The binding affinity for ATP was high and estimation of binding was without interference from other sites on the membranes. The results demonstrate an antagonism between Na\(^+\) and K\(^+\) in the control of binding of ATP, explain some anomalies of kinetics, and indicate the relative importance of various parts of the ATP molecule for binding.

**METHODS**

**Materials**—Membranes containing (Na\(^+\) + K\(^+\))-ATPase from guinea pig kidney cortex, and [\(\gamma\)-\(^32\)P]ATP were prepared according to the methods of Post and Sen (5, 6). The enzyme was purified with NaI by the following modification of the method of Nakao et al. (7). The NaI solution contained 4 mM NaI, 4 mM H\(_2\)Na\(_2\)ATP, 1 mM \(\beta\)-mercaptoethanol, and 5 mM H\(_2\)NaEDTA. The pH was adjusted to 8.2 with solid Tris. At 0° equal volumes of this solution and of the membrane suspension (4 to 6 mg of protein per ml in 10 mM imidazole-HCl buffer, pH 7.4) were mixed and allowed to stand for 45 min. Then the membranes were washed three times by centrifugation and resuspension in 10 mM imidazole-HCl buffer (pH 7.4). They were finally resuspended in 30 mM Tris-CDTA buffer (pH 7.6) containing 1 mM \(\beta\)-mercaptoethanol. This latter buffer was used throughout the experiments if not otherwise indicated. The specific activity of the enzyme was about 1 to 2 units per mg of protein and the sensitivity to (Na\(^+\) + K\(^+\)) was about 98%. Only membranes treated with NaI were used in all of the experiments.

\(\beta,\gamma\)-Methylene ATP and \(\alpha,\beta\)-methylene ADP were obtained from Miles Laboratories, Kankakee, Illinois. Other nucleotides were obtained from Sigma. CDTA was obtained from Matheson, Coleman and Bell, Norwood, Ohio.

**Binding Measurements**—Unless otherwise stated, binding of [\(^32\)P]ATP to membranes was estimated in the flow-dialysis apparatus of Colowick and Womack (8), purchased from Bel-Art Products, Pequannock, New Jersey. Two sizes of dialysis cell were used in different experiments. The upper dialysis cell (0.5 or 1.0 ml) contained about 2 mg of membrane protein and 0.2 to 0.5 \(\mu\)M [\(^32\)P]ATP, containing about 7 \(\times\) 10\(^5\) cpm. The membrane between the chambers was Visking cellulose dialysis casing. Buffer at 0° was pumped through the lower chamber (1.0 or 3.0 ml). Ten fractions of the effluent were collected after each addition to the upper chamber. Aliquots from the last 3 even-numbered fractions in each group of 10 were counted in a thin window counter. In intervening aliquots, hydrolysis of [\(^32\)P]ATP was estimated by adsorption of ATP on charcoal (9). The counts in the even-numbered fractions were corrected.

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The dissociation constant was 0.22 for one, to the phosphorylation capacity of this enzyme, (b) and potassium ion-stimulated adenosine triphosphatase (a) because binding capacity of the membranes was equal, one for one, to the phosphorylation capacity of this enzyme, (b) because binding was inhibited by pretreatment of the membranes with the specific inhibitor, ouabain, and (c) because binding was inhibited by potassium ion with antagonism of this inhibition by sodium ion, which had no direct effect. The dissociation constant was 0.22 \(\mu\)M. Other nucleotides displaced ATP including, in order of decreasing effectiveness: deoxyadenosine triphosphate, \(\beta,\gamma\)-methylene adenosine triphosphate, cytidine triphosphate, and inosine triphosphate. Monovalent cations reduced the binding affinity at progressively higher cation concentrations, as follows: potassium, rubidium, thallous, ammonium, and cesium ions. Lithium ion had no effect. Binding was stable between pH 5.6 and 7.6, but declined sharply above pH 8.0. In the presence of potassium ion alone, there appeared to be one or more binding sites on this enzyme with a much lower affinity for adenosine triphosphate. These may be regulatory sites.

