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GEL ELECTROPHORETIC IDENTITY OF THE $(Na^+ + Mg^{2+})$- AND $(Na^+ + Ca^{2+})$-STIMULATED PHOSPHORYLATIONS OF RAT BRAIN ATPase

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SUMMARY

The classical E_2-P intermediate of $(Na^+ + K^+)$-ATPase dephosphorylates readily in the presence of K^+ and is not affected by the addition of ADP. To determine the significance in the reaction cycle of $(Na^+ + K^+)$-ATPase of kinetically atypical phosphorylations of rat brain $(Na^+ + K^+)$-ATPase we compared these phosphorylated components with the classical E_2-P intermediate of this enzyme by gel electrophoresis. When rat brain $(Na^+ + K^+)$-ATPase was phosphorylated in the presence of high concentrations of Na^+ a proportion of the phosphorylated material formed was sensitive to ADP but resistant to K^+. Similarly, if phosphorylation was carried out in the presence of Na^+ and Ca^{2+} up to 300 pmol/mg protein of a K^+-resistant, ADP-sensitive material were formed. If phosphorylation was from [γ-^{32}P]CTP up to 800 pmol ^{32}P/mg protein of an ADP-resistant, K^+-sensitive phosphorylated material were formed. On gel electrophoresis these phosphorylated materials co-migrated with authentic Na^+-stimulated, K^+-sensitive, E_2-P-phosphorylated intermediate of $(Na^+ + K^+)$-ATPase, supporting suggestions that they represent phosphorylated intermediates in the reaction sequence of this enzyme.

INTRODUCTION

In the reaction cycle of the Na^+ pump or $(Na^+ + K^+)$-ATPase (ATP phosphohydrolase, EC 3. 6. 1. 3.) the membrane particles containing this enzyme are phosphorylated by the γ-phosphate of ATP in the presence of Na^+ and Mg^{2+} [1]. On addition of K^+ the phosphoenzyme formed reacts readily with water, releasing the phosphate as inorganic phosphate and regenerating free enzyme [1]. Further studies have suggested that this reaction cycle consists of a sequence of phosphorylated intermediates; an initial transient intermediate formed after phosphorylation from ATP and a second intermediate formed when this phosphorylation produces a configurational change in the enzyme. These intermediates are called E_1-P and E_2-P, respectively, and both the formation of E_1-P and its transition to E_2-P require Mg^{2+} [2].

While the E_2-P intermediate may be identified by phosphorylating this enzyme in the presence of Na^+, Mg^{2+} and [^{32}P]ATP, the transient E_1-P form of

the phosphoenzyme proved difficult to identify in native $(Na^+ + K^+)$-ATPase [2]. One approach to this problem was that of Tobin et al. [3], who suggested that divalent cations other than Mg^{2+} might catalyze formation of E_1-P but prevent its transition to E_2-P. In this way the E_1-P form of the phosphoenzyme might be isolated and its kinetic properties investigated.

By substituting Ca^{2+} for Mg^{2+} in their phosphorylation system Tobin et al. [3–5] demonstrated a phosphorylated intermediate in a rat brain $(Na^+ + K^+)$-ATPase preparation which had many of the properties expected of E_1-P. In particular the phosphoenzyme formed under these conditions reacted readily with ADP (ADP-sensitive) and poorly with K^+ (K^+-resistant) or ouabain [3–5]. Based on these and other kinetic considerations it was suggested that the phosphoenzyme formed in the presence of Ca^{2+} was the transient E_1-P form of the $(Na^+ + K^+)$-ATPase [3–5].

Because the specific activity of the rat brain $(Na^+ + K^+)$-ATPase preparations on which these experiments performed was very low, it remained possible that the Ca^{2+}-dependent phosphorylation was spurious and involved components of these membrane preparations other than $(Na^+ + K^+)$-ATPase. In particular, it was not possible to demonstrate the E_1-P to E_2-P change in membrane preparations phosphorylated in the presence of Ca^{2+} [5]. Also, Knauf and coworkers have recently shown that in the presence of Ca^{2+} at least two different red cell membrane components are phosphorylated from [γ-^{32}P]ATP [6]. Because of these considerations, we compared the gel electrophoresis patterns of $(Na^+ + K^+)$-ATPase preparations phosphorylated from [γ-^{32}P]ATP in the presence of Mg^{2+} or Ca^{2+}. In addition, we also compared the gel electrophoresis patterns of a ADP-resistant, K^+-sensitive phosphorylated material formed in the normal reaction cycle of rat brain $(Na^+ + K^+)$-ATPase and the phosphorylated material formed when these membranes are phosphorylated from [γ-^{32}P]CTP[7]. The results suggest that the same polypeptide is phosphorylated in all cases and the data are consistent with the hypothesis that the phosphorylated intermediate formed in the presence of Ca^{2+} is similar to or closely related to the E_1-P intermediate of the $(Na^+ + K^+)$-ATPase [3–5].

