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STUDIES ON THE PARTIAL REACTIONS CATALYZED BY THE $(Na^+ + K^+)$-ACTIVATED ATPase

I. EFFECTS OF SIMPLE ANIONS AND NUCLEOSIDE TRIPHOSPHATES ON THE ALKALI-CATION SPECIFICITY OF THE *p*-NITROPHENYLPHOSPHATASE

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SUMMARY

1. The K^+-dependent *p*-nitrophenylphosphatase activity of the $(Na^+ + K^+)$-activated ATPase complex is stimulated by the addition of $Na^+ + ATP$. Since oligomycin blocks the $(Na^+ + ATP)$-stimulation, but not the K^+-dependent activity, the existence of a "K^+-sensitive site" and a "Na^+-sensitive site" is indicated. The object of this work was to learn more about these sites through kinetic studies.

2. The "K^+-sensitive site" responded to Li^+, Rb^+, and Cs^+; but the "Na^+-sensitive site "showed absolute specificity for Na^+.

3. The order of cation specificity of the "K^+-sensitive site" ($K^+ = Rb^+ > Cs^+ > Li^+$), and the absolute specificity of the "Na^+-sensitive site" remained constant under conditions (*e.g.*, change in the major anion of the assay medium) which had been used to demonstrate the changing specificity of another alkali-cation-activated enzyme (AMP deaminase).

4. The "Na^+-sensitive site" could be demonstrated only in the presence of certain nucleoside triphosphates (ATP, ITP and CTP). Nucleoside diphosphates, nucleoside monophosphates, pyrophosphate and orthophosphate had no activating effects in the presence of Na^+.

5. A variety of simple anions were found to have inhibitory effects on the enzyme. Because of this, and due to the impurity of the enzyme, the complex kinetic data were of little value for mechanistic interpretations.

6. Na^+ inhibited the enzyme both in the presence and absence of an activator cation. From the kinetic data it was not possible to determine if this inhibition was exerted at the same "Na^+-sensitive site" that is involved in the $(Na^+ + ATP)$-activation of the enzyme.

INTRODUCTION

Preparations of $(Na^+ + K^+)$-activated ATPase (ATP phosphohydrolase, EC 3.6.1.3) from various sources also catalyze the K^+-stimulated hydrolysis of

several simple organic phosphates[1,2]. Previous studies on the K^+-activated *p*-nitrophenylphosphatase of these preparations have shown that : (1) The K^+-activated *p*-nitrophenylphosphatase, like the $(Na^+ + K^+)$-activated ATPase, is inhibited by cardiac glycosides[1,2]. (2) In contrast to $(Na^+ + K^+)$-activated ATPase, the K^+-activated *p*-nitrophenylphosphatase is not inhibited by oligomycin[3,5]. (3) In the presence of sub-optimal concentrations of K^+, *p*-nitrophenylphosphatase activity is further stimulated by the simultaneous presence of ATP and Na^+ (ref. 4). (4) The extra stimulation caused by Na^+ + ATP is blocked by oligomycin[5,6].

The different responses of the K^+-activated *p*-nitrophenylphosphatase and the $(Na^+ + K^+ + ATP)$-activated *p*-nitrophenylphosphatase to oligomycin indicate the existence of two distinct ion-sensitive sites on the enzyme: The "K^+-sensitive site" and the "Na^+-sensitive site". It should be emphasized that these two sites are identified on the basis of studies with the *p*-nitrophenylphosphatase activity of the membrane preparations. If it is assumed, as we feel it must be[7], that this activity is intimately related to the $(Na^+ + K^+)$-activated ATPase and the physiological process of ATP-dependent ion translocation, it becomes important to investigate further the properties of these two ion-sensitive sites. Therefore, the objects of the present studies were to do certain kinetic studies on the *p*-nitrophenylphosphatase activity in the hope of determining (a) the alkali-cation specificities of the "Na^+-sensitive" and the "K^+-sensitive" sites; (b) the nature of the effect of ATP on these two sites; and (c) the possible existence and mechanism of interaction between these two sites and the active site. Two recent reports[4,8] contain the results of experiments similar to some of those presented in this paper. However, parts of our data which are either at variance with or in addition to previous observations lead us to some different interpretations.

