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# SODIUM AND POTASSIUM ION-DEPENDENT ADENOSINE TRIPHOSPHATASE OF MAMMALIAN BRAIN

## INTERACTIONS OF MAGNESIUM IONS WITH THE PHOSPHATASE SITE

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### Summary

Kinetic parameters are reported for $Mg^{2+}$, $Na^+$ and $K^+$ as activators of the *p*-nitrophenylphosphatase activity associated with ($Na^+ + K^+$)-ATPase (ATP-phosphohydrolase, EC 3.6.1.3) of beef brain. In each case the phosphatase reaction is activated at low concentrations of the cation and inhibited by higher concentrations. The concentrations of cation that produce half-maximal activation and half-maximal inhibition are increased as the concentration of either of the other two cations is increased. These second ligand effects are all saturable functions. The apparent binding constant that characterizes the effect on activation is closely similar to that acting upon the inhibitory phase in each case.

### Introduction

($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) couples hydrolysis of ATP to cell $Na^+$ efflux and $K^+$ influx, and is regulated in a complex manner by these ligands [1,2]. In addition, $Mg^{2+}$ is essential to the ATPase, but is not known to be transported by this system [3]. The overall ATPase reaction is probably a continuous cyclic process, which can be studied as a series of partial reactions [2,4—6]:

$$E_1 + ATP \overset{Na^+, Mg^{2+}}{\rightleftharpoons} E_1P + ADP \quad (1)$$

$$E_1P \overset{Mg^{2+}}{\rightleftharpoons} E_2P \quad (2)$$

$$E_2P \overset{K^+}{\rightarrow} E_2 + P_i \quad (3)$$

$$E_2 \rightleftharpoons E_1 \quad (4)$$

Reactions 1—3 can be measured experimentally. Reaction 4 is a return to the original conformation and has not been observed directly. $K^+$-dependent hydrolysis of artificial substrate such as *p*-nitrophenylphosphate seems to be related to the $K^+$-dependent dephosphorylation of the $(Na^+ + K^+)$-ATPase [7]. $Mg^{2+}$ is absolutely required for exchange, phosphorylation reactions [4,5,7], but high-affinity ATP binding does not require $Mg^{2+}$ [8,9]. $Mg^{2+}$ effects on the partial reactions are complex. Optimal concentrations for exchange and phosphorylation are <0.1 mM; higher concentrations cause inhibition [4,6]. However, millimolar concentrations of $Mg^{2+}$ are optimal to catalyze the phosphatase and ATPase reactions. $Mg^{2+}$ seems to oppose the action of reagents such as oligomycin, which are believed to stabilize the $E_1$ enzyme [10]. $Mg^{2+}$ is also required for ouabain binding [2]. These findings have been taken as evidence that $Mg^{2+}$ stabilizes the "$E_2$" enzyme conformation [2,11]. Recently evidence for two different types of $E_2$ has been reported [12].

The investigation of $Mg^{2+}$ effects is complicated by its chelation with ATP. Most kinetic studies of the overall $(Na^+ + K^+)$-ATPase reaction assume MgATP to be the actual substrate, and have been carried out under conditions in which that form predominates [13]. The differing $Mg^{2+}$ requirements and ATP requirements for the different partial reactions and experimental conditions support the possibility that there is more than one $Mg^{2+}$ site. Hydrolysis of *p*-nitrophenylphosphate has a relatively high $Mg^{2+}$ requirement, though ATP need not be present and *p*-nitrophenylphosphate chelates little $Mg^{2+}$.

The experiments reported here were designed to measure the interactions of $Mg^{2+}$ with the ATPase under simplified conditions. Initial velocity measurements of the *p*-nitrophenylphosphatase reaction were performed under conditions that minimize or eliminate the effects of interaction with other ligands.

## Materials and Methods

Fresh beef brains were obtained from Frederick County Products and stored in liquid $N_2$. The preparation of NaI-treated deoxycholate microsomes rich in $(Na^+ + K^+)$-ATPase has been described [14].

The assay for $K^+$-dependent *p*-nitrophenylphosphatase is based upon the spectrophotometric measurement of *p*-nitrophenol [15]. The reactions were routinely performed at pH 9 at 25°C for 15 min, conditions which minimize ATPase activity and are linear with respect to the phosphatase activity. The usual buffer was 0.05 M Tris · HCl. Concentrations of other ligands are described in the appropriate figure legends or text.

$Na^+$-dependent ATP-ADP transphosphorylation was determined by incubating a reaction mixture containing 10 $\mu$g enzyme with 1.25 mM [$^{14}$C]ADP and 5 mM ATP (as Tris salts) in 0.05 M Tris · HCl buffer at pH 9.0, 25°C for 1 h. $MgCl_2$ was varied and $Na^+$-dependent activity was taken as the increment of [$^{14}$C]ATP formed in the presence of 125 mM NaCl. Nucleotides were counted after separation by thin-layer chromatography [4].

Computer fitting of kinetic data to equations used an iterative least-squares method, called MLAB, which has been described [15,16]. Information supplied with the best fitting parameters includes the normal error for each parameter, showing how much it can change without significantly affecting the variance of

the data from the simulation, the dependency of each parameter on the values of other parameters, and the root means squared error of the simulation from the data. Root means squared error, dependency, and parameter normal error values were generally extremely low for all of the simulations shown here. The equations were chosen to fit the data (i.e. obtain minimal root means squared error) with the fewest parameters possible.