The activity of the sodium and potassium ion pump in plasma membranes remains as a (Na\(^+\) + K\(^+\))-stimulated ATPase after isolation of the disrupted membranes (1-3). The reaction sequence of (Na\(^+\) + K\(^+\))-ATPase includes Na\(^+\)-dependent phosphorylation and K\(^+\)-dependent dephosphorylation of a carboxyl group on a protein (1, 2, 4). This paper reports studies of an earlier step in the reaction sequence, the binding of ATP.

* This work was supported by Grant 5R01 HE-01974 from the National Heart and Lung Institute, National Institutes of Health, United States Public Health Service.

1. The abbreviations used are: (Na\(^+\) + K\(^+\))-ATPase, sodium and potassium ion-stimulated adenosine triphosphatase; CDTA, (1,2-cyclohexylenedinitrilo)tetraacetic acid; 3',5'-cyclic AMP, cyclic adenosine 3',5'-monophosphate.
for 3 to 8% hydrolysis. At the end of each experiment a large excess of unlabeled ATP was added to the upper dialysis cell to drive off all the [32P]ATP from the binding sites, so that the radioactivity in the effluent corresponded then to 100% free [32P]ATP. At the end of an experiment less than 2% of the total [32P]ATP had been lost from the upper chamber.

ATP binding was also measured by a rapid mixing-centrifuging method. Membranes were suspended at 0° in 30 mM Tris-CDTA (pH 7.6) containing K+, or no cation in the control. Of this suspension, 1.0 ml was mixed with 10 μl of [32P]ATP (Tris salt) to give a final concentration of 3 to 15 μM and was centrifuged immediately at 90,000 × g for 20 min. After careful removal of the supernatant by decantation and wiping the wall of the container, the sediment was resuspended in fresh chloroform-methanol-formic acid (4:2:1) and an aliquot was dried and counted, giving the amount of [32P]ATP in the sediment. In order to estimate unbound [32P]ATP in the sediment, unlabeled ATP was added to control tubes to a final concentration of 10 mM prior to addition of labeled ATP. Counts in this sediment came almost entirely from unbound ATP, since the fraction of nucleotides bound was negligibly small; unbound ATP was subtracted from total ATP to obtain bound ATP. The total amount of free ATP was estimated by subtraction of the bound ATP from the total amount of ATP added. In the presence of Na+, this method was unsatisfactory probably because too much 32P was split from the [32P]ATP and remained adsorbed on the membranes.

Preparation of (Na+ + K+)-ATPase Inhibited by Ouabain. Ouabain was bound to the enzyme before flow dialysis. The membranes were incubated in a solution containing 0.4 mM MgCl2, 1.0 mM Tris-PO4 (pH 7.4) and 1 mM ouabain overnight at 4°. Prior to the ATP binding, the membranes were centrifuged down, resuspended in 30 mM Tris-CDTA (pH 7.6) containing 1 mM ouabain at 0°. This ouabain-Tris-CDTA buffer was also pumped through the lower chamber of the dialysis cell. To dissociate ouabain from the enzyme, the membranes were centrifuged down and incubated at 37° for 20 min in ouabain-free Tris-CDTA buffer (10, 11).

Phosphorylation Capacity of (Na+ + K+)-ATPase—The procedure was that of Post and Sen (6) carried out at 0°. To correct for the loss of radioactivity due to repeated washings and spontaneous splitting, three sets of four parallel samples each were washed one, two, or three times, respectively. For each set the time between phosphorylation and the end of washing was recorded. Extrapolation to zero time gave the initial amount of phospho-enzyme. Each washing cycle required about 100 min and the loss of phospho-enzyme was about 4% per hour.