MATERIALS AND METHODS

The enzyme from rat brain was prepared by the method of SKOU[9]. The *p*-nitrophenylphosphatase and ATPase activities were assayed as described before[5]. The standard assay medium for *p*-nitrophenylphosphatase contained 40 mM Tris–HCl (pH 7.4), and 4 mM *p*-nitrophenylphosphate, 4 mM $MgCl_2$, 25 mM KCl, and an appropriate amount of enzyme in a final volume of 2.5 ml. For the ATPase activity the standard assay medium contained 40 mM Tris–HCl (pH 7.4), 2 mM ATP, 2 mM $MgCl_2$, 100 mM NaCl, 30 mM KCl and the enzyme, in a final volume of 2.5 ml. Under these conditions the *p*-nitrophenylphosphatase activity of a typical preparation was 6 μmoles of *p*-nitrophenol liberated per mg of protein per h, and the ATPase activity was 60 μmoles of P_i formed per mg protein per h. About 90% of both activities could be inhibited by 1 mM ouabain. All substrates and nucleoside phosphates were obtained from Sigma Chemical Co. (St. Louis, Mo.). When these were supplied as sodium or potassium salts they were converted to the Tris salts by passage through Dowex-50 in Tris form. α,β-Methylene-adenosinetriphosphate and β,γ-methylene-adenosinetriphosphate were purchased from Miles Laboratories, Inc. (Elkhart, Ind.).

RESULTS

Effects of anions

It is known that either ATP or NaCl has an inhibitory effect on *p*-nitrophenylphosphatase[11]. Before examining the details of the activating effects of the simultaneous presence of ATP and NaCl on the K^+-sensitive process, it was appropriate to consider the nature of the inhibitory effects of each agent. Since it has been reported[11,12] that pyrophosphate, orthophosphate, and several nucleotides also inhibit *p*-nitrophenylphosphatase activity, we considered the possibility that *p*-nitrophenylphosphatase may be inhibited by a variety of anions, and that the observed inhibition of the enzyme by NaCl may also be due to the effect of the chloride ion. Fig. 1 shows the effects of varying Cl^- concentrations, added as Tris–HCl, NaCl, and $(CH_3)_4NCl$, on the enzyme activity in the presence and absence of a fixed concentration of K^+. The data show that although all three salts inhibit the enzyme activity, NaCl is clearly more effective than Tris–HCl and $(CH_3)_4NCl$. One must conclude, therefore, that at least part of the inhibitory effect of NaCl is due to Na^+. The question of the possible inhibitory effect of Cl^- cannot be answered from the data of Fig. 1 alone. It could be argued that the inhibitions obtained with organic chlorides are due to the weak inhibitory effects of organic cations on the activity. Against this argument are the results of experiments presented in Fig. 2. Here the effects of varying concentrations of Na^+ and K^+, in the presence of various anions, on the enzyme activity are shown. It is evident from the results that the apparent inhibitory effect of Na^+ is dependent on the nature of the salt used. With sulfate and nitrate salts stronger inhibitory effects are obtained. It is also apparent that the maximum activation obtained with K^+, and the apparent decrease in the activating ability of K^+ with increasing K^+ concentrations are also dependent on the nature of the K^+ salt used. From the results of Figs. 1 and 2, and in conjunction