## Results

*Substrate activation*

Potassium nitrophenylphosphatase activity as a function of nitrophenylphosphate concentration was measured at a fixed $K^+$ concentration (10 mM) and various $Mg^{2+}$ concentrations (Fig. 1). These curves are approximately hyperbolic. Both $V_{app.}$ and $[S]_{0.5V}$ increase with increasung $Mg^{2+}$ (Fig. 2). The $Mg^{2+}$ effects on $V_{app}$ and $[S]_{0.5V}$ are clearly independent because the $V_{app.}$ effect is half saturated at 0.34 mM $Mg^{2+}$ and is approximately hyperbolic, whereas the $[S]_{0.5V}$ effect is sigmoid and requires more than 10 times higher $Mg^{2+}$ to produce half-saturation (Table I).

*$Mg^{2+}$ activation*

Nitrophenylphosphatase activity has been measured as a function of $Mg^{2+}$ concentration at fixed substrate (10 mM) and various fixed $K^+$ concentrations (Fig. 3). As previously reported [17], $Mg^{2+}$ activation is biphasic. The secondary phase of $Mg^{2+}$ inhibition becomes less apparent as the $K^+$ concentration is increased (Fig. 3). These data are fitted to Eqn. 5 as discussed below. The kinetic analysis is summarized in Fig. 4, $V_{app.}$, $[Mg^{2+}]_{0.5A}$, and $[Mg^{2+}]_{0.5I}$ all increase with increasing $K^+$ concentration. The effects of $K^+$ on the latter two parameters are roughly parallel (Table II).

Nitrophenylphosphatase activity was also measured as a function of $Mg^{2+}$ concentration at various fixed $Na^+$ concentrations with constant substrate (10 mM) and constant $K^+$ (1 mM). These data are shown in Fig. 5. The similarity to the data of Fig. 3 is evident. The principal difference is that, under these condi-

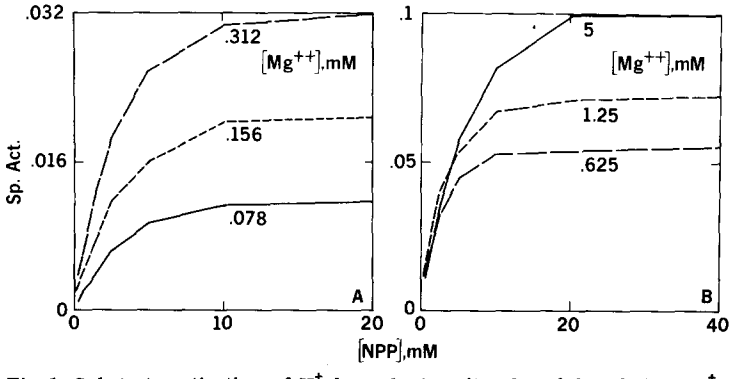


Fig. 1. Substrate activation of $K^+$-dependent *p*-nitrophenylphosphatase; $K^+$ = 10 mM. (a) ———, $Mg^{2+}$ = 0.078 mM; - - - - - -, 0.156 mM; - - - - - -, 0.312 mM. (b) - - - - - -, $Mg^{2+}$ = 0.625 mM; - - - - - -, 1.25 mM; ———— ——, 5 mM.

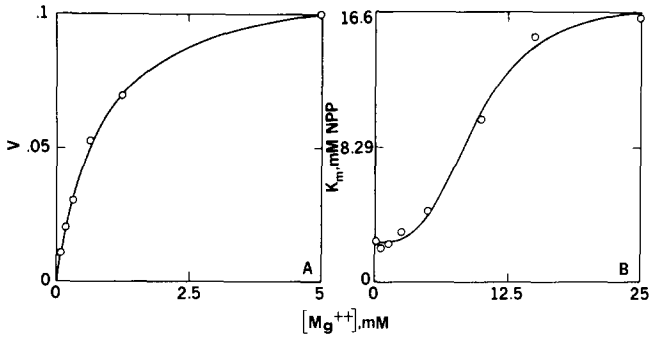


Fig. 2. Effect of $Mg^{2+}$ on constants for substrate activation of $K^+$-dependent *p*-nitrophenylphosphatase, $K^+$ = 10 mM. a, $V$; b, $K_m$ (mM *p*-nitrophenylphosphate). See tables.

tions, increasing $Na^+$ decreases $V_{app.}$. $Na^+$ activation of the beef brain phosphatase is insignificant in the absence of nucleotide [15]. The experiment of Fig. 5 was repeated in the presence of 50 $\mu$M ATP and the data are shown in Fig. 6. The effect of $Na^+$ on the $Mg^{2+}$ parameters remains qualitatively the same; the effect of ATP appears as an increase of $V_{app.}$ with increasing $Na^+$ in contrast to the $Na^+$ inhibition seen in Fig. 5. The data of Figs. 5 and 6 were fit to Eqn. 5 and the effects of $Na^+$ on $Mg^{2+}$ parameters are summarized in Figs. 7 and 8. In

TABLE I

ACTIVATION PARAMETERS

For $K^+$-dependent nitrophenylphosphatase, except for the $Mg^{2+}$ exchange constants. Results are expressed in units of mol/l.

| | | $K_a$ | $K_i$ | Source of data |
|---|---|---|---|---|
| | | Potassium | | |
| Zero | $Mg^{2+}$ | $2.6 \cdot 10^{-4}$ | $1.6 \cdot 10^{-2}$ | Fig. 10 |
| Infinite | $Mg^{2+}$ | $2.2 \cdot 10^{-3}$ | $9.2 \cdot 10^{-2}$ | Fig. 10 |
| | | Sodium | | |
| Zero | $Mg^{2+}$ | $3.4 \cdot 10^{-4}$ | $8 \cdot 10^{-3}$ | Fig. 13 |
| Infinite | $Mg^{2+}$ | $5 \cdot 10^{-3}$ | $>10^{-1}$ | Fig. 13 |
| | | Magnesium | | |
| Zero | $K^+$ | $8 \cdot 10^{-5}$ | $1.9 \cdot 10^{-3}$ | Fig. 4 |
| Infinite | $K^+$ | $8 \cdot 10^{-4}$ | $1.3 \cdot 10^{-2}$ | Fig. 4 |
| Zero | $Na^+$ | $2.1 \cdot 10^{-4}$ | $3.5 \cdot 10^{-3}$ | Fig. 7 |
| Infinite | $Na^+$ | $2.9 \cdot 10^{-3}$ | $1.2 \cdot 10^{-1}$ | Fig. 7 |
| ADP-ATP exchange * (125 mM $Na^+$) | | $5 \cdot 10^{-6}$ | $1 \cdot 10^{-4}$ | Text |
| | | Nitrophenyl phosphate | | |
| Zero | $Mg^{2+}$ | $2.4 \cdot 10^{-3}$ | — | Fig. 2 |
| Infinite | $Mg^{2+}$ | $1.7 \cdot 10^{-2}$ | — | Fig. 2 |