Measurement of (Na+ + K+)-ATPase Activity and Protein—One milliliter of incubation medium for estimating total ATPase activity contained 100 μmoles of NaCl, 25 μmoles of KCl, 4 μmoles of MgCl2, 4 μmoles of ATP, and 10 to 20 μg of membrane protein in 30 mM imidazole glycylglycine (pH 7.4). The nucleotides were added as the Tris salts. Another similar medium contained 0.5 μmoles of ouabain in place of NaCl and KCl to estimate (Na+ + K+)-insensitive ATPase activity. (Na+ + K+)-ATPase activity was the difference between these activities. The reaction was terminated after 20 min at 37° by adding an equal volume of 0.8% sodium dodecyl sulfate. This reagent has two advantages. First, it denatures the enzyme at neutral pH, at which ATP is stable for hours, and second, it completely dissolves the particulate membranes and prevents clogging of the tubing of the Technicon AutoAnalyzer. Inorganic phosphate was determined on a Technicon AutoAnalyzer by the method of Yee (12). One unit of enzyme splits 1 μmole of ATP per min at 37°.

Protein was estimated by Miller’s modification (13) of the method of Lowry et al. (14).

RESULTS

Dissociation Constant of ATP Binding Site on Membranes—The dissociation constant for a ligand is usually determined from plotting binding data according to Scatchard (15) or Klotz (16). The flow dialysis method of Colowick and Womack (8) yields the necessary data in one short procedure with one enzyme sample and a varying ligand concentration (Fig. 1). The data of ATP binding to membranes fell on a straight line in a Scatchard plot (Fig. 2). There was a slight deviation from linearity at the highest ATP concentration possibly due to binding at another site. There was one type of site with a dissociation constant, K, for ATP equal to 2.2 × 10−7 M (S.D. = ±0.66 × 10−7, nine experiments). The high concentration of Tris-CDTA minimized splitting of ATP during the experiment. CDTA itself did not influence binding of ATP over a concentration range of 5 to 50 mM. At 30 mM CDTA less than 8% of the ATP was split at the end of the experiments.

Ratio of ATP Binding Site to (Na+ + K+)-ATPase Phosphorylation Capacity—The intercept of a Scatchard plot on the abscissa yields the concentration of a binding site. In order to relate binding to (Na+ + K+)-ATPase the quantity of the site was compared with the quantity of (Na+ + K+)-ATPase phospho-protein. The quantity of the phospho-enzyme was corrected for loss during isolation. The ratio of ATP binding site per mg of membrane protein to the quantity of 32P on the phos-
Fig. 2. Scatchard plot of ATP binding data obtained by flow dialysis in the absence of alkali metal and free magnesium ions. The upper dialysis cell contained 4.5 mg of membrane protein in 1 ml of buffer. From the slope the dissociation constant, $K$, was 0.29 µM. From the intercept on the abscissa the amount of binding was 263 pmoles of ATP per mg of protein.

Inhibition of ATP Binding by Ouabain—Cardioactive steroids such as ouabain are highly specific inhibitors of (Na$^+$ + K$^+$)-ATPase. When ouabain was bound to (Na$^+$ + K$^+$)-ATPase by an established procedure (10, 11), the binding of ATP to the membranes was prevented. When ouabain was dissociated from the enzyme by warming in the absence of Mg$^{++}$ (10, 11), the binding of ATP was partially restored (Fig. 3). Perhaps restoration was incomplete because binding of ouabain made the enzyme unstable.

Differential Action of Na$^+$ and K$^+$ on Binding of ATP—Addition of Na$^+$ alone increased binding slightly (Table I). Estimation of the dissociation constant for ATP in the presence of 50 or 100 mM NaCl showed no significant change from a control in the absence of NaCl. Addition of K$^+$ alone at a concentration of 3 mM drove ATP off the binding site. Further addition of Na$^+$ reversed the action of K$^+$ and allowed ATP to return to the binding site (Fig. 4). A 1.3-fold excess of Na$^+$ concentration over K$^+$ concentration restored binding to half of the maximal level at an ATP concentration of 0.63 µM. Li$^+$ up to a concentration of 140 mM did not substitute for Na$^+$ in producing this effect (Fig. 5).