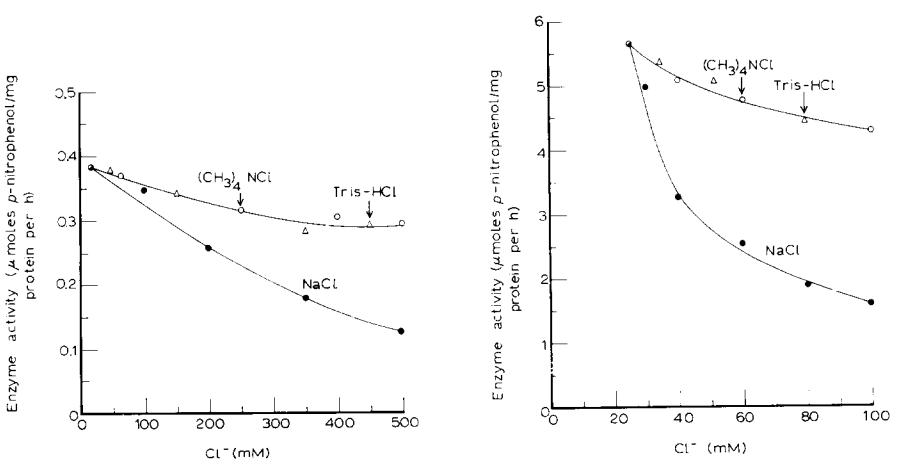

Fig. 1. Effects of varying concentrations of chloride (added as NaCl, ●; $(CH_3)_4NCl$, ○; and Tris–HCl, △) on *p*-nitrophenylphosphatase activity in the absence of an activator monovalent cation (Fig. 1a) and in the presence of 5mM K^+ (Fig. 1b). Tris–HCl was added as a buffered solution at pH 7.4. In calculating the chloride concentration for each point, the initial concentration of chloride due to the presence of a fixed amount of Tris–HCl (pH 7.4) in all assay tubes was taken into account. All reaction mixtures contained 4 mM substrate and 4 mM Mg^{2+}.

with previous observations[10,12] on the effects of nucleotides and orthophosphate on the enzyme, it seems reasonable to assume that a variety of anions have inhibitory effects on *p*-nitrophenylphosphatase activity. Although it seems that in general anions with higher negative charges have greater inhibitory effects, the different effects of Cl^- and NO_3^- observed from our data, and the fact that GTP and CTP are not as effective as ATP[11], indicate that factors other than charge are also important.

Comparison of the data of Fig. 1a with those of Fig. 1b shows that any of the tested chloride salts is a more effective inhibitor when the activity is measured in the presence of K^+. It may be concluded, therefore, that the inhibitory effects of Na^+ and anions on the *p*-nitrophenylphosphatase are more pronounced when the activator cation (K^+) is present. The reason for this phenomenon is not clear.

Ion specificity of "K⁺-sensitive" site in the presence of various anions

The data of the preceding section which indicated that anions have an influence on *p*-nitrophenylphosphatase activity, raised the question as to whether the order of alkali-cation specificity of this enzyme is altered in the presence of different anions. It should be recalled that the ion specificity of another alkali-cation-activated enzyme (AMP deaminase of erythrocytes) is profoundly affected by various simple anions[13]. The effects of varying concentrations of K^+, Na^+, Li^+, Rb^+ and Cs^+ on enzyme activity in the presence of Cl^-, SO_4^{2-} and NO_3^- were tested. Fig. 3 shows the results obtained with chloride salts. Although the activating effect of each cation is affected by the anion (Fig. 2b), it was found that regardless of the nature of the anion the order of cation specificity is $K^+ = Rb^+ > Cs^+ > Li^+ > Na^+$. The cation specificity of the enzyme was also studied when the enzyme activity was partially inhibited by either ATP or orthophosphate. The order of specificity remained as shown in Fig. 3.