* In molar total $[Mg^{2+}]$.

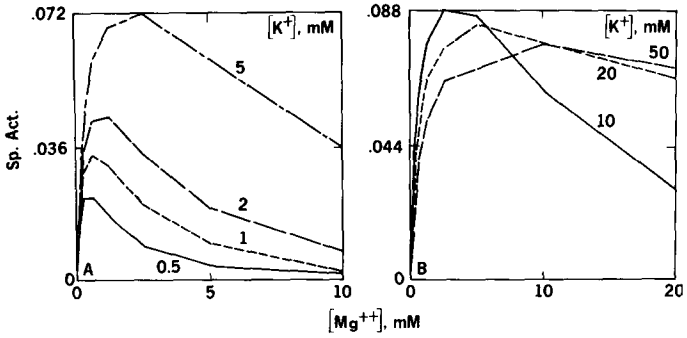


Fig. 3. $Mg^{2+}$ activation of $K^+$-dependent nitrophenylphosphatase at the $K^+$ concentrations shown ( in mM). $Mg^{2+}$ = 0.039, 0.078, 0.156, 0.312, 0.625, 1.25. 2.5, 5, 10 and 20 mM. Conditions are otherwise as described in Materials and Methods.

the presence of nucleotide, all three parameters increase roughly in parallel. $Na^+$ inhibition in the absence of nucleotide is only partial. The relief of $Mg^{2+}$ inhibition by high $Na^+$ is so marked as to make measurement of a $[Mg^{2+}]_{0.5I}$ difficult.

*$K^+$ activation*

Activation of nitrophenylphosphatase by $K^+$ at fixed substrate concentration (10 mM) was measured at various $Mg^{2+}$ concentrations (Fig. 9). Inhibition by high $K^+$ is more apparent at the lower $Mg^{2+}$ concentrations. The data were fit to Eqn. 5, which is discussed below, and the variations of $V_{app.}$, $[K^+]_{0.5V}$ and $[K^+]_{0.5I}$ with $Mg^{2+}$ concentrations are shown in Fig. 10. These parameters all increase with increasing $Mg^{2+}$ and are half-saturated at about 1 mM $Mg^{2+}$.

*$Na^+$-$K^+$ interaction at low $Mg^{2+}$ concentrations*

We have reported the complex interactions of $Na^+$ and $K^+$ as nitrophenylphosphatase activators. In these earlier experiments the nitrophenylphosphatase activity was measured in the presence of 10 mM $Mg^{2+}$. This is sufficient to effectively minimize the inhibitory effects of high levels of $Na^+$ and $K^+$. Figs. 11

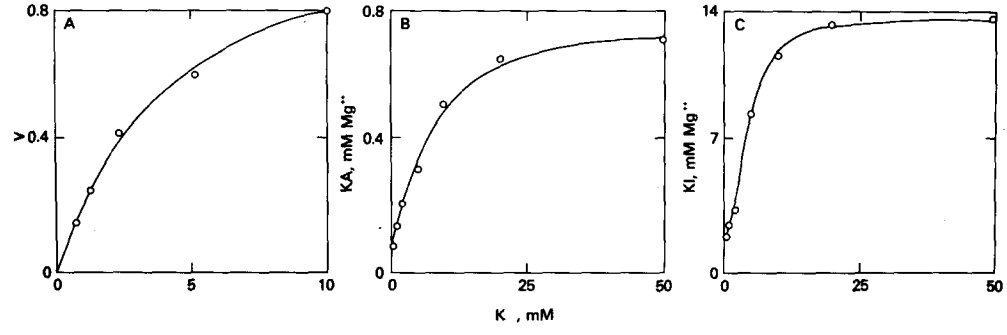


Fig. 4. Constants for Eqn. 5 describing $Mg^{2+}$ activation of $K^+$-dependent *p*-nitrophenylphosphatase as a function of $K^+$. A, *V* in μmol (*p*-nitrophenol liberated/mg protein per min); B, $K_a$ (mM $Mg^{2+}$); C, $K_i$ (mM $Mg^{2+}$). See tables and text.

