Further Studies on the Activation of Acetic Thiokinase by Magnesium and Univalent Cations [1, 2]

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The activation of acetate by acetic thiokinase has been shown to proceed through 2 steps (2, 18):

ATP + acetate $\rightleftharpoons$ Adenyl acetate + PP_i I

Adenyl acetate + CoA $\rightleftharpoons$ Acetyl CoA + AMP II

Von Korff (16) studied the over-all reaction and reported that univalent cations were required for the synthesis of acetyl CoA from ATP, acetate and CoA. K^+, Rb^+, and NH_4^+ at concentrations of 0.04 M gave maximum activity while Na^+ and Li^+ strongly inhibited the reaction. Hiatt and Evans (6) reported a similar effect of univalent cations upon the overall reaction catalyzed by acetic thiokinase from plants. The inhibitory effect of Na^+ and Li^+ was not due to competition between these ions and activating univalent cations (6). Pyruvate kinase from animal tissues (8), formate activating enzyme from erythrocytes (4), yeast aldehyde dehydrogenase (5), and phosphotransacetylase (15) are other enzymes which are activated by K^+ but are inhibited by Na^+. Incorporation of amino acids into protein by cell-free extracts of pea roots is promoted by K^+ and inhibited by Na^+, Li^+, NH_4^+, and Rb^+ (17). The nature of the Na^+ inhibition of these enzymes was not studied in detail.

The overall acetic thiokinase reaction requires Mg^{++} (1, 7, 12) and Berg (2) reported that Mg^{++} is required for only the first step in the reaction sequence. The effect of univalent cations on the individual steps in the reaction sequence has not been studied with either plant or animal acetic thiokinase.

In this paper experimentation on the effect of cations on acetic thiokinase has been extended to study their effect on individual steps of the reaction sequence. It was considered that these studies would present a better understanding of the role of univalent cations in enzyme reactions and of the antagonism between Na^+ and activating cations in certain enzyme systems.

Materials and Methods

Enzyme Extracts. Acetic thiokinase from *Spinacia oleracea* L. and condensing enzyme from pig heart were prepared as previously reported (6). Glucose-6-phosphate dehydrogenase and yeast hexokinase were purchased from Nutritional Biochemicals Corporation and were dialyzed for 12 hours against 0.001 M Tris buffer at pH 7.4 before use.

Other Materials. ATP, CoA (75% purity), TPN, and oxaloacetic acid were obtained from the Nutritional Biochemicals Corporation. DPN and AMP were obtained from the Sigma Chemical Company. Adenyl acetate was prepared from AMP and acetic anhydride according to the procedure described by Berg (3) except that the pH was adjusted with solid Tris instead of KOH. Other chemicals were of reagent grade and were obtained from commercial sources. The Tris salts of DPN, ATP, and PP_i were prepared by passing solutions of the sodium salts of these compounds through an exchange column containing Dowex 50 resin (Tris cycle). Tris salts of other reagents were prepared by neutralizing the respective acids to pH 7.5 with Tris.

Standard Assay Procedures. The overall reaction catalyzed by acetic thiokinase was assayed by measuring the synthesis of citrate in a system involving the coupling of acetic thiokinase with condensing enzyme. The standard reaction mixture in a volume of 1 ml contained the following constituents: 100 μmoles Tris buffer adjusted to pH 8.0 with HCl; 10 μmoles Tris acetate; 2.5 μmoles $MgCl_2$; 50 μmoles KCl; 1.5 μmoles Tris oxaloacetate; 5 μmoles ATP; 0.1 μmole CoA; 20 to 30 units of condensing enzyme and acetic thiokinase extract usually containing between 1 and 2 mg of protein. The reaction mixture was incubated for 20 minutes at 30° and the reaction was stopped by adding 1 ml of 15% trichloroacetic acid. Denatured protein was removed by centrifugation and citrate was determined by the method of Natelson et al. (13). A control containing no CoA was carried through the procedure.

The first and second steps in the reaction sequence were assayed by measuring the conversion of adenyl acetate to ATP and acetyl CoA, respectively. Since adenyl acetate is apparently tightly bound to the enzyme (18), the first step in the reaction (equation I) could not be assayed by measuring adenyl acetate formation from ATP. The conversion of adenyl acetate to ATP (equation I) was assayed by measuring the reduction of TPN spectrophotometrically in a system coupling ATP synthesis with hexokinase and glucose-6-phosphate dehydrogenase essentially as described by Berg (2). The standard reaction mixture in a volume of 1 ml contained the following

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constituents: 100 μmoles Tris buffer, pH 7.4; 5 μmoles $MgCl_2$; 10 μmoles Tris PP_i; 0.5 μmole adenyl acetate; 25 μmoles glucose; 0.5 μmole TPN; 0.3 mg hexokinase; 4 μg glucose-6-phosphate dehydrogenase; and acetic thiokinase extract containing 1 to 2 mg of protein. The reaction mixture was incubated for 15 minutes at 30° and the reaction was stopped by adding 5 ml of 0.5 M sodium phosphate buffer, pH 7.2. TPN reduction was determined with a Beckman DU spectrophotometer at a wavelength of 340 mμ and activity is expressed as change in optical density at this wavelength. A reaction mixture containing no adenyl acetate was used as a control. The quantity of hexokinase and glucose-6-phosphate dehydrogenase present was 20 to 30 times the amount required to insure that acetic thiokinase was the limiting enzyme. The rate of utilization of ATP by hexokinase and glucose-6-phosphate dehydrogenase was unaffected by Na^+, K^+, and adenyl acetate. A 25% inhibition of ATP utilization by 10^{-2} M Tris pyrophosphate did not affect the assay of acetic thiokinase by the coupled system.

