limiting step in the reaction sequence of (Na + K) ATPase in the presence of Mg, Na and K.

The value of k_3 in our eel tissues at 0°C with 25 mM potassium was greater than that in brain tissue at 15°C with 0.6 mM potassium (4.43 sec^{-1}), as determined by Kanazawa et al (1973). In light of these differences in experimental methodology, the fact that the values for k_3 fall within the same range suggests that the turnover

of the phosphoenzyme in the presence of Mg, Na and K is a function of temperature and potassium concentration.

The linear relationships (see figures 23 and 16) between the rate of P_i release and the level of nucleotide-protein and phosphoprotein complexes confirm and extend the findings of Neufeld and Levy (1970) who showed that the rate of inorganic phosphate release was proportional to the level of phosphoenzyme in the presence of Mg, Na and K. These authors further calculated that the hydrolytic rate should be proportional to the level of a postulated E·ATP complex, a prediction that is confirmed by the by the experiments presented above.

The plot of values of v versus [ATP] in figure 8 is a Michaelis-type function that can be fitted to the equation,

$$v = \frac{k_{cat}\ E_t\ [ATP]}{K_m(app) + [ATP]} \qquad (3)$$

where k_{cat} is derived from the equation,

$$[ES]_{total} \xrightarrow{k_{cat}} E + \text{Products} \qquad (4)$$

where the values for the apparent affinity constant (K_m), and for V_{max} can be evaluated graphically from the parameters of the reciprocal plot of v versus [ATP], and where those of k_{cat} and E_t (total enzyme) can be evaluated as described below:

$$V_{max} = k_{cat} E_t = 3{,}100 \text{ pmoles/mg/sec} \quad (5)$$

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} = 8.7 \text{ sec}^{-1} \quad (6)$$

$$E_t = E_{free} + E_{bound} = 355 \text{ pmoles/mg} \quad (7)$$

If the enzymatically active (Na + K) ATPase is taken to be of molecular weight of 250,000 Daltons (Kepner and Macy, 1968), then a pure preparation of the enzyme would contain 4,000 pmoles of enzyme per mg of protein, and the estimated value of E_t accounts for approximately 9% of the total microsomal protein in our preparation (355/4,000 x 100%).

The estimate of 355 pmoles/mg for E_t is nearly the same as the estimated value of the maximal level of E·P in the presence of Mg, Na and K (400 pmoles/mg). If we assume that there is one mole of phosphate binding sites per mole of enzyme (in the presence of Mg, Na and K), then the maximal binding of phosphate with our preparation is consistent with an enzyme purity of approximately 9-10%. However, the maximal binding of phosphate in the presence of Mg and Na (approximately 1,000 pmoles/mg) suggests either that the enzyme is about 25% pure or that there is more than one mole of phosphate binding sites per mole of enzyme in the presence of Mg and Na.

If the microsomal preparation of the eel electric organ is no more than 10% pure (i.e. if only 10% of the microsomal protein is composed of (Na + K) ATPase), then the above calculations imply that there is no more than one phosphate binding site per molecule of the (Mg + Na + K)-dependent form of the enzyme; and that there are two to three such binding sites per molecule of the (Mg + Na)-dependent form of the enzyme. This type of binding arrangement fits with the description of half-of-the-sites reactivity, described below.

The value of K_m(apparent) of 40 μM denotes the apparent affinity of the enzyme for the substrate in the postulated Michaelis sequence with two reactive intermediates. The real affinity (K_s) is that defined by the conventional equation,

$$K_s = \frac{[E_{free}]\ [ATP]}{[E \cdot ATP]} = \frac{k_{-1}}{k_1} \qquad (8)$$

From this equation, and from equations 1 through 7, it can be shown that:

$$K_s = (1 + k_2/k_3)\ K_m = 312\ \mu M \qquad (9)$$

which means that the degree of dissociation of the E·ATP complex (at equilibrium) is approximately eightfold greater than that implied by the K_m (app).