MATERIALS AND METHODS

1. ATPase preparation and labeling

Rat brain ATPase, prepared as described by Tobin et al. [8] was used throughout. Its protein content was assayed by the method of Lowry et al. [9] and its ATPase activity as described by Tobin et al. [3]. Total ATPase activity varied between 150 and 300 μmol P_i/mg protein per h and more than 95% of the activity was ouabain sensitive. [γ-^{32}P]ATP was obtained from New England Nuclear, Boston, Mass. and [γ-^{32}P]CTP was obtained from ICN Ltd, Irvine, Calif. Labeling of the enzyme from [γ-^{32}P]ATP or [γ-^{32}P]CTP was performed as described by Tobin et al. [3]. In kinetic experiments (Fig. 2) the amount of phosphoenzyme was estimated by millipore filtration as described previously [3]. In gel electrophoresis experiments the enzymes were washed twice by alternate centrifugation and resuspension in a trichloroacetic acid/ATP/P_i solution as described previously [10]. Final resuspension prior to electrophoresis was in 1 ml of 2% sodium dodecylsulfate with 1% mercaptoethanol.

2. Gel electrophoresis of phosphorylated membranes

Polyacrylamide gels at pH 2.4 were prepared as described by Avruch et al. [11] and

allowed 1 h to polymerize. The gels were then placed in an ISCO gel electrophoresis apparatus and approx. 28 mg of crystalline sucrose and 3 μl of basic fuschin (0.05 %) added to each tube. 100 μl of the phosphorylated rat brain enzyme preparation was applied to each tube. Gels were run at 5.5 V/cm in a 0.1 M phosphate buffer (pH 2.4) containing 1 g of sodium dodecylsulfate per liter. When the tracking dye approached the end of the tube the gels were removed, measured and the position of the tracking dye marked. One gel of each pair was cut with a Bus-Dahlborg razor blade battery and each slice placed in 10 ml of water. The Cerenkov radiation produced was counted in a Beckman LS 100 liquid scintillation spectrometer with an efficiency of about 30 % and plotted as cpm [11]. Uncut gels were stained with Coomassie Brilliant Blue, placed in a diffusion destainer for 1–3 days and then stored in 7 % acetic acid until ready for photography. Gel electrophoresis experiments were repeated at least four times and in each case typical experiments are presented.

RESULTS

Fig. 1 shows the staining patterns and distribution of ^{32}P obtained when rat brain $(Na^+ + K^+)$-ATPase preparations were phosphorylated in the presence of [γ-^{32}P]ATP and Na^+ with either Ca^{2+} or Mg^{2+} as the divalent cation. The maximal