Comparison of Monovalent Inorganic Cations—In the stimulation of (Na$^+$ + K$^+$)-ATPase, Na$^+$ is unique and all other alkali metal cations substitute for K$^+$, as do also NH$_4^+$ and Tl$^+$. In binding experiments Li$^+$ had no effect over a concentration range
The apparent inhibitory cation dissociation constant decreased with membrane preparation injected into the apparatus. In all cases the increasing ion concentration, probably because of multiple, 6) was due to differences in the amount of the viscous membrane preparation analogous to that used later in Table II. In this calculation it was calculated for a single binding site according to an equation for calculating. 5 to 160 mM. At high enough concentrations all cations. KC1 (6 mM) was added to dissociate the ATP-enzyme complex. Then LiCl or KC1 was added progressively to obtain total cation concentrations as indicated. The rate of dialysis of free [32P]ATP was estimated by measuring the appearance of radioactivity in the dialysate in a steady state. The concentration of ions was identical on both sides of the membrane. For further details see “Methods.” High concentrations of both ions produced a similar slight restoration of binding. The 10% reduction in the dialysis rate at the end of the experiment might be due to the increase in ionic strength (compare Table I).

**FIG. 6.** Effect of monovalent cations, except Na+, on ATP binding to kidney membranes. The total concentration of [32P]ATP was 0.22 μM in all experiments. The same membrane preparation was used in the same concentration with every ion except Tl+. With Tl+, chloride was replaced by acetate. For further details see “Methods.” ▲—▲, Tl+; ○—○, Rb+; O—O, K+; @—@, NH4+; A—A, Cs+; X—X, Li+.

**FIG. 5.** Insensitivity to Li+ of K+ inhibition of ATP binding. Membrane protein (2.4 mg) in 1 ml of CDTA buffer was equilibrated with 100 pmoles of [32P]ATP in the absence of inorganic cations. KC1 (6 mM) was added to dissociate the ATP-enzyme complex. Then LiCl or KC1 was added progressively to obtain total cation concentrations as indicated. The rate of dialysis of free [32P]ATP was estimated by measuring the appearance of radioactivity in the dialysate in a steady state. The concentration of ions was identical on both sides of the membrane. For further details see “Methods.” High concentrations of both ions produced a similar slight restoration of binding. The 10% reduction in the dialysis rate at the end of the experiment might be due to the increase in ionic strength (compare Table I).

**Table II**

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Dissociation constant</th>
<th>Relative activity of (Na+ + K+)-dependent nucleotidase %</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>2 x 10^-4</td>
<td>100</td>
</tr>
<tr>
<td>ADP</td>
<td>2 x 10^-4</td>
<td>49</td>
</tr>
<tr>
<td>dATP</td>
<td>1 x 10^-3</td>
<td>2.3</td>
</tr>
<tr>
<td>α,β-Methylene ATP</td>
<td>2 x 10^-4</td>
<td>&lt;0.6</td>
</tr>
<tr>
<td>CTp</td>
<td>6 x 10^-6</td>
<td>2.3</td>
</tr>
<tr>
<td>CDp</td>
<td>7 x 10^-6</td>
<td>&lt;0.6</td>
</tr>
<tr>
<td>TTP</td>
<td>3 x 10^-4</td>
<td>2.4</td>
</tr>
<tr>
<td>GTP</td>
<td>4 x 10^-4</td>
<td>&lt;0.6</td>
</tr>
<tr>
<td>UTP</td>
<td>1 x 10^-4</td>
<td>2.4</td>
</tr>
<tr>
<td>AMP</td>
<td>6 x 10^-4</td>
<td>&lt;0.6</td>
</tr>
<tr>
<td>AMP</td>
<td>6 x 10^-4</td>
<td>&lt;0.6</td>
</tr>
<tr>
<td>3',5'-cyclic AMP</td>
<td>&gt;5 x 10^-4</td>
<td></td>
</tr>
</tbody>
</table>