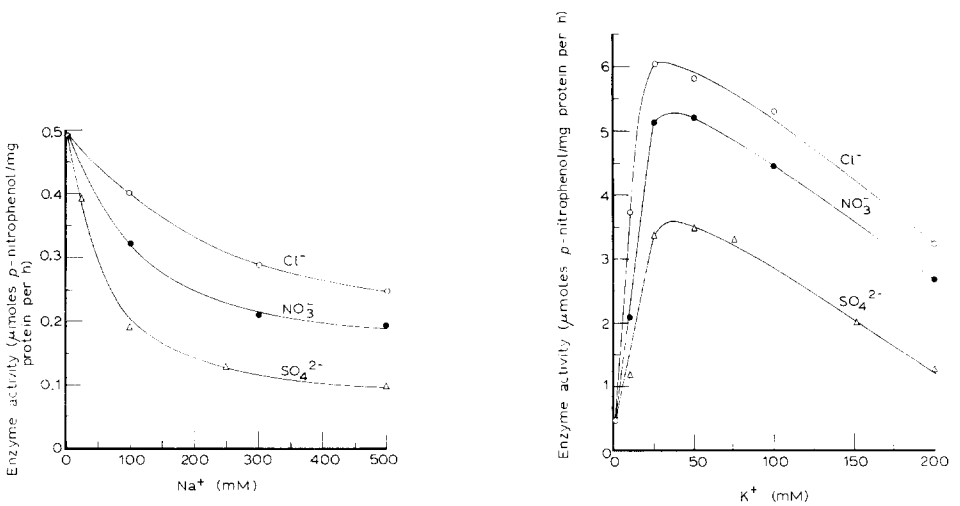

Fig. 2. Effects of varying concentrations of Na^+ (Fig. 2a), and K^+ (Fig. 2b) on the *p*-nitrophenylphosphatase activity in the presence of various anions. The buffers used were 40 mM Tris–HCl (pH 7.4), 40 mM Tris–H_2SO_4 (pH 7.4), and 40 mM Tris–HNO_3 (pH 7.4). Na^+, K^+, and Mg^{2+} were added as appropriate salts. All reaction mixtures contained 4 mM substrate and 4 mM Mg^{2+}. ○, Cl^-; ●, NO_3^-; △, SO_4^{2-}.

Ion specificity of the "K^+-sensitive site" in the presence of Na^+ and nucleotides

The simultaneous presence of Na^+ and ATP has a stimulating effect on the activity only when an activator cation (K^+) is present. A more detailed examination of the interactions of Na^+, K^+ and ATP on the enzyme activity was attempted. Fig. 4 shows the effect of varying concentrations of K^+ on the enzyme activity in

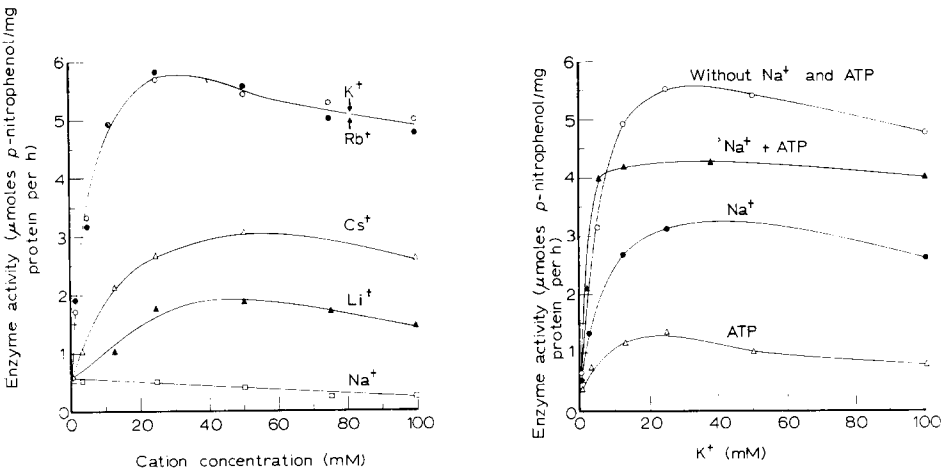

Fig. 3. Effects of varying concentrations of Li^+ (▲), Na^+ (□), K^+ (○), Rb^+ (●), and Cs^+ (△) on *p*-nitrophenylphosphatase activity. Reaction conditions were the same as described for Fig. 2. Cations were present as chloride salts.

Fig. 4. Effects of varying concentrations of K^+ on *p*-nitrophenylphosphatase in the presence of: 0.4 mM Mg^{2+}; ●, 4 mM Mg^{2+} + 20 mM Na^+; △, 4 mM Mg^{2+} + 0.1 mM ATP; ▲, 4 mM Mg^{2-}, 20 mM Na^+ + 0.1 mM ATP. Substrate concentration was 4 mM.