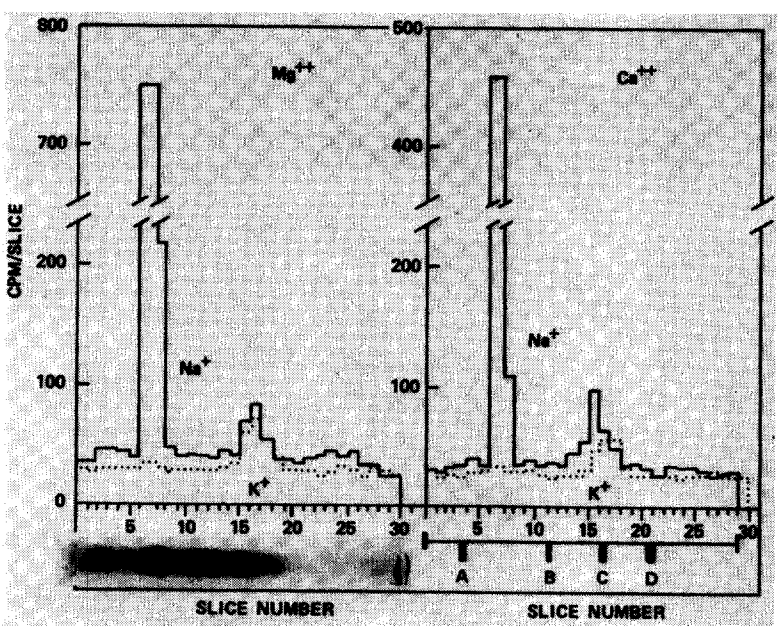

Fig. 1. Gel electrophoresis of rat brain ATPase phosphorylated in the presence of Mg^{2+} or Ca^{2+}. Rat brain ATPase was phosphorylated from [γ-^{32}P]ATP in the presence of 100 mM Na^+ or 100 mM K^+ and either 1 mM Mg^{2+} or 1 mM Ca^{2+}. Phosphorylation was for 5 s at 0 °C and denaturation, washing and electrophoresis were performed as described in Materials and Methods. Mg^{2+}-stimulated labeling was 500.6 pmol ^{32}P/mg protein, while Ca^{2+}-stimulated labeling was 247 pmol ^{32}P/mg protein. The solid lines show cpm observed in each slice when labeling was performed in the presence of Na^+, the dotted lines labeling in the presence of K^+. The lower panels show the staining patterns of the ATPase preparations (right-hand panel) or migration distances of thyroglobulin (A), ovalbumin (B), catalase (C) or lysozyme (D) standards.

incorporation of ^{32}P into the gel slice occurred in slice No. 7 and the relative labeling in each peak (the Ca^{2+} peak was 58 % of the Mg^{2+} peak) corresponds well with our previous experience in which the $(Na^+ + Ca^{2+})$-stimulated phosphoenzyme usually averaged about 60% of the level of the $(Na^+ + Mg^{2+})$-stimulated phosphoenzyme [3–5]. This peak of maximal ^{32}P incorporation ran fractionally behind the darkest staining band in these gels, and this was the only point on these gels at which Na^+ stimulated ^{32}P labeling significantly above that observed in the presence of K^+. Comparison of these peaks of labeling with the migration of known molecular weight standards indicates that the molecular weight of the phosphorylated material is about 95 000. The data suggest that the Na^+-stimulated increment in ^{32}P incorporation into these membranes observed in the presence of Ca^{2+} involves a polypeptide of similar molecular weight to that involved in the Na^+- and Mg^{2+}-dependent ^{32}P incorporation.

One unusual characteristic of the Na^+-stimulated phosphorylation of rat brain $(Na^+ + K^+)$-ATPase has been the relative resistance of this material to dephosphorylation by K^+ [3]. Fig. 2 shows an experiment which illustrates this point. In this experiment rat brain ATPase was phosphorylated from [γ-^{32}P]ATP in the presence of Na^+ and Mg^{2+}. 4 s after formation the phosphoenzyme was challenged by the addition of K^+, with or without added nucleotides. In the absence of added nucleotides up to 100 mM K^+ only partly dephosphorylated the enzyme,

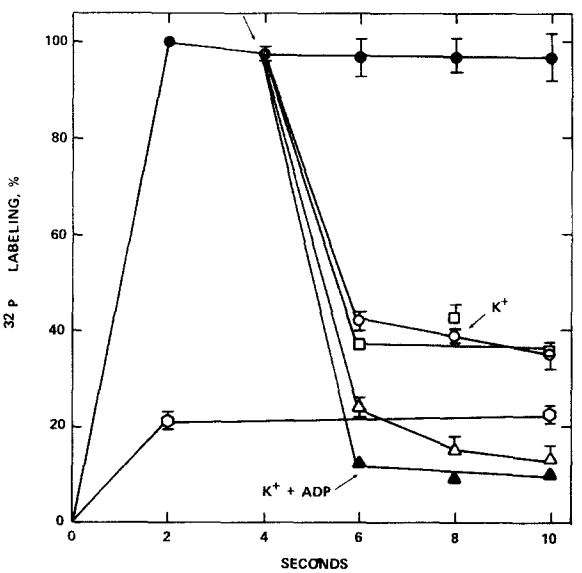

Fig. 2. K^+-resistant phosphorylation of rat brain ATPase. The solid circles (●—●) show the steady-state levels of phosphoenzyme in the presence of 100 mM Na^+, 1 mM Mg^{2+} and 0.05 mM [γ-^{32}P]ATP at 0 °C. The open circles (○—○) and open squares (□—□) show the steady-state levels of the phosphoenzyme when 25 mM or 100 mM K^+ were added at 4 s. The open triangles (△—△) show labeling when 10 mM ATP was added with 24 mM K^+ and the solid triangles (▲—▲) show labeling when 10 mM ADP was added with 25 mM K^+. The hexagons (⬡—⬡) show labeling of these membranes in the presence of 100 mM K^+. All points are calculated as a percentage of labeling in the presence of Na^+ at 2 s which averaged 717±13 pmol ^{32}P/mg protein. All points are the means ±S.E. of four separate experiments with different enzyme preparations.

reducing labeling to about 40 % of that observed in the presence of Na^+. This observation is in contrast with the behavior of guinea pig kidney ($Na^+ + K^+$)-ATPase, where labeling in the presence of 167 mM Na^+ was essentially completely discharged by 3 mM K^+ [1].