TABLE II

CATION INTERACTION PARAMETERS

Molar concentration of the indicated ligand necessary to produce a half-maximal change in the activation parameters of the second ligand.

| Effect | To increase $V$ | To decrease affinity |
|---|---|---|
| $Mg^{2+}$ on substrate | $3.4 \cdot 10^{-4}$ | $3.9 \cdot 10^{-3}$ |
| $Mg^{2+}$ on $K^+$ | $3.8 \cdot 10^{-4}$ | $1.7 \cdot 10^{-3}$ |
| $Mg^{2+}$ on $Na^+$ | $4.9 \cdot 10^{-4}$ | $1.8 \cdot 10^{-3}$ |
| $K^+$ on $Mg^{2+}$ | $5 \cdot 10^{-3}$ | $6 \cdot 10^{-3}$ |
| $Na^+$ on $Mg^{2+}$ | — | $2.4 \cdot 10^{-2}$ |
| $Na^+$ on $Mg^{2+}$ (with ATP) | $4.3 \cdot 10^{-3}$ | $6 \cdot 10^{-3}$ |

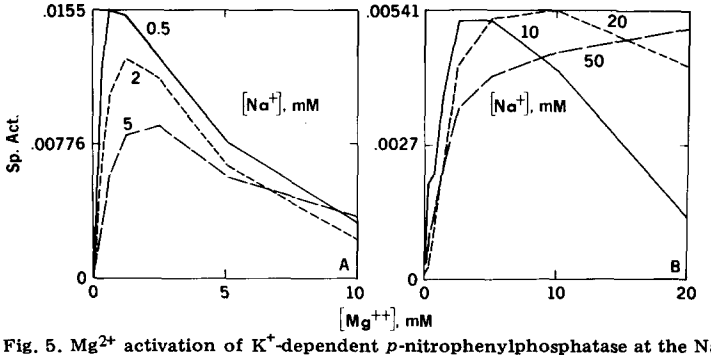


Fig. 5. $Mg^{2+}$ activation of $K^+$-dependent *p*-nitrophenylphosphatase at the $Na^+$ concentrations shown. $K^+$ = 1 mM; conditions otherwise are as described in Materials and Methods.

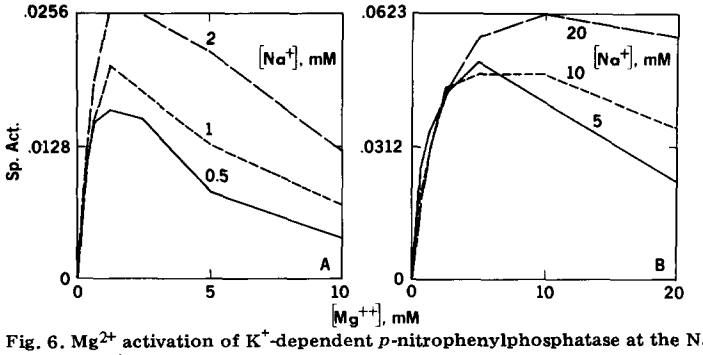


Fig. 6. $Mg^{2+}$ activation of $K^+$-dependent *p*-nitrophenylphosphatase at the $Na^+$ concentration shown; ATP = 0.05 mM, $K^+$ = 1 mM; conditions otherwise are as in Materials and Methods.

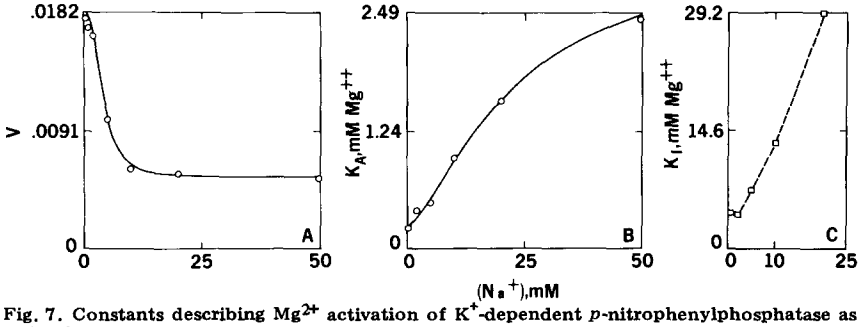


Fig. 7. Constants describing $Mg^{2+}$ activation of $K^+$-dependent *p*-nitrophenylphosphatase as a function of $Na^+$; $K^+$ = 1 mM. A, $V$; B, $K_a$ (mM $Mg^{2+}$). See tables and text.

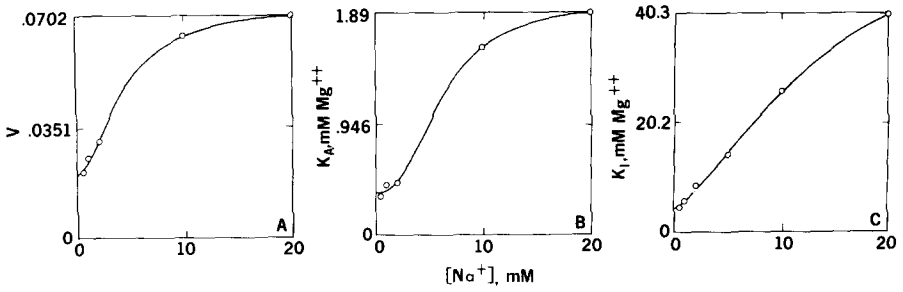


Fig. 8. Constants describing $Mg^{2+}$ activation of $K^+$-dependent *p*-nitrophenylphosphatase as a function of $Na^+$ with ATP = 0.05 mM and $K^+$ = 1 mM. A, *V*; B, $K_a$ (mM $Mg^{2+}$); C, $K_i$ (mM $Mg^{2+}$).

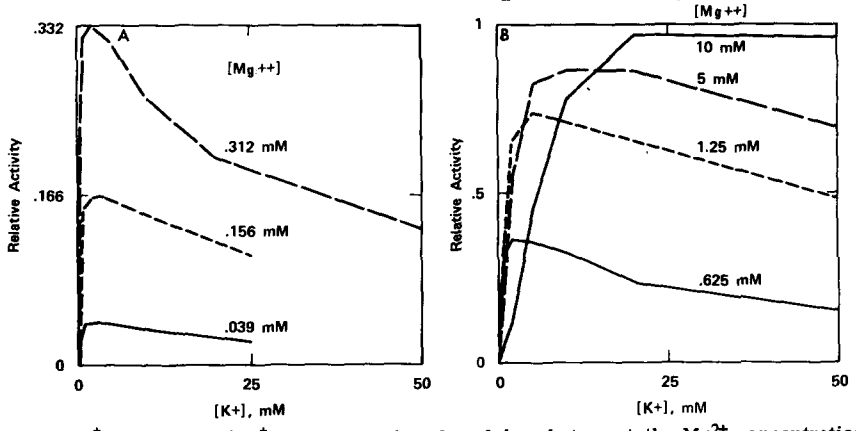


Fig. 9. $K^+$ activation of $K^+$-dependent nitrophenylphosphatase at the $Mg^{2+}$ concentrations shown. $K^+$ = 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, and 50 mM. Conditions are otherwise described in Materials and Methods.