The conversion of adenyl acetate and CoA to acetyl CoA (equation II) was assayed by coupling acetyl CoA synthesis with condensing enzyme and measuring the formation of citrate. The standard reaction mixture in a volume of 1 ml contained the following constituents: 100 μmoles Tris buffer, pH 7.4; 50 μmoles KCl; 1.5 μmoles Tris oxaloacetate; 0.1 μmole CoA; 2 μmoles adenyl acetate; 20 to 30 units of condensing enzyme and acetic thiokinase extract containing 2 to 4 mg of protein. The mixture was incubated for 10 minutes at 30° and the reaction was stopped by adding 1 ml of 15% trichloroacetic acid. After centrifugation to remove denatured protein, a 1-ml aliquot was assayed for citrate by the method of Natelson et al. (13).

Hexokinase was assayed by measuring TPN reduction in a system coupling glucose-6-phosphate synthesis from glucose and ATP with glucose-6-phosphate dehydrogenase. The cuvette contained the following constituents in a volume of 3.0 ml: 200 μmoles Tris buffer, pH 7.4; 2.5 μmoles $MgCl_2$; 50 μmoles glucose; 0.5 μmole TPN; 5 μmoles ATP; 1 μg glucose-6-phosphate dehydrogenase; and hexokinase. The reaction was started by adding ATP and the optical density change at a wavelength of 340 mμ was followed with a Beckman DU spectrophotometer. Glucose-6-phosphate dehydrogenase was assayed by the same procedure except that hexokinase was present in excess.

Condensing enzyme was assayed according to the optical procedure of Ochoa (14). One unit of condensing enzyme is defined as that amount which gives an optical density change of 0.01 per minute under standard assay conditions. Protein was determined by the Folin-phenol method of Lowry et al. (11) using bovine albumin as the standard.

Results

Effects of Adenyl Acetate and Pyrophosphate Concentration on ATP Synthesis. Maximum enzyme activity was obtained with an adenyl acetate concentration of 5×10^{-4} M (fig 1). Although not shown in figure 1, enzyme activity was reduced markedly by adenyl acetate at concentrations greater than 10^{-3} M. A PP_i concentration of 10^{-2} M was optimum for the enzyme and an excess of PP_i inhibited the reaction.

Magnesium Requirement for ATP Synthesis. The effect of Mg^{++} on ATP synthesis from adenyl acetate and PP_i is shown in figure 2. Separate experiments indicated that hexokinase and glucose-6-phosphate were present in such excess quantities that their activity did not limit the reaction rate at Mg^{++} concentrations greater than 2.5×10^{-4} M. The results shown in figure 2 are, therefore, due only to the effect of Mg^{++} on ATP synthesis. Maximum activity was produced by a Mg^{++} concentration of 5×10^{-3} M. Increasing PP_i to twice the optimum concentration resulted in a twofold increase in the concentration of Mg^{++} required for maximum activity.

Univalent Cation Inhibition of ATP Synthesis. Univalent cations inhibited the synthesis of ATP from adenyl acetate and PP_i (fig 3). In the presence of 2.5×10^{-3} M Mg^{++}, NaCl at concentrations of 0.1 M and 0.2 M inhibited enzyme activity by 55% and 70%, respectively. Potassium inhibited the reaction less than sodium.

The effect of 0.1 M NaCl and 0.1 M KCl on ATP synthesis at various concentrations of Mg^{++} was determined. When these data are plotted according to the method of Lineweaver and Burk (9) the projected lines intersect at the ordinate (fig 4) indicating that the inhibition of Na^+ and K^+ is due to competition of these ions with Mg^{++}. Table I shows the effect of several univalent cations on ATP synthesis from adenyl acetate at 2 levels of $MgCl_2$.

Effect of Adenyl Acetate Concentration on Synthesis of Acetyl CoA from Adenyl Acetate and CoA. Maximum synthesis of acetyl CoA from adenyl acetate (equation II) was produced by an adenyl acetate concentration of approximately 3×10^{-3} M (fig 5). This concentration of adenyl acetate was six-fold