If unlabeled ATP was added with the K^+ a spontaneous decay of this K^+ resistant phosphoenzyme was exposed, and if ADP was added with K^+ this labile material was rapidly dephosphorylated. This unexpected behavior of a Na^+- and Mg^{2+}-stimulated phosphoenzyme [2] prompted us to examine this material by gel electrophoresis. Fig. 3 shows that labeling of rat brain ATPase phosphorylated in the presence of Na^+, Mg^{2+} and K^+ co-migrated with the Na^+-stimulated phosphoenzyme, consistent with the idea that the phosphorylated material formed under these conditions is also a phosphorylated intermediate of the ($Na^+ + K^+$)-ATPase.

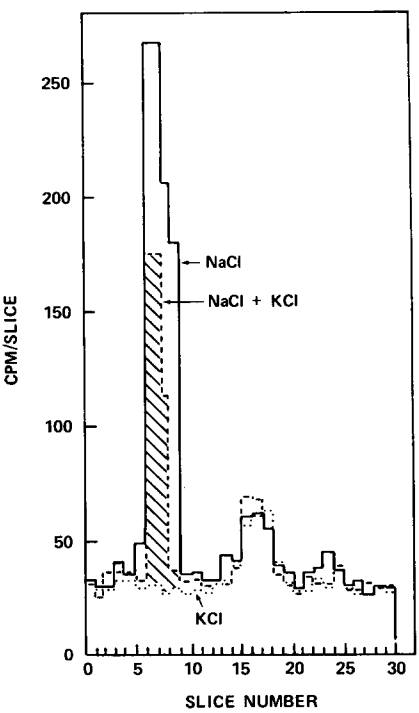

Fig. 3. Gel electrophoresis of the Na^+-stimulated K^+-resistant phosphorylation of rat brain ATPase. Rat brain ($Na^+ + K^+$)-ATPase was phosphorylated from [γ-^{32}P]ATP in the presence of 1 mM Mg^{2+}, 0.1 mM [γ-^{32}P]ATP and either 100 mM Na^+ or 100 mM K^+ or these cations combined. The labeling reaction was stopped at 5 s and the denatured phosphoenzyme either millipore filtered or washed as described in Materials and Methods. Labeling was 725 pmol ^{32}P/mg protein in the presence of Na^+, 317 pmol ^{32}P/mg protein in the presence of Na^+ plus K^+ and 160 pmol ^{32}P/mg protein in the presence of K^+. After washing, 100-μl samples of the phosphoenzyme were subjected to gel electrophoresis. The solid line shows the distribution of counts obtained with 100 mM Na^+ in the reaction mixture, the dashed line labeling with 100 mM K^+ in the reaction mixture. The hatched column shows the distribution of counts when labeling was performed in the presence of 100 mM each of Na^+ and K^+. About 83 % of the counts applied to the gel were recovered in gel slices Nos 6, 7 and 8. The proportion of counts in the hatched columns (36 % of Na^+ stimulated labeling) corresponds with 28 % of K^+-resistant phosphoenzyme put on the gels.

One of the early observations concerning the phosphorylated intermediate of $(Na^+ + K^+)$-ATPase was that under certain conditions nucleotides other than ATP (i.e. CTP, ITP, UTP) did not interfere with the formation of the phosphoenzyme from ATP, and because of this it was suggested that these nucleotides were unable to phosphorylate this enzyme [12]. Reexamining this hypothesis Tobin et al. [7] showed that [γ-^{32}P]CTP phosphorylated rat brain ATPase preparations to the same extent as [γ-^{32}P]ATP and that this phosphoenzyme had kinetic characteristics similar to the phosphoenzyme formed from [γ-^{32}P]ATP. Fig. 4 shows that the ADP