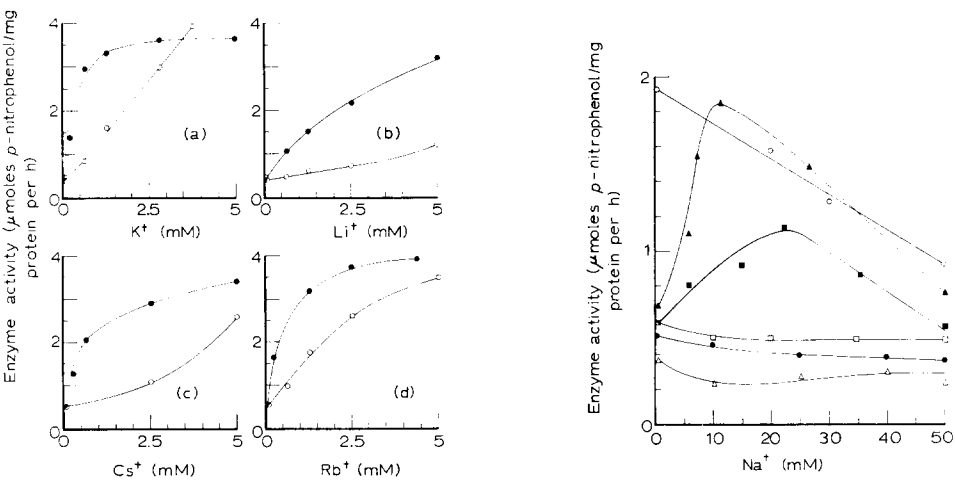

Fig. 5. Effects of varying concentrations of K^+ (Fig. 5a), Li^+ (Fig. 5b); Rb^+ (Fig. 5c), and Cs^+ (Fig. 5d) on *p*-nitrophenylphosphatase activity in the presence (●) and absence (○) of 20 mM Na^+ + 0.1 mM ATP. All reaction mixtures contained 4 mM substrate and 4 mM Mg^{2+}.

Fig. 6. Effects of varying concentrations of Na^+ on *p*-nitrophenylphosphatase activity in the presence of K^+ alone, ATP alone, and K^+ + ATP. All reaction mixtures contained 4 mM substrate and 4 mM Mg^{2+}. 0,2 mM K^+; □, 0.2 mM K^+; △, 0.1 mM ATP; ●, without K^+ and ATP; ▲, 2 mM K^+ + 0.1 mM ATP; ■, 0.2 mM K^+ + 0.1 mM ATP.

the presence of Na^+ alone, ATP alone, Na^+ + ATP, and in the absence of Na^+ and ATP. It is evident that at any K^+ concentration the enzyme activity is greater in the presence of Na^+ + ATP than those in the presence of either Na^+ alone or ATP alone. From the data of Fig. 4 it may seem that the effect of the simultaneous presence of Na^+ and ATP is only to overcome partially the inhibitory effects of either ATP or Na^+. That this is not the case becomes evident by examining the data at K^+ concentrations below 5 mM (Fig. 5a). Under these conditions the activity in the presence of Na^+ and ATP is even greater than that obtained with K^+ alone. The first question of interest arising from the above data is on the nature of the activating effect of Na^+ + ATP on the K^+-activated *p*-nitrophenylphosphatase. From the examination of data of Figs. 4 and 5a it should be evident that the presence of Na^+ + ATP affects both the apparent K_m for K^+ and the v_{max}. A more detailed analysis and interpretation of the kinetic data is not possible (see DISCUSSION) due to the complexity of the situation caused by the presence of two inhibitor anions (ATP and Cl^-), one inhibitor cation (Na^+), and an activator (K^+). However, since it is certain that at low concentrations of K^+ the activating effect of this ion is somehow increased in the presence of Na^+ + ATP, it became of interest to determine if the activating effects of other cations on the activity are also affected by the presence of Na^+ + ATP. Figures 5b, 5c and 5d show the effects of varying concentrations of Li^+, Cs^+ and Rb^+ on the enzyme activity in the presence and absence of Na^+ + ATP. It is quite clear that the apparent K_m values of all tested ions, like that of K^+, are decreased in the presence of Na^+ + ATP. It is also evident that the order of cation specificity of the K^+-sensitive site in the presence of Na^+ + ATP is the same as that in the absence of Na^+ + ATP ($K^+ = Rb^+ > Cs^+ > Li^+$).

Ion specificity of the "Na^+-sensitive" site

Fig. 6 shows the effect of various concentrations of Na^+ on the enzyme activity in the presence and absence of fixed concentrations of K^+ and ATP. The results of similar experiments in which Na^+ and K^+ concentrations were held constant and ATP concentration was changed have been presented before[5]. These data confirm the previous observations on the necessity of the simultaneous presence of Na^+ and ATP for the maximal activation of the enzyme in the presence of low K^+ concentrations. In addition, the data show that the concentration of Na^+ at which maximal activation is obtained depends on the K^+ concentration used. From these and the previous results[5] it is evident that for the clear demonstration of the activating effect of the simultaneous presence of Na^+ and ATP on the K^+-activated *p*-nitrophenylphosphatase it is necessary to choose a proper ratio of Na^+ and K^+, and relatively low concentrations of K^+ and ATP.