**ATP Binding in Presence of K+**—In order to estimate the affinity of the membranes for ATP in the presence of a saturating concentration of K+, it was necessary to use much higher concentrations of ATP. Under these conditions, a centrifugation method became preferable for measuring the small fraction of ATP bound. In the presence of K+, sites with a much lower affinity for ATP were indistinctly detectable. The number of these sites appeared to be greater than in the absence of K+. Binding at these sites also was greatly reduced by pretreatment of the (Na+ + K+)-ATPase with ouabain as above (Fig. 7). The affinity for ATP in the absence of K+ was about 2- to 3-fold cooperative binding sites. The range of these constants was K+ and Rb+ > 0.1 to 0.4 mM, Tl+ > 0.2 to 0.6 mM, NH4+ > 1.0 to 3.5 mM, and Cs+ > 3 to 6 mM. The relative effectiveness of these ions in inhibiting (Na+ + K+)-ATPase is similar (compare Fig 2 in Reference 18). Tl+ is not a strong inhibitor (compare Fig. 1 in Reference 19). At high concentrations these ions inhibit the activity of the enzyme by competing with Na+. In these experiments no Na+ was present. For dephosphorylation of (Na1 + K1)-ATPase the relative sequence of apparent affinities is different, namely, Tl+ > Rb+ > K+ > Cs+ > NH4+ > Li+.^2

[^2]: R. L. Post, unpublished experiments.

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C. Hegyvary and R. L. Post 5237

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5238 Binding of ATP to (Na$^+$ + K$^+$)-ATPase

Vol. 246, No. 17

Fig. 7. Scatchard plot of binding of ATP to membranes in the presence of K$^+$. Data were obtained by the mixing-centrifuging method. Each sample contained 1.3 mg of protein in 30 mM Tris-CDTA (pH 7.24). Ouabain was bound to the membranes beforehand by the procedure described under “Methods.” Control, membranes with 2 mM Tris-PO$_4$ and without inorganic cations (△△); membranes in 100 mM KCl (□□□); ouabain-treated membranes, control (△△△); ouabain-treated membranes in 100 mM KCl (○○○). The binding of ouabain to (Na$^+$ + K$^+$)-ATPase was probably incomplete. The dissociation constant in the absence of inorganic cations was 0.6 μM; in the presence of K$^+$ it was about 30 μM. The number of binding sites appears to be about 2-fold greater in the presence of K$^+$ than in its absence.

Fig. 8. Competition between [32P]ATP and other nucleotides (unlabeled) for binding to a single membrane preparation. In each experiment first 0.7 μmole of [32P]ATP was added to 4.3 mg of membrane protein in a volume of 1 ml. Then increasing amounts of an unlabeled nucleotide were added. The fraction of [32P]ATP bound (ordinate) is plotted against the concentration of the unlabeled nucleotide (abscissa). 6-6, ATP; □□□, ADP; △△△, dATP; ○○○, α,β-methylene ATP; ▲▲▲, dADP; #---¤, α,β-methylene ADP; CTP; ITP; GTP; UTP; TTP; AMP; and 3',5'-cyclic AMP (Fig. 8). The dissociation constant for binding of each nucleotide was calculated and the relative (Na$^+$ + K$^+$)-dependent nucleotide triphosphatase activity of the same membrane preparation was estimated. All nucleotides which were substrates for (Na$^+$ + K$^+$)-nucleotidase displaced ATP from the binding site (Table II). Inorganic phosphate up to a concentration of 20 mM had no effect on ATP binding.

ATP binding did not change between pH 5.8 and pH 7.6, but sharply decreased above pH 8.0 (Fig. 9).

Exposure to heat or digestion with phospholipase A (20) or trypsin (21) inactivated both (Na$^+$ + K$^+$)-ATPase activity and ATP binding completely (not shown).