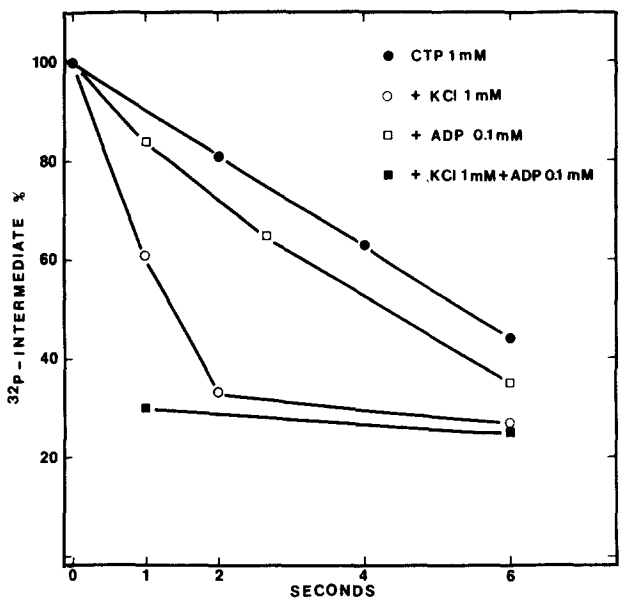

Fig. 4. Sensitivity of [γ-^{32}P]CTP-dependent phosphorylated intermediate to ADP and K^+. About 200 μg of rat brain enzyme was incubated with 100 mM Na^+, 1 mM Mg^{2+} and 0.05 mM [γ-^{32}P]ATP at 0 °C for 5 s. After 5 s (indicated as zero time) the phosphoenzyme was isolated by adding 1 mM unlabeled CTP (solid circles ●—●) or 1 mM CTP plus 0.1 mM ADP (open squares □—□) or CTP plus 1 mM K^+ (open circles ○—○). The solid squares (■—■) show labeling when 0.1 mM each of ADP and 1 mM K^+ were added to the enzymes. Labeling is expressed as a percentage of that observed at 5 s in the absence of unlabeled CTP, which was 537.8 pmol ^{32}P/mg protein. All points are single experimental determinations.

and K^+ sensitivity of the CTP-dependent phosphorylated intermediate of $(Na^+ + K^+)$-ATPase is consistent with this material being E_2-P in type and in Fig. 5 we compared the gel electrophoresis patterns of rat brain ATPase phosphorylated from [γ-^{32}P]-ATP and [γ-^{32}P]CTP. These materials showed similar migration patterns, supporting suggestions that the phosphorylated intermediate formed from [γ-^{32}P]CTP is the same as that formed from [γ-^{32}P]ATP [7].

DISCUSSION

These results show that phosphorylation of rat brain $(Na^+ + K^+)$-ATPase preparations from [γ-^{32}P]ATP resulted in the phosphorylation of a membrane

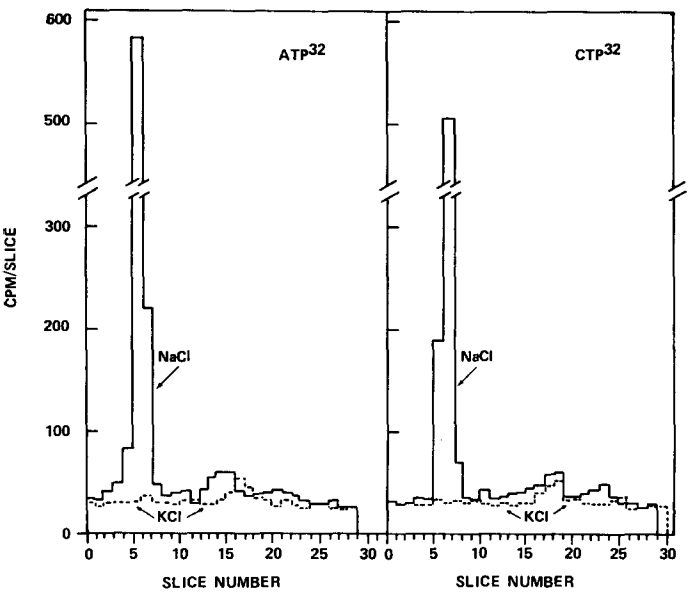

Fig. 5. Gel electrophoresis of labeling of rat brain ATPase from [γ-^{32}P]CTP. Rat brain enzyme was phosphorylated in the presence of Na^+, Mg^{2+} and [γ-^{32}P]CTP. Labeling of these membranes averaged 767.8 pmol ^{32}P/mg protein from [γ-^{32}P]ATP and 797.1 pmol ^{32}P/mg protein from [γ-^{32}P]-CTP. The left-hand panel shows the pattern of labeling obtained with [γ-^{32}P]ATP, the right-hand panel that obtained with [γ-^{32}P]CTP. About 61 % of the counts placed on the gels were recovered in slices Nos 4, 5, 6 and 7.