The experiments described above are relevant to the question of the ion specificity of the "Na^+-sensitive site". The data suggest that in order to determine if other cations can act like Na^+ in activating the enzyme in the presence of ATP and K^+, it is not sufficient to replace Na^+ with fixed concentrations of other cations. It seemed possible that other cations may be able to replace Na^+ at concentrations other than the maximal effective Na^+ concentration, or at ATP concentrations different from those used in Fig. 6. Therefore, an extensive study was performed to determine the possible effects of Rb^+, Cs^+ and Li^+ on the ATP-dependent modification of the

K^+-activated *p*-nitrophenylphosphatase. In none of the experiments was there any evidence that Na^+ at the "Na^+-sensitive site" could be replaced by any other cation.

Effects of other nucleotides and anions on the "Na^+-sensitive site"

In experiments similar to those of Figs. 4–6 the effects of different concentrations of various nucleoside triphosphates, diphosphates and monophosphates on the enzyme activity were studied. In agreement with our previous preliminary observations[5], and the more extensive data of others[4], of the nucleoside triphosphates only ATP, ITP and CTP were found to be effective. In our preparation ADP, like other nucleoside diphosphates and monophosphates, showed no activating effect (contrast YOSHIDA *et al.*[4]).

Two structural analogues of ATP (α,β-methylene-adenosinetriphosphate and β,γ-methylene-adenosinetriphosphate), pyrophosphate, and orthophosphate were also tested for their possible activating effects. Over a wide range of concentrations ($1 \cdot 10^{-6} - 1 \cdot 10^{-2}$ M) none were found to be effective.

Experiments similar to those of Figs. 4–6, but with the chloride salts completely replaced by sulfate or nitrate, were also performed. Under these conditions no significant changes in the activating effects of $Na^+ + ATP$ were observed.

Ion specificity of the $(Na^+ + K^+)$-activated ATPase

Although data on the specificity of several $(Na^+ + K^+)$-activated ATPase preparations have been published[1], for the purpose of comparison of the specificities of the *p*-nitrophenylphosphatase and ATPase activities, it was important to determine the alkali-cation specificity of the ATPase of the preparation used in the present studies. In agreement with previous results it was found that while no other cation can replace Na^+, in its presence all other tested cations are effective activators. The order of specificity being $K^+ = Rb^+ > Cs^+ > Li^+$.

DISCUSSION

The data presented here show, in agreement with previous observations[11,12], that the "K^+-sensitive site" of the p-nitrophenylphosphatase has a "broad-band" specificity. It responds to all the tested alkali cations with the possible exception of Na^+. The order of its cation specificity being $K^+ = Rb^+ > Cs^+ > Li^+ > (Na^+?)$. This order is the same as that obtained for the specificity of the "K^+-sensitive site" of the $(Na^+ + K^+)$-activated ATPase of the same preparation. Detailed studies on the formation and breakdown of "phosphorylated enzyme"[1] have not been performed with the particular preparation used in these studies. However, such studies with other preparations have shown that other alkali cations can replace K^+ in its influence on the breakdown of phosphorylated enzyme, and that the order of specificity of cations for this reaction is also the same as the order mentioned above[14]. These facts give further support to the assumption that the "K^+-sensitive site" identified in studies on *p*-nitrophenylphosphatase is the same as the "K^+-sensitive site" of the $(Na^+ + K^+)$-activated ATPase.

Our studies also show that a "Na^+-sensitive site" of the *p*-nitrophenylphosphatase which can be operationally identified in the presence of ATP and K^+, and by its sensitivity to oligomycin[5,6], has an absolute specificity for Na^+. Again, this

is in agreement with the absolute specificity of the "Na^+-sensitive site" of the ($Na^+ + K^+$)-activated ATPase, and that of the Na^+-dependent formation of the phosphorylated enzyme[1,14]. That the above three operationally determined Na^+ sites are the same is not, however, intuitively obvious. One could assume that the activation of K^+-dependent phosphatase by $Na^+ + ATP$ is due to the formation of phosphorylated enzyme which causes a change in the enzyme more favorable to K^+-dependent hydrolysis. The fact that ($Na^+ + ATP$)-activation of *p*-nitrophenylphosphatase is observed only in the presence of low concentrations of K^+ would give some support to this assumption. However, as discussed elsewhere[5,6], there are serious objections to correlating the above mentioned modification of the enzyme with phosphorylation *per se*. Alternatively, it could be envisioned that the primary role of the "Na^+-sensitive site" is to produce, in the presence of ATP, a modification in the enzyme complex which is (a) necessary if phosphorylated enzyme is to be formed and (b) favorable, but not necessary, to the subsequent K^+-dependent hydrolysis of either the phosphorylated enzyme or *p*-nitrophenylphosphate.