DISCUSSION

These kidney membranes had a site which bound ATP with high affinity (Figs. 1 and 2). The site was on (Na$^+$ + K$^+$)-ATPase, since (a) the quantity of binding sites was the same as the capacity to form the phospho-protein of (Na$^+$ + K$^+$)-ATPase (“Results”), (b) binding was inhibited by pretreatment with ouabain, a specific inhibitor of (Na$^+$ + K$^+$)-ATPase (“Results”), (c) binding was inhibited by K$^+$ with antagonism of this inhibition by Na$^+$ (Fig. 4), and (d) monovalent cation specificity in inhibition of binding corresponded to specificity in the inhibition of (Na$^+$ + K$^+$)-ATPase (Fig. 6). Furthermore, no nucleotide which is a substitute for ATP in (Na$^+$ + K$^+$)-ATPase failed to bind (Table II) and inactivation of (Na$^+$ + K$^+$)-ATPase by heating or digestion with phospholipase A or trypsin abolished binding.

Influence of Nucleotide Structure on Binding—The group most influential for binding was the amino group in position 6 of the
purine ring. Replacement of this group with a hydroxyl group reduced the affinity about 1500-fold (compare ATP with ITP in Table II). In addition, displacement of the purine ring with a pyrimidine ring (compare ATP with CTP) or loss of the β-phosphate (compare ADP with AMP) reduced the affinity about 100-fold (compare ATP with ADP). Such information may be helpful in the design of a system for purification of this enzyme by affinity chromatography.

**Relationship between Binding Affinity and Effectiveness of Nucleotides as Substrates**—The relationship is still too complex for effective analysis. Complexity is shown by considerations of the following sort. In Table II the activity estimations were made at a nucleotide concentration of 4 mM with 4 mM MgCl₂, 100 mM NaCl, and 25 mM KCl. The binding data suggest that ITP should be bound under these conditions; the effect of Mg⁺⁺ is not predictable but the ratio of Na⁺⁺ to K⁺⁺ is relatively favorable and the dissociation constant is less than the added nucleotide concentration by a factor of 10. Finally, ITP can phosphorylate the enzyme (22, 23). However, the rate of splitting of ITP was only 2.4% of that of ATP. As further evidence of complexity it may be pointed out that in the presence of a low concentration of Na⁺⁺ ATP can stimulate the K⁺⁺-dependent p-nitrophenyl phosphatase activity of this enzyme, although at high concentrations both ATP and Na⁺⁺ are inhibitors (2). There may well be several steps in the reaction sequence or several kinds of sites on the enzyme at which nucleotides act (1, 2).

**Relationship of Metal Ions to ATP Binding**—Highly effective binding in the absence of Mg⁺⁺, as in these experiments, denies an earlier hypothesis, based on kinetic evidence (3), that the complex of Mg⁺⁺ with ATP is the only true substrate. In the kinetic experiments inhibition by free ATP or free Mg⁺⁺ requires other explanations. That ATP bound in the absence of Mg⁺⁺ is a precursor of the phospho-enzyme is shown by a pulse experiment (Fig. 14 in Reference 24). In this experiment [¹⁴C]ATP was added to the enzyme in the presence of Na⁺⁺ and absence of Mg⁺⁺. Following a chase of unlabeled ATP and the missing Mg⁺⁺, a transient pulse of [¹⁴C]phospho-enzyme appeared.

K⁺ and Na⁺ control the binding of ATP through their action on the enzyme rather than through formation of complexes with ATP. The dissociation constants of K⁺-ATP and Na⁺-ATP are the same and are about 4.4 mM (25). This concentration is higher than concentrations of 0.9 mM Na⁺⁺ and 0.9 mM K⁺⁺ which influenced binding to the enzyme in Fig. 4. The action of Li⁺⁺ is more decisive, however. The dissociation constant of Li⁺-ATP appears to be about 6-fold lower than that of the other two ions (26). Suppose that K⁺ removes ATP from the enzyme by forming K⁺-ATP, which would not be bound. Then excess Li⁺⁺ will easily displace K⁺ from ATP to form Li⁺⁺-ATP. Li⁺⁺ alone did not influence binding (Fig. 6), so that Li⁺⁺ should have antagonized the dissociating action of K⁺ according to this hypothesis. Yet addition of Li⁺⁺ to an ATP enzyme complex previously dissociated with K⁺ had only the same slight tendency to favor recombination as did the addition of an equivalent concentration of K⁺ in place of Li⁺⁺ (Fig. 5). Consequently the action of K⁺ is on the enzyme and not on the ATP. It is likely that Na⁺ and K⁺ induce alternative conformations of the dephospho-enzyme with multiple differences in properties as illustrated by sensitivity to inhibition by Be⁺⁺ (27, 28). K⁺ enhanced sensitivity to Be⁺⁺ and Na⁺ diminished it.