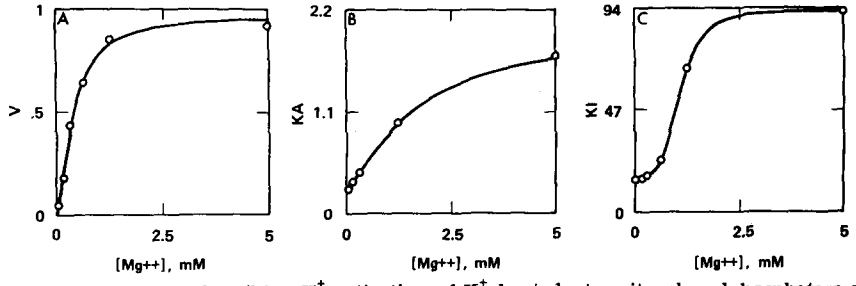


Fig. 10. Constants describing $K^+$ activation of $K^+$-dependent *p*-nitrophenylphosphatase as a function of $Mg^{2+}$. A, *V*; B, $K_a$ in mM $K^+$; C, $K_i$ in mM $K^+$. See tables and text.

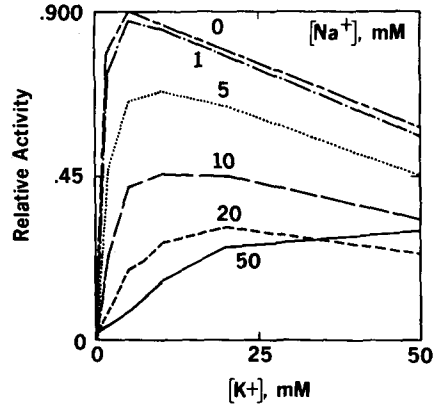


Fig. 11. $K^+$ activation of $K^+$-dependent *p*-nitrophenylphosphatase at the $Na^+$ concentrations shown; $Mg^{2+}$ = 1.25 mM; $K^+$ activation as in Fig. 3.

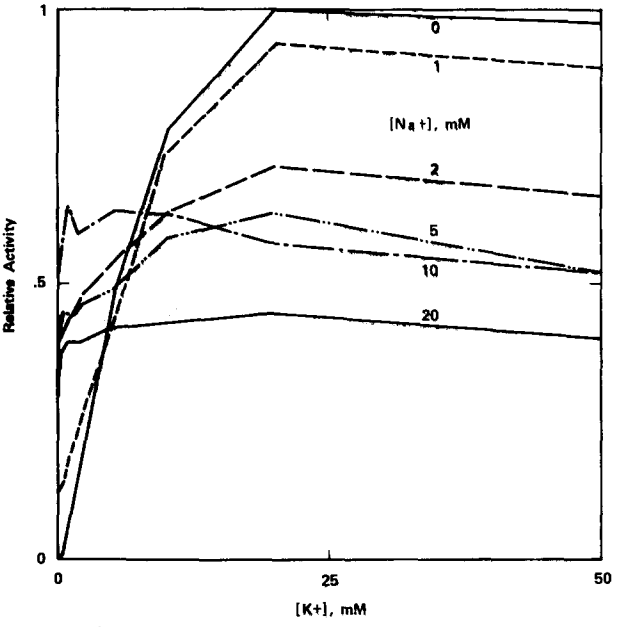


Fig. 12. $K^+$ activation of $K^+$-dependent *p*-nitrophenylphosphatase at the $Na^+$ concentrations shown; ATP = 0.1 mM; $Mg^{2+}$ = 1.25 mM.

and 12 show the $K^+$ activation curves obtained at various fixed levels of $Na^+$ in the presence of 10 mM nitrophenylphosphate and 1.25 mM $Mg^{2+}$. At this lower level of $Mg^{2+}$, $K^+$ inhibition becomes evident at concentrations greater than 10 mM (Figs. 8 and 10; compare Fig. 3A of ref. 15). Increasing $Na^+$ increases both $[K^+]_{0.5A}$ and $[K^+]_{0.5I}$. In the presence of ATP, increasing $Na^+$ transforms an increasing proportion of the enzyme into a form with high $K^+$ affinity (compare Fig. 11 with Fig. 3B of ref. 15). At lower $Mg^{2+}$ levels this transformation occurs at lower $Na^+$ concentration and $Na^+$ exerts a much larger inhibition on the $V_{app}$.

*ATP-ADP exchange*

$Na^+$-dependent ATP-ADP exchange is a biphasic function of $Mg^{2+}$ concentration. Apparent $Mg^{2+}$ affinity for activation, obtained by fitting the exchange role as a function of $Mg^{2+}$ to Eqn. 5, is over 10-fold greater than for phosphatase activation. Concentrations of $Mg^{2+}$ that activate phosphatase inhibit exchange (see Table I).

## Discussion

These studies demonstrate that increasing concentrations of $Mg^{2+}$, $Na^+$ and $K^+$ produce activation of the *p*-nitrophenylphosphatase reaction followed by inhibition in each case. The shape of the activation curves for a given cation is strongly influenced by the concentration of each of the others. Previously these interactions between $Na^+$ and $K^+$ have been considered in terms of mutual com-

petitive interactions [7,15,18—20]. The present results implicate $Mg^{2+}$ in a similar phenomenon.