An original objective of this work was to find out if kinetic studies and determinations of various kinetic parameters of *p*-nitrophenylphosphatase may be used to draw any conclusions on the nature of the interactions of Na^+, K^+ and ATP with the enzyme. We feel that the portion of our kinetic data that is presented in this paper is sufficient to show the limitations of this approach. Examination of our data would reveal that they are in general agreement with those presented by others[4,8]. Many of the kinetic curves, such as those in Figs. 4 and 5, could be presented in the form of Hill plots and analyzed in terms of allosteric processes. On this basis it may be concluded that Na^+, K^+, and ATP are allosteric modifiers of the enzyme. While such conclusions (as discussed in detail by ROBINSON[8]) are in agreement with our suggestion (based mainly on studies with oligomycin[5,6]) that there is a modifying site on the enzyme, we hesitate to interpret the kinetic data in the context of allosteric processes. The reason for this may become clear if specific data are discussed. Consider the simplest set of kinetic data that are presented here: namely the effects of varying concentrations of a single activating cation on the enzyme activity at fixed substrate and Mg^{2+} concentrations. It is evident that at lower ranges of action concentrations the curves are sigmoid in shape (Fig. 5). However, a closer look at the data (Figs. 2 and 3) would show that all activation curves have sharp peaks. As indicated in RESULTS the inhibition at higher activator cation concentrations is due to the inhibitory effects of the anions that are by necessity added with the activator cation. In addition it should be recalled that (a) the substrate is an anion, and there is direct evidence that it also inhibits the activity[8,11]; and (b) the enzyme source is a membrane fraction containing a variety of phospholipids which are suspected to have profound effects on the enzyme activity[15,17]. Obviously in such a complex situation the designation of an activator cation as an allosteric modifier on the basis of the sigmoid shape of a very limited portion of the data is at best dubious.

In addition to the activating effect of Na^+ at the above-mentioned"Na^+-sensitive site", our data show inhibitory effects of Na^+ on *p*-nitrophenylphosphatase activity. These effects are apparent in the absence of K^+ (Figs. 1a, 2a), in the presence of K^+ (Fig. 1b), and in the presence of K^+ and ATP (Fig. 6). Inhibitory effects of higher concentrations of Na^+ on the ($Na^+ + K^+$)-activated ATPase are well known.

The inhibition of pump activity by Na^+, from the same side of the membrane as that where K^+ activates the pump, has also been demonstrated[18]. In view of these facts the consideration of the site of the above inhibitory effects of Na^+ on *p*-nitrophenylphosphatase becomes relevant. The obvious possibilities are that these effects are due to the interaction of Na^+ with (a) the "K^+-sensitive site"; (b) the same "Na^+-sensitive site" that is involved in the activating effect of Na^+; and (c) site or sites that are different from the above two. Due to the complexity of kinetics of the enzyme, as discussed above, a choice between the various alternatives cannot be made until additional evidence becomes available.

An interesting outcome of the present studies is the finding that the order of the ion specificity of the "K^+-sensitive site" and the absolute specificity of the "Na^+-sensitive site" remain remarkably constant under a variety of conditions which had been used previously to demonstrate the changing ion-specificity of the alkali-cation-activated AMP deaminase[13,19]. Also noteworthy is the observation that the nucleoside triphosphate which is required for the demonstration of the "Na^+-sensitive site" of the *p*-nitrophenylphosphatase cannot be replaced by the tested analogues of ATP. Again, this is in contrast to the ability of these compounds to mimic the effects of ATP on AMP deaminase[20]. On the basis of our studies on AMP deaminase we had previously suggested that perhaps the modifying effects of ATP on the $(Na^+ + K^+)$-activated ATPase complex could be explained by the same type of general mechanism that was postulated to explain the effects of ATP on the ion-specificity of AMP deaminase[19]. Our present results show that such a comparison may not be justified.

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