The value of K_s in our preparation was about 1500 times greater than the value of 0.2 μM found by Hegyvary and Post (1971) utilizing equilibrium dialysis in a Mg-free system. Assuming that both of these findings are valid, we will have to propose that there are two forms of the same E·ATP complex (one formed in the absence of magnesium, and one formed in the presence of magnesium), or that there are two different E·ATP subunits.

Robinson (1974a) has attempted to reconcile the apparent existence of two ATP binding sites with different affinities on the basis of a postulated reaction mechanism known as half-of-the-sites-reactivity, first proposed by Stein et al (1973). Assuming that the (Na + K) ATPase exists as a dimer of two identical subunits, each of molecular weight of 100,000, Robinson proposes that at low concentrations of ATP, the substrate binds to one subunit with high affinity, and with little or no subsequent hydrolytic activity. This occurs in the absence of magnesium, as in the experiments of Hegyvary and Post (1971) as well as Norby and Jensen (1971), or in the presence of magnesium, as suggested by the low K_m of sodium dependent phosphorylation (Kanazawa et al, 1967). High affinity ATP binding with little hydrolytic activity seems to occur even in the presence of Mg, Na and K-since potassium inhibits the overall hydrolysis under these conditions at low initial concentrations of ATP

(Siegel and Goodwin, 1972). At higher concentrations of ATP, the first subunit binds ATP with high affinity (and allows phosphorylation in the presence of sodium), while the second subunit binds ATP with a much lower affinity, inducing significant hydrolytic activity at the first subunit. The actual hydrolytic activity would proceed at only one subunit at a time, with hydrolytic activity alternating between the two subunits.

Jorgensen (1974) has calculated that the maximum number of ATP binding sites is equal to one-half the maximum number of phosphate binding sites; he further assumes that there are two large polypeptides involved in binding and phosphorylation and that while only one can bind ATP at any given time, both can be phosphorylated. This is also consistent with the findings of Kyte (1975) which suggest that the large enzymatic subunit exists in a dimer form in the native membrane. The concept of each subunit of the dimer binding ATP at different times is also consistent with the model of half-of-the sites-reactivity.

The K_s value calculated for the (Na + K) ATPase reaction sequence in the presence of Mg, Na and K, 310 μM, is not too different from the concentration of ATP within the cell. This calculation is therefore

in support of the concept that a low affinity binding of ATP may be related to the hydrolytic activity of the enzyme, (Na + K) ATPase in vitro, and to the activity of the sodium pump in vivo.

It is important to reemphasize that, in the steady state, the (Mg + Na + K)-dependent phosphoenzyme has kinetic characteristics consistent with the overall hydrolysis, while Kanazawa et al (1967) have shown that the K_m for the formation of the (Mg + Na)-dependent phosphoenzyme at 0^oC is different from that of the concomitant hydrolysis. Taborsky (1974) states that: "the basic requirement any enzymatic mechanism must meet if some postulated component step is to be valid in the framework of a mechanistic hypothesis is that the kinetic features of the step fit into the kinetics of the overall reaction." The above findings, along with those of Kanazawa, suggest that under the conditions utilized, the (Mg + Na)-dependent phosphoenzyme formation and hydrolysis are not directly related, while the (Mg + Na + K)-dependent phosphoenzyme formation and hydrolysis are directly related.

The findings of a relationship between the intermediary complexes and the overall hydrolysis in the presence of Mg, Na and K (under steady state conditions) fulfill the

requirements of an in-vivo steady state system-insofar as the enzyme is constantly supplied with substrate and is presumably in the "physiologically optimal" conformation dictated by the presence of both sodium and potassium in the environment.