Two different hypothetical explanations may be considered: (i) inhibition may result from binding to a site that is spatially distinct from the activating site; (ii) activation and inhibition may be manifestations of interaction at a single site for a given ligand. Case I would be supported if activation and inhibition parameters could be modified independently as described by the product of separate functions:

$$v = V((K_a/M)^m + 1)^{-1} \cdot ((M/K_i)^n + 1)^{-1} . \quad (5)$$

Case II appears more consistent with the data as reported here because there are nearly parallel changes in activation and inhibition parameters produced by altering second ligands. More than one mechanism can be envisioned for biphasic actions of ligands at a single site. For example, if the enzyme is oligomeric, maximal activation might occur at partial occupancy and inhibition at full occupancy.

A simpler mechanism for case II is one arising from an ordered reaction sequence. If the dissociation of one of the products, e.g. phosphate, must be preceded by dissociation of a ligand, e.g. $Mg^{2+}$, the rate equation for an ordered Bi Ter mechanism [21] reduces to

$$v = V((K_a/M) + 1 + (M/K_i))^{-1} . \quad (6)$$

In this equation $K_i$ is the product of a number of constants that includes $K_a$. Thus any factor that modifies $K_a$ will change $K_i$ proportionately.

The curves generated by Eqns. 5 and 6 are identical so that the two models are not easily distinguished by the shapes of the activation curves. In the discussion of the interactions of different pairs of cations that follows, the constants have been derived by fitting the data to Eqn. 5.

*Cation interaction with substrate parameters*

Previous studies of the phosphatase reaction catalysed by *Electrophorus* ATPase [22] demonstrate that the apparent substrate affinity increases as either $Na^+$ or $K^+$ is increased, which could be described by an equation of the form

$$v = V[1 + K_s(1 + K_a/A)]^{-1} . \quad (7)$$

This implies a mechanism that requires activator binding before substrate is bound.

The $Mg^{2+}$ effects on substrate parameters do not follow this pattern. Tables I and II and Fig. 2 summarize the effects of $Mg^{2+}$ on activation of $K^+$-dependent *p*-nitrophenylphosphatase by substrate. $Mg^{2+}$ constants are corrected for $Mg^{2+}$-$K^+$ competition at the constant $K^+$ level of 10 mM in these experiments, using

$$K_{true} = \frac{K_{(K^+ \text{ on } Mg^{2+})}}{K_{(K^+ \text{ on } Mg^{2+})} + [K^+]} \times K_{obs} . \quad (8)$$

Increasing $Mg^{2+}$ increases $V$ and decreases substrate affinity (Fig. 2). The effect of $Mg^{2+}$ on $V$ is half-maximal at 0.34 mM when corrected for the competitive effect of the 10 mM $K^+$ present.

The effect of $Mg^{2+}$ on substrate affinity is half-maximal at 3.9 mM. Increasing $Mg^{2+}$ appears to titrate the enzyme from a form with relatively high substrate affinity (apparent $K_s$ at 0 $Mg^{2+}$ = 2.4 mM) to a form with lower substrate affinity (apparent $K_s$ at infinite $Mg^{2+}$ = 17 mM). Over the range of $Mg^{2+}$ concentrations that increases $V$ there is only a small effect on the apparent $K_s$.

*Interactions between cation pairs*

Each of the biphasic $Mg^{2+}$ activation curves in Fig. 3 was fit to Eqn. 5. $V$, $K_a$, and $K_i$ were then replotted as functions of $K^+$ concentration (Fig. 4). Each is a saturable function of $[K^+]$. The effect of $[K^+]$ on each of the $Mg^{2+}$ parameters can be characterized from these plots (Tables I and II). Increasing $[K^+]$ from zero to infinity increases $K_a$ 10-fold, and $K_i$, 7-fold. Half-maximal $K^+$ effects on all three parameters are attained at about 5 mM.

The reciprocal effects of $Mg^{2+}$ on $K^+$ parameters have been evaluated in the same manner (Fig. 10). In this case the half-maximal $[Mg^{2+}]$ for effects on $K_a$ and $K_i$ is distinctly higher than that for the $V$ effect (Table II). However, the overall range of modulation of the affinity constants is again 6—10-fold. $Na^+$ activation requires the presence of some $K^+$. In the absence of ATP, the $Na^+$ activation is too small to examine quantitatively. However, $Na^+$ does have significant effects on the $Mg^{2+}$ parameters in the absence of ATP (Fig. 7): increasing $Na^+$ increases $K_a$ more than 10-fold and increases $K_i$ about 30-fold (Table I).

$Na^+$ decreases $V$, but is only a partial inhibitor. This effect cannot be explained solely on the basis of $Na^+$-$K^+$ or $Na^+$-$Mg^{2+}$ competition. High $Na^+$ may result in a reaction pathway having a slower rate, possibly through stabilization of an intermediate, or may convert the enzyme to a form having a lower affinity for *p*-nitrophenylphosphate or for $K^+$, and thus less activity at the constant $K^+$ or substrate level used.