The low affinity binding of ATP in the presence of K⁺ (Fig. 7) may help to explain some kinetic anomalies. The $K_m$ with respect to ATP for (Na⁺⁺ + K⁺⁺)-ATPase activity increases with increasing concentration of K⁺ (29). In the usual assay system it is about 0.3 mM (4), which is about 1000-fold higher than the dissociation constant for binding in Table II or the $K_m$ for phosphorylation (4) in the absence of K⁺⁺. The $K_m$ for (Na⁺⁺ + K⁺⁺)-ATPase activity is only about 10-fold higher than that for ATP binding to the presence of K⁺ (Fig. 7). The high $K_m$ suggests that ATP ordinarily combines with a K⁺ form of the enzyme and that disassociation of K⁺ is a rate-limiting step in (Na⁺⁺ + K⁺⁺)-ATPase activity. This is because K⁺ must dissociate before Na⁺ can combine with the enzyme and catalyze transphosphorylation (30). The low ATP affinity of the K⁺ form of the enzyme also helps to explain inhibition of Na⁺⁺-sensitive ATPase by K⁺ (34–35). With the ATP concentration reduced to about 1 μM, a ouabain-sensitive Na⁺⁺-dependent ATPase activity persists, which is partially inhibited by K⁺. In this case inhibition due to impairment of binding of ATP overrides acceleration of dephosphorylation. Czerwinski, Gitelman, and Welt (32) and Neufeld and Levy (33, 34) preferred to interpret the phenomenon as that of a separate enzyme. Kanazawa, Saito, and Tomomura (36) included inhibition by K⁺ in a reaction sequence for a single enzyme on the basis of transient kinetic experiments of phosphorylation and dephosphorylation.

**Possible Feature in Mechanism of Binding**—Levels of pH above 8 impaired binding of ATP at a low concentration without corresponding inhibition of activity at a high concentration (Fig. 9). ATP itself does not have an ionizable group in this range of pH. On the enzyme protein a suitable group would be an alpha-aminogroup. In other proteins such groups participate in conformational changes associated with ligand binding, for instance, in hemoglobin (37) and in chymotrypsin (38).

**Possible Function of Control of ATP Binding in Cell Physiology**—Control of ATP binding by K⁺, specifically by intracellular K⁺, may adjust the distribution of ATP consumption under conditions of a poor supply of ATP, such as anoxia or cell injury. When the concentration of ATP falls, the ion pump consumes less ATP as long as the intracellular K⁺ concentration remains high. A high K⁺ concentration is necessary for high gradients of K⁺ and Na⁺ across the plasma membrane and these gradients energize the control of cell volume (39) and other functions. When the gradient is dissipated and the cell is in danger of osmotic lysis, then the low concentration of K⁺ allows the ion pump to bind ATP and to function again slowly to prevent death of the cell by lysis.

**Addendum**—After this paper was submitted for publication, similar papers by Norby and Jensen appeared (40, 41). Our experiments confirm their estimation of a binding site with a dissociation constant of 0.12 μM, which increased in the presence of K⁺. Our estimate of the increase was greater than theirs probably because of less interference from hydrolysis of ATP in our experiments. Our experiments also support their conclusion that the 6-amino group of the purine ring and the beta-phosphate group in ATP are essential for tight binding.

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