component with a molecular weight about 95 000. Phosphorylation of this component was stimulated by Na^+ and if K^+ was substituted for Na^+ in the reaction mixture this material did not label above background. The phosphorylation of this component was labile at pH 7.0 in that it was not detected on gels run at this pH, but was more stable at pH 2.4 under the conditions reported here. These observations and the detailed studies of Avruch et al. [11] on the conditions for formation and breakdown of this component make it likely that this component is a phosphorylated intermediate in the reaction cycle of $(Na^+ + K^+)$-ATPase. The conditions chosen to stimulate formation of this material, i. e. Na^+ and more than 1 mM Mg^{2+}, and the kinetic characteristics of this material detailed elsewhere [3–5], make it likely that the bulk of this material is the E_2-P intermediate in the reaction cycle of this enzyme.

This ability to determine the electrophoretic mobility of the E_2-P form of the phosphoenzyme allowed us to compare its electrophoretic pattern with the electrophoretic patterns of putative phosphorylated intermediates either formed under atypical conditions or whose kinetic behavior was atypical. Since a widely accepted [13] reaction cycle for the $(Na^+ + K^+)$-ATPase postulates phosphorylation at the same active site on this enzyme throughout the reaction cycle of this enzyme [2], it may be expected that any phosphorylated intermediate of this enzyme will co-migrate with the E_2-P form of the $(Na^+ + K^+)$-ATPase, irrespective of the conditions under which it was formed or its kinetic behavior. On the other hand, deviation from a co-migration pattern would suggest that the phosphorylated material

involved did not take part in the reaction cycle for the $(Na^+ + K^+)$-ATPase as proposed by Post and coworkers [2].

The results show that when this enzyme was phosphorylated in the presence of Ca^{2+} instead of Mg^{2+} the phosphoenzyme formed co-migrated with that formed in the presence of Mg^{2+}. The amount of Ca^{2+}-dependent material co-migrating with the Mg^{2+}-dependent material was also sufficient to account for the Ca^{2+}-dependent labeling of these membranes. The results support previous suggestions that this material is similar to or closely related to the postulated E_1 -P intermediate of the $(Na^+ + K^+)$-ATPase [3–5].

When rat brain ATPase was phosphorylated in the presence of high concentrations of Na^+, a proportion of the intermediate formed was quite resistant to K^+ (Fig. 2). Because this material was resistant to K^+ it fell outside the operational definition of E_2-P, which is [2] that the enzyme-phosphate bond be readily hydrolyzed in the presence of K^+. This material, however, turned over at about the same rate as E_2-P and was sensitive to ADP (Fig. 2). Again on electrophoresis this material co-migrated with the E_2-P intermediate of $(Na^+ + K^+)$-ATPase, supporting the idea that this K^+-resistant material is also an intermediate in the reaction cycle of $(Na^+ + K^+)$-ATPase. Its kinetic characteristics suggest that it is the postulated E_1-P intermediate in the reaction cycle of this enzyme whose existence in native rat brain $(Na^+ + K^+)$-ATPase can be demonstrated simply by raising the concentration of Na^+ in the reaction system (3–5].

The nucleotide specificity of $(Na^+ + K^+)$-ATPase has been controversial [13] largely because of discrepancies between the ability of different substrates to phosphorylate this enzyme and their ability to support turnover of this enzyme. While it has long been known that nucleotide substrates other than ATP are poorly hydrolyzed by this enzyme [14] it has only recently become apparent that these nucleotides are fully able to phosphorylate this enzyme [12, 15]. In demonstrating phosphorylation of these membranes from [γ-^{32}P]CTP Tobin et al. [7] argued from the kinetic characteristics of the phosphorylated material that this material was an intermediate in the reaction cycle of the $(Na^+ + K^+)$-ATPase. This CTP-dependent phosphoenzyme was shown (Fig. 5) to have the same pattern of ADP resistance and K^+ sensitivity as the Na^+-, Mg^{2+}- and [^{32}P]ATP-dependent phosphorylated intermediate of rat brain $(Na^+ + K^+)$-ATPase [3–5] and Fig. 5 shows that the labeling of these membranes from [γ-^{32}P]CTP comigrates with [γ-^{32}P]ATP-dependent labeling. These data thus further support suggestions that nucleotides other than ATP are capable of forming the phosphorylated intermediates of this enzyme [7].

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