In the presence of ATP, $Mg^{2+}$ effects on $Na^+$ activation are similar to its effects on $K^+$ activation without ATP (cf. Figs. 6 and 9). $Mg^{2+}$ increases $V$ (Table II) and decreases apparent monovalent cation affinities (Table I). For both $Na^+$ and $K^+$, the $Mg^{2+}$ effect on $V$ is characterized by an apparent $K_i$ of 0.4

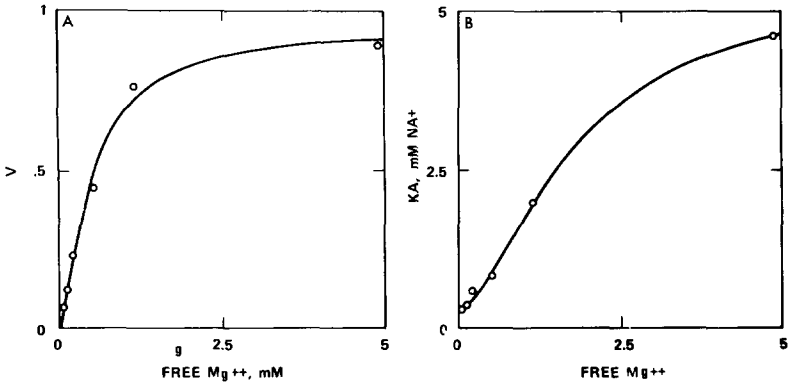


Fig. 13. Constants describing $Na^+$ activation as a function of $Mg^{2+}$ with ATP = 0.05 mM, $K^+$ = 0.5 mM, A $V$; B, $K_a$ (mM $Na^+$), See tables and text.

mM. In contrast, the $Mg^{2+}$ effects on monovalent cation affinities are described by constants 5-fold larger. Thus two distinct mechanisms must be involved. An analogous dichotomy is seen in the effects of $Mg^{2+}$ on substrate parameters (see above).

This parallel variation of $K_a$ and $K_i$ for each of the cations suggests as the most probable hypothesis that each cation must dissociate from the enzyme at some point in each catalytic cycle.

*Relation of cation interactions to a transport mechanism*

Different experimental approaches have defined a variety of cation sites and conformational states of the $(Na^+ + K^+)$-ATPase and it is important to attempt to reconcile these operationally defined entities to the confines of a transport model.

In addition to the major $E_1$ and $E_2$ conformations, different forms of $E_2$ have been defined [12] in terms of their ability to interact with $K^+$. High concentrations of $Mg^{2+}$ transform the $K^+$-sensitive form to one that is $K^+$ insensitive. Transient kinetic studies have given some indication of the existence of a kinetically significant subsequent to the hydrolysis of $E_2P$ [23]. In the forward direction of the ATPase cycle, phosphate appears to dissociate before $K^+$ [24]. Thus the $K^+$-insensitive forms may be those containing bound $K^+$ and stabilized by the concomitant binding of $Mg^{2+}$ and/or phosphate.

In the presence of relatively high $Mg^{2+}$ concentrations, two different conformations have also been defined in terms of their different $K^+$ affinities as manifest by activation of the phosphatase [15]: the lower affinity "T" form is converted to the high affinity "R" form by $Na^+$, $K^+$, or phosphorylation. Employing different techniques, Robinson [25] has defined high-affinity "$\beta$" and lower affinity "$\alpha$" sites for $K^+$. In addition he has characterized the $K^+$ inhibition of phosphatase activity as resulting from an interaction at a very low affinity "$\gamma$" site [25] corresponding, in our formulation, to inhibition at high cation concentration due to the necessity of ordered association and dissociation.

Among the points to be considered are (i) the minimum number of classes of cation-binding sites that will accommodate the observations; (ii) a mechanism to account for the ligand interactions; (iii) a correlation among the conformational states defined by the different experimental approaches.

Discussion of models is hampered by uncertainty as to the subunit structure of the enzyme. It is reasonable to assume that the catalytic subunits exist as dimers [26,27]. ATP binding kinetics demonstrate high- and low-affinity binding sites [28,29]. Evidence for high- and low-affinity $Mg^{2+}$ sites also exists [30], as is reflected by the $Mg^{2+}$ activation of ADP-ATP exchange (see Table I). The parameters of $Mg^{2+}$ activation of the nitrophenylphosphatase appear to correspond to the low-affinity site of the ATPase. The two $Mg^{2+}$ sites may be functionally associated with the two ATP sites. Several proposals for "half-of-sites" mechanisms have been advanced on the basis of these observations [29,31,32]. In any event it appears that both $Mg^{2+}$ and MgATP are effective at the low-affinity $Mg^{2+}$ site [29].

Fig. 14 outlines relationships among cation binding events that appear to be consistent with both the ATPase and nitrophenylphosphatase reaction kinetics.

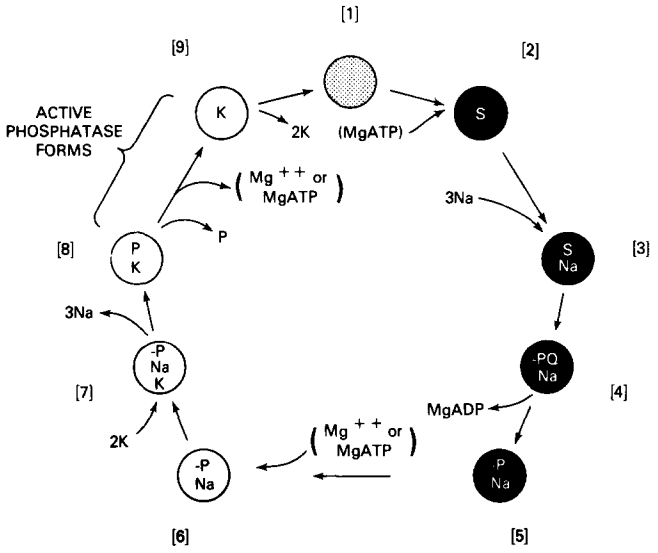


Fig. 14. Hypothetical sequence of cation binding in the ($Na^+ + K^+$)-ATPase and $K^+$-dependent phosphatase mechanisms. Major intermediates are indicated by circles: blck circles correspond to E 1 conformations; open circles correspond to $E_2$ conformations. The unliganded enzyme (step 1) probably exists as an equilibrium of both forms. Ligands shown within the reaction circle are intracellular. See text for further discussion.