Although the chemical nature of intermediary complexes observed in the absence of potassium might be identical to that in the presence of all three ions, the ion-dependent conformation and the levels of these enzyme complexes are different from the corresponding properties in the presence potassium. Therefore, it is difficult to correlate the level of an intermediate under one condition (e.g. in the presence of Mg and Na) with the rate of hydrolysis under another condition (e.g. in the presence of Mg, Na and K). Mardh (1974) has been able to show that the rate of formation of the (Mg + Na)-dependent phosphoenzyme is consistent with the rate of dephosphorylation by potassium. This finding does support the notion that the (Mg + Na)-dependent phosphoenzyme can be a precursor for the hydrolytic release of P_i induced by potassium. However, in the steady state, our observations, as well as those of Post (1965) and Kanazawa et al (1970) suggest that the breakdown of the (Mg + Na + K)-dependent phosphoenzyme is the rate-limiting step for the release of inorganic phosphate in the hydrolysis of ATP by (Na + K) ATPase.

C. The Acid-Precipitated Phosphoenzyme; E_1P and E_2P forms

The phosphoenzyme formed in the presence of Mg and Na seems to be qualitatively identical to the phosphoenzyme formed in the presence of Mg, Na and K, and there is little reason to doubt that both phosphoenzymes are in some way related to the hydrolytic activity of the (Na + K) ATPase. It is therefore worthwhile to clarify the nature of these phosphoenzymes on the basis of the E_1P and E_2P terminology.

The (Mg + Na)-dependent phosphoenzyme is thought to be a mixture of the E_1P and E_2P forms, with the E_2P form predominating, as expressed by the high sensitivity of this phosphoenzyme to dephosphorylation by potassium. The (Mg + Na + K)-dependent phosphoenzyme is thought to be predominantly the E_1P form, as potassium will have induced the breakdown of any E_2P phosphoenzyme.

Mardh (1975) and Kuriki (1976) have both shown that, in the presence of high sodium concentrations, the phosphoenzyme will be predominantly in the E_1P configuration, and it is thought that sodium accelerates the reaction, $E_2P \longrightarrow E_1P$. In experiments carried out in our laboratory (not shown), both the (Mg + Na)-dependent and the (Mg + Na + K)-dependent phosphoenzyme were reduced by the presence of ADP in the reaction mixture, testifying to the presence of the E_1P enzyme configuration under both sets of ionic conditions.

Robinson (1974a) has hypothesized that at low ATP concentrations, where the ATP is bound by one enzyme subunit with low affinity, activities of the E_1 enzyme might be elicited; that is, Na-dependent ATPase activity, and (Mg + Na)-dependent ADP:ATP exchange. In the presence of high levels of ATP, where the substrate would be occupying both high and low affinity sites, E_2 enzyme activities would be elicited; that is, (Na + K)-dependent hydrolysis and the binding of ouabain.

D. Effects of Ouabain

Ouabain is a potent and specific inhibitor of the sodium pump and of the (Na + K)-dependent ATPase activity. The amount of ouabain bound to the enzyme correlates with the ATPase activity, and the binding is most rapid to a phosphorylated form of the enzyme (Sen et al, 1969). Ouabain reduces the sodium-dependent phosphorylation of the enzyme by ATP at $0^{o}C$ and at higher temperatures (Skou and Hilberg, 1969; Post et al, 1965: Fahn et al, 1968) and blocks the potassium-dependent dephosphorylation (Charnock et al, 1963; Ahmed and Judah, 1965; Sen et al, 1969); it also seems to interfere with the ADP:ATP exchange reaction (Siegel and Josephson, 1972). Once bound, the ouabain is thought to produce a dephosphoenzyme which is resistant to re-phosphorylation by ATP (Sen et al, 1969).

Table III presents data on the level of phosphoenzyme formed from paired aliquots of native and ouabain-treated microsomes under the designated ionic conditions. Each level of phosphoenzyme is shown with the corresponding hydrolytic rate.

In the presence of Mg and Na and in that of Mg, Na and K, the effect of ouabain was to reduce the level of phosphoenzyme as well as the concomitant hydrolytic rate. These effects can be explained by a block of the complexation of ATP with the enzyme, or by a blockade of the breakdown of the nucleotide-protein complex. However, the data in figure 22 clearly show that ouabain reduces the level of the nucleotide-protein, and this ultimately leads to decreased phosphorylation and inorganic phosphate release.