The unliganded enzyme (1), may exist in equilibrium of $E_1$ and $E_2$ states: $K^+$ binding may stabilize $E_2$ and ATP may stabilize $E_1$ [15]. ATP binding may occur with or without $Mg^{2+}$ [8,9] and the order of $Na^+$ and ATP binding may be random [33]. Following phosphorylation to (4), ADP rapidly dissociates [37]. The equilibrium between $E_1P$ and $E_2P$ is regulated by the low-affinity $Mg^{2+}$ site (6). Because $Na^+$ concentration determines the level of phosphoenzyme, it is probable that $Na^+$ does not dissociate before $K^+$ is bound (7).

The role of $K^+$ in catalyzing hydrolysis of $E_2P$ is well-documented [34]. Evidence for a relatively stable complex (8) between $E_2$ and orthophosphate comes from the demonstrated reversal of the ATPase reaction [35], NMR studies [36], and transient kinetics [23,34]. The relative effects of $Rb^+$, $K^+$ and $Li^+$ on steady-state levels of EP and on rates of hydrolysis of $E_2P$ are taken to indicate that inorganic phosphate dissociates before $K^+$ (9) [24]. We propose that the nitrophenylphosphatase reaction consists of obligatory transitions between forms 8 and 9 of Fig. 14.

From the evidence given in this paper, $Mg^{2+}$, at the low-affinity site, appears to participate in a sequential association-dissociation cycle during the course of this cycle. In addition to being required for catalytic activity, $Mg^{2+}$ may stabilize the complex between $E_2$ and inorganic phosphate, (8), and thus inhibit the phosphatase at high concentrations.

## References

1 Albers, R.W. (1967) Annu. Rev. Biochem. 36, 727—756
2 Albers, R.W., Koval, G.J. and Siegel, G.J. (1968) Mol. Pharmacol. 4, 324—336
3 Glynn, I.M. and Karlish, S.J.P. (1975) Ann. Rev. Physiol. 37, 13—55

4 Fahn, S., Koval, G.J. and Albers, R.W. (1966) J. Biol. Chem. 241, 1882–1889
5 Fahn, S., Koval, G.J. and Albers, R.W. (1968) J. Biol. Chem. 243, 1993–2002
6 Stahl, W.L. (1968) J. Neurochem. 15, 499–509
7 Albers, R.W. and Koval, G.J. (1972) J. Biol. Chem. 247, 3088–3092
8 Hegyvary, C. and Post, R.L. (1971) J. Biol. Chem. 246, 5234–5240
9 Norby, J.G. and Jensen, J. (1971) Biochim. Biophys. Acta 233, 104–116
10 Robinson, J.D. (1971) Mol. Pharmacol. 7, 238–246
11 Post, R.L. (1973) in Mechanisms in Bioenergetics (Azzone, G.F., ed.), p. 203, Academic Press, New York
12 Post, R.L., Toda, G. and Rogers, F.N. (1975) J. Biol. Chem. 250, 691–701
13 Robinson, J.D. (1974) Biochim. Biophys. Acta 341, 232–247
14 Nakao, T., Tashima, Y., Nagano, K. and Nakao, M. (1965) Biochem. Biophys. Res. Commun. 19, 755–758
15 Swann, A.C. and Albers, R.W. (1975) Biochim. Biophys. Acta 382, 437–456
16 Knott, A.D. and Reese, D.K. (1972) Proc. On-Line Conf. 1, 497
17 Nagai, K., Izumi, F. and Yoshida, H. (1966) J. Biochem. 59, 295–303
18 Skou, J.C. (1974) Biochim. Biophys. Acta 339, 234–245
19 Skou, J.C. (1974) Biochim. Biophys. Acta 339, 246–257
20 Skou, J.C. (1974) Biochim. Biophys. Acta 339, 258–273
21 Cleland, W.W. (1963) Biochim. Biophys. Acta 67, 104–172
22 Albers, R.W. and Koval, G.J. (1973) J. Biol. Chem. 248, 777–784
23 Froehlich, J.P., Albers, R.W., Koval, G.J., Goebel, R. and Berman, M. (1976) J. Biol. Chem. 251, 2186–2188
24 Post, R.L., Hegyvary, G. and Kume, S. (1972) J. Biol. Chem. 247, 6530–6540
25 Robinson, J.D. (1975) Biochim. Biophys. Acta 384, 250–264
26 Dahl, J.L. and Hokin, L.E. (1974) Annu. Rev. Biochem. 43, 327–356
27 Kyte, J. (1972) J. Biol. Chem. 247, 7642–7649
28 Neufeld, A.H. and Levy, H.M. (1969) J. Biol. Chem. 244, 6493–6497
29 Robinson, J.D. (1976) Biochim. Biophys. Acta 429, 1006–1019
30 Fahn, S., Hurley, M.R., Koval, G.J. and Albers, R.W. (1966) J. Biol. Chem. 241, 1890–1895
31 Repke, K.R.H. and Schoen, R. (1973) Acta Biol. Med. Ger. 31, K19–30
32 Stein, W.D., Lieb, W.R., Karlish, S.J.D. and Eilam, Y. (1973) Proc. Natl. Acad. Sci. U.S. 70, 275–278
33 Mardh, S. and Post, R.L. (1977) J. Biol. Chem. 252, 633–638
34 Mardh, S. (1975) Biochim. Biophys. Acta 391, 448–463
35 Tanaguchi, K. and Post, R.L. (1975) J. Biol. Chem. 250, 3010–3018
36 Grisham, C.H. and Mildvan, A.S. (1974) J. Biol. Chem. 249, 3187–3197
37 Karlish, S.J.P., Yates, D.W. and Glynn, I.M. (1976) Nature 263, 251–253