Jorgensen (1974) has shown that the number of ATP binding sites is equal to the number of ouabain binding sites on the enzyme, and Hansen et al (1971) have shown the same relationship, as well as that ouabain inhibits the functions of the enzyme (Na + K) ATPase by preventing the binding of ATP. These data are, of course, consistent with our findings on the effects of ouabain on the level of the acid-precipitated nucleotide-protein complex.

As mentioned above, one of the known effects of

Reaction Velocity (pmoles/ mg/ sec)

Ionic Conditions	Mg		Mg + Na		Mg + Na + K	
Enzyme State	native	ouabain	native	ouabain	native	ouabain
Incubation Time (seconds) 5	68	69	214	77	1,356	235
10	82	115	306	78	1,342	268

Phosphoenzyme Level (pmoles/ mg)

Ionic Conditions	Mg		Mg + Na		Mg + Na + K	
Enzyme State	native	ouabain	native	ouabain	native	ouabain
Incubation Time (seconds) 5	29	32	925	57	112	32.3
10	34	42	962	57	97	42.8

Table III. The effect of ouabain on the reaction velocity (upper table) and the phosphoenzyme level (lower table) under varied ionic conditions.

ouabain is to block the potassium-induced dephosphorylation of the phosphoenzyme, and this would be translated into an increased level of phosphoenzyme in the presence of Mg, Na, K and ouabain over that observed in the presence of Mg, Na and K alone. However, reference to figure 15 and Table III will establish that such was not the case in our experiments. Ouabain decreased the level of phosphoenzyme formed in the presence of Mg, Na and K to that observed in the presence of Mg alone. In order to realize that ouabain did, in actuality, decrease the potassium-induced dephosphorylation, it is necessary to compare the rate constants of dephosphorylation in the presence of Mg, Na, K and ouabain and in that of Mg, Na and K (Table IV).

The apparent rate constant of dephosphorylation, v/EP is calculated for various reaction conditions in Table IV. The ouabain-induced changes in v/EP were as follows:

(i) little or no change in the presence of Mg alone;
(ii) a five-fold increase in the presence of Mg and Na; and
(iii) a one-third decrease in the presence of Mg, Na and K.

The ouabain-induced decrease in v/EP for the reaction in the presence of Mg, Na and K shows that ouabain inhibited the potassium-induced dephosphorylation step as well as inhibiting the phosphorylation (and binding of ATP) steps.

Reaction Velocity/ Phosphoenzyme Level (v/ EP in seconds^{-1})

Ionic Conditions	Mg		Mg + Na		Mg + Na + K	
Enzyme State	native	ouabain	native	ouabain	native	ouabain
Incubation Time (seconds) 5	2.34	2.17	0.231	1.35	12.1	7.27
10	2.41	2.74	0.318	1.35	13.8	6.26

Table IV. Calculated value of the apparent rate constant of dissociation for the phosphoenzyme complex in the steady state (for native and ouabain-treated microsomes) in the presence of various ions. The reaction conditions are described in table III, and the above calculations were made utilizing the data in table III.

The ouabain-induced inhibition of the (Mg + Na)-dependent ATPase activity could not be ascribed to a block in the dephosphorylation of the EP complex, as the apparent rate constant for this reaction is plainly increased by ouabain. Rather, the inhibition of ATPase activity can be ascribed to a block in the re-phosphorylation of the ouabain-treated enzyme.

In a paper by Sen et al (1969), one of the figures (figure 6) shows that treatment with ouabain gives rise to a (Mg + Na)-dependent phosphoenzyme that is less stable than its non-ouabain-treated control. Although the authors claim that figure 5 in this same paper shows that the ouabain-treated (Mg + Na)-dependent phosphoenzyme is more stable than the non-ouabain-treated counterpart, closer inspection of this experiment reveals that this conclusion is faulty. Both the native and the ouabain-treated phosphoenzymes require the same period of time to fall to half their original levels when the phosphoenzyme is chased with non-radiolabeled ATP; furthermore, the efficacy of an ATP chase would be enhanced in the case of the native phosphoenzyme, as the ATP can easily phosphorylate this enzyme, while the ouabain-treated phosphoenzyme will be refractory to interaction with the cold ATP molecule.

Our experiments demonstrating a ouabain-induced decrease in the stability of the (Mg + Na)-dependent phosphoenzyme are therefore consistent with the

experiments of Sen et al described above.

E. The Heat-Precipitated Phosphoenzyme Complex

It has been established that intermediary phosphoenzyme complexes of (Na + K) ATPase are produced when the enzyme reacts with ATP. However, the recovery of these complexes in acid-precipitated enzyme preparations is not necessarily complete as the technique of acid precipitation is based on the assumption that the phosphorylated intermediates associated with the reaction pathway will be stable after exposure to conditions of low pH. The technique of acid precipitation ignores the possibility that acid-labile enzyme-phosphate linkages might also be formed during the course of these reactions, and no information relative to these complexes would be available via the current experimental approach. For instance, histidyl-phosphate linkages have been observed, and these would be acid-labile and alkali-stable (Feldman and Butler, 1969; Stevens-Clark et al, 1968).

It seems likely that the intermediates of the (Na + K) ATPase reaction would be most stable at or near neutral pH, since the hydrolytic reaction shows optimal activity at these pHs. The experimental procedure of terminating the hydrolytic reaction by rapid exposure of the

reaction vessel to a boiling water bath has uncovered the existence of a phosphoenzyme complex that is maximally stable between pH 6 and 8, suggesting that it is uniquely different from the acyl and ester phosphates.

Nagano et al (1967) and Alexander and Rodnight (1974) have utilized a neutral SDS solubilization procedure to terminate the hydrolytic reaction at non-acid pH. This procedure produced an enzyme-phosphate complex similar, if not identical to the acid-precipitated phosphoenzyme complex. However, solubilization by SDS is a fairly vigorous procedure, and weak phosphoenzyme linkages might well have been destroyed. This is particularly applicable to the heat-precipitated phosphoenzyme complex which is destroyed by SDS solubilization and therefore cannot be visualized through SDS polyacrylamide gel electrophoresis.

The heat-precipitated phosphoenzyme complex is labile to most procedures that successfully solubilize the membrane-bound enzyme. Attempts to utilize mild solubilization procedures, like Triton X and Tween detergents at varied pHs, in order to isolate the enzyme-phosphate complex via a Sephadex G-50 chromatographic column were unsuccessful, as the enzyme-phosphate linkage apparently could not withstand the detergent treatment.

On the basis of its lability at acid pH, we can conclude that the heat-precipitated phosphoenzyme complex is neither an acyl-phosphate nor either a seryl- or threonyl-phosphate complex. On the basis of its lability at alkaline pH, we can conclude that it is not a histidyl-phosphate complex. Further experimental work is required before more solid conclusions can be drawn on the chemical nature of the heat-precipitated phosphoenzyme complex.

The possible position of the heat-precipitated phosphoenzyme complex in the reaction scheme for the hydrolysis of ATP can best be understood in terms of the experiments utilizing ADP and inorganic phosphate to chase the labeled phosphoenzyme and the experiments utilizing radio-labeled inorganic phosphate to "back-label" the enzyme.

The fact that a five-fold excess of ADP (over the original concentration of [γ-^{32}P] ATP used) produces a marked decrement in the level of phosphoenzyme-with the phosphoenzyme remaining at a constant, reduced level with time-suggests that some equilibrium has been reached in which any ADP-reactive phosphoenzyme that is being formed is simultaneously being chased, while a steady-state level of ADP-insensitive phosphoenzyme is maintained.

A possible explanation for the fact that the effect of an ADP chase on the (Mg + Na + K)-dependent heat-precipitated phosphoenzyme was greater than the effect of an ATP chase is that the affinity of the phosphoenzyme for ADP is greater than the affinity of the free enzyme for ATP; that is, while the effect of an ATP chase is to compete with the radiolabeled ATP molecule for phosphate binding sites on the free enzyme, the effect of an ADP chase is for the ADP to bind with the E·P complex, and drive the reaction toward the formation of ATP by phosphorylating ADP. This would mean that the exchange reaction would have to be significantly faster than the phosphorylation reaction. Blostein (1970) has shown that at 0°C Na-activated hydrolysis is slower than the concomitant rate of ADP:ATP exchange. However, it may be somewhat naive to apply a similar explanation to a reaction where the hydroysis reaction is at near-maximum velocity in the presence of potassium (as well as magnesium and sodium).

The fact that all of the phosphoenzyme was quickly chased by a five-fold excess of inorganic phosphate suggests that all of the enzyme phosphate is exchangeable with inorganic phosphate, and that it is therefore formed via a low energy linkage. This idea is also supported by the inhibition of phosphorylation by

P_i equimolar with the [γ-^{32}P] ATP in the heat-terminated reaction.

Inhibition of the formation of the heat-precipitated, (Mg + Na + K)-dependent phosphoenzyme by inorganic phosphate is not necessarily an unequivocal test for a low energy phosphoenzyme bond like E_2P. Since the acid-precipitated (Mg + Na)-dependent phosphoenzyme is thought to be of the E_2P confirmation because of its high sensitivity to dephosphorylation by potassium, it, too, should show sensitivity to inorganic phosphate in the reaction mixture. Figure 35, however, shows that the inclusion of inorganic phosphate in the reaction of the (Na + K) ATPase with [γ-^{32}P] ATP did not reduce the level of phosphoenzyme observed after acid precipitation (in the presence of Mg and Na). However, the concentration of inorganic phosphate used in these experiments was equivalent to the concentration of [γ-^{32}P] ATP, and the effect was only characterized at one time period. It would be worthwhile to repeat these experiments with higher concentrations of inorganic phosphate and as a function of time.

It should be restated that, under similar reaction conditions to those for the heat-precipitated reactions, inorganic phosphate did not reduce the labeling of the acid-precipitated phosphoenzyme (figure 35). Albers et al (1963) showed that a thousand-fold excess of inorganic phosphate did not reduce the labeling of the (Na + K)

ATPase from [γ-^{32}P] ATP (in a reaction terminated by acid precipitation). Hexum (1970) showed that inorganic phosphate is a non-competitive inhibitor of the hydrolytic reaction with a K_I of 23 mM, suggesting that very high concentrations of inorganic phosphate would be required in order to observe effects on the reaction steps. It should, however, be mentioned that Post (1975) has shown that inorganic phosphate can inhibit acid-precipitated phosphoenzyme formation by keeping the enzyme in an E_2P configuration; this enzyme state is thought to accept phosphorylation from [γ-^{32}P] ATP at a very slow rate.

Support for the idea that the heat-precipitated phosphoenzyme is a low energy complex similar to the E_2P phosphoenzyme conformation comes from the observation that it is possible to back-label the enzyme with inorganic phosphate under varied ionic conditions (in a reaction terminated by heat precipitation), a step that is theoretically energy independent. Post (1975) has shown that it is possible to back-label the enzyme in an acid-terminated reaction under certain ionic conditions and has correlated this back-labeled phospho-enzyme with the E_2P phosphoenzyme conformation. In our hands (not shown) the back-labeling reaction terminated by acid precipitation was successful only in the case where the enzyme had been pre-treated with ouabain,

supposedly locking it into an E_2 conformation receptive to exchange with inorganic phosphate.

It is important to note at this point the recent experiments by Racker's group (Kuruki and Racker, 1976) that have shown that the simple step of ion binding to the (Na + K) ATPase of the eel electric organ can induce conformational changes in the protein with a concomitant release of energy. If this is indeed the case, then we cannot assume that binding of inorganic phosphate from the "tail" end of the reaction is an energy-independent step. Rather, this back-labeling reaction, as well as the chase from the forward direction of the reaction by inorganic phosphate could well signal the formation of high (?) energy enzyme-phosphate bonds. This could also serve to explain the fact that addition of ADP chases the heat-precipitated phosphoenzyme, a step that theoretically requires the existence of a high energy enzyme-phosphate bond.

The efficacy of the heat-terminated back-labeling reaction suggests that it may be possible to produce the same phosphoenzyme complex (after heat precipitation) utilizing either ATP or P_i as the substrate. However, two factors mitigate against the assumption that the phosphoenzyme formed from P_i is similar to that formed from ATP in the heat-terminated reaction. Firstly, the

level of the back-labeled phosphoenzyme is the same whether the reaction is carried out in the presence of Mg and Na or Mg, Na and K, while the phosphorylation by ATP is favored in the presence of Mg, Na and K. Secondly, the presence of ATP does not reduce the ultimate level of back-labeled phosphoenzyme observed; this ATP would be expected to phosphorylate the enzyme with non-radio-labeled phosphate, thereby reducing the level of the back-labeled E·P.

In order to begin to establish a relationship between the phosphoenzymes formed in the back-labeling reaction and those formed in the forward reaction from ATP, it will be necessary to at least carry out an analysis of the pH stability and hydroxylamine sensitivity of the back-labeled phosphoenzyme complex in order to compare its chemical characteristics with those established for the heat-precipitated phosphoenzyme formed from ATP.

ADDENDUM to DISCUSSION

It should be noted that a number of phosphoenzyme complexes have been mentioned in the literature of the (Na + K) ATPase field with properties different from those of the well-characterized acid-precipitated acyl-phosphoenzyme complex. It is important to distinguish the heat-precipitated phosphoenzyme complex from these other phosphoenzymes. Skou (1969) observed an hydroxylamine-insensitive phosphoenzyme that he characterized as the "stable binding" of the (Na + K) ATPase; Rodnight (1966) and Nagano (1967) found that the Mg-dependent, acid-precipitated phosphoenzyme was hydroxylamine-insensitive; and Mardh (1972) observed a non-acyl-phosphate phosphoenzyme. However, the "stable-binding" phosphoenzyme of Skou and the Mg-dependent phosphoenzyme observed by Rodnight and Nagano do not show an ATP-induced turnover, while the (Mg + Na + K)-dependent, heat-precipitated phosphoenzyme does show a chase by ATP (figure 34). The non-acyl-phosphate phosphoenzyme observed by Mardh when most of the ATP had been hydrolyzed was not sensitive to ouabain, while the (Mg + Na + K)-dependent, heat-precipitated phosphoenzyme formation was reduced by ouabain.

In order to further characterize the heat-precipitated phosphoenzyme complex in terms of the E_1P and E_2P complexes of the reaction scheme described in the Introduction, it would be worthwhile to utilize various chemical agents that are thought to "lock" the enzyme into one or another of these conformations as experimental probes. For instance, oligomycin, NEM, or calcium as well as the compound, quercetin (Kuriki and Racker, 1976) are thought to lock the enzyme into an E_1P conformation. After treating the enzyme with one of these compounds, and forming the phosphoenzyme with [γ-^{32}P] ATP, it would be worthwhile to examine the effects of an ADP and P_i chase in the heat terminated reaction, as well as examining the pH stability and hydroxylamine sensitivity of the heat-precipitated phosphoenzyme. Similarly, ethacrynic acid is thought to lock the enzyme into an E_2P conformation (Banerjee et al, 1971), and the same experimental procedure would be applied with this compound.

It would also be of interest to determine whether treatment of the enzyme with the above mentioned chemical compounds would have effects on the characteristics of the back-labeling reaction, and its sensitivity to ATP and ADP.

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