Table I

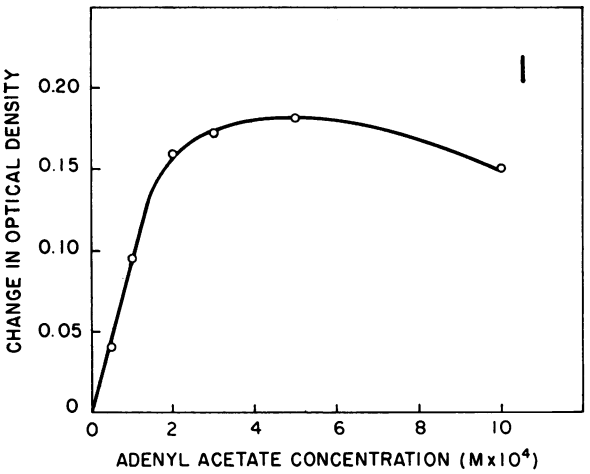
The Effect of 0.1 M Concentrations of Several Univalent Cation Chlorides on ATP Synthesis from Adenyl Acetate and PP_i at 2 Levels of $MgCl_2$

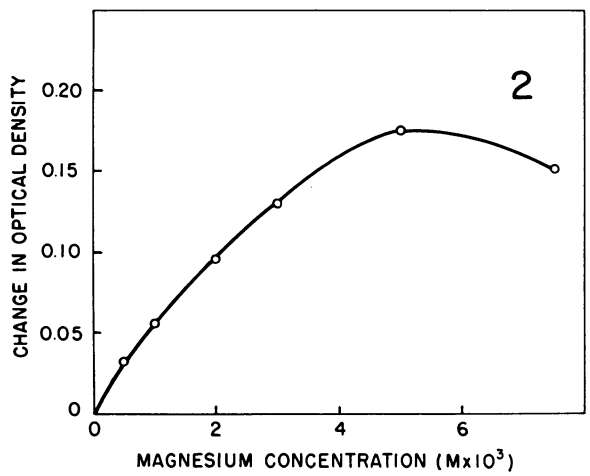
Standard assay conditions were used except that univalent cations and $MgCl_2$ were varied as indicated. The dialyzed enzyme extract added to each reaction mixture contained 1.5 mg of protein.

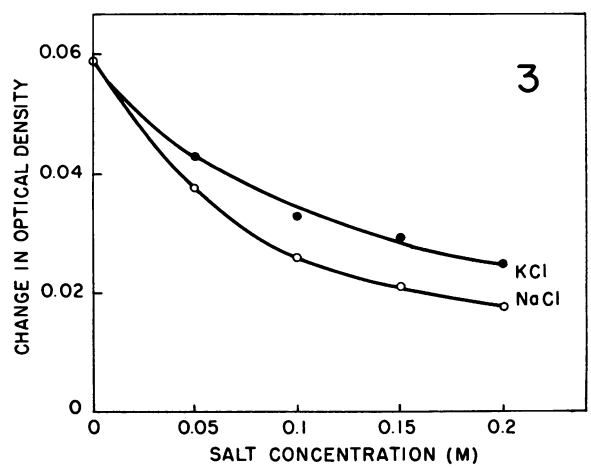
Cation added	Activity in presence of 2.5×10^{-3} M Mg	Activity in presence of 5×10^{-3} M Mg
	Change in OD at 340 mμ	
None	0.074	0.132
K	0.052	0.127
Rb	0.048	0.115
Na	0.042	0.096
Li	0.035	0.045
Cs	0.036	0.055

greater than the optimum for ATP synthesis (equation I). Adenyl acetate at 10^{-2} M slightly inhibited acetyl CoA synthesis.

Effect of Univalent Cations and Magnesium on Synthesis of Acetyl CoA from Adenyl Acetate and CoA. An absolute requirement for K^+ was exhibited in the synthesis of acetyl CoA from adenyl acetate and CoA (fig 6). When Rb^+ was substituted for K^+ a similar curve was obtained. Na^+ did not replace K^+ in the activation of acetic thiokinase nor did Na^+ inhibit the reaction in the presence of 0.05 M K^+. Mg^{++} up to a concentration of 5×10^{-3} M did not affect the reaction rate either in the presence or absence of K^+.







Discussion

Nearly all transphosphorylation reactions involving ATP require the presence of Mg ions and it has become more or less routine procedure to add this ion when assaying these reactions. It is generally assumed that the reactive species is a Mg^{++} complex of ATP. In addition a number of enzyme reactions involving nucleoside triphosphates show an absolute requirement, or a stimulation by, univalent cations. This requirement is usually met by K^+ but in most cases Rb^+ and NH_4^+, and in some cases Na^+, will substitute for K^+. The majority of reactions which involve ATP have not been tested for univalent cation requirement and such a requirement could easily be missed since many of the reagents are commonly used in the form of their sodium or potassium salts. A wide variety of enzymes, some not involving phosphorylated substances, have been found to require K^+ and no common reaction pattern for the basis of K^+ requirement has yet emerged.

The activation of acetate by acetic thiokinase provides a good system for studying the role of inorganic ions in transphosphorylation since the reaction proceeds by 2 steps which can be assayed independently. Mg^{++} is required in the first step of the reaction sequence (equation I), but not in the synthesis of acetyl CoA from adenyl acetate and CoA. This agrees with the findings of Berg (2). The increase in Mg^{++} requirement with increasing PP_i concentration suggests, but does not prove, that a Mg-PP_i complex may be the active form of the substrate. The overall reaction will not proceed in the absence of Mg^{++}. These findings lend further support to the proposal that the true substrate in the forward reaction of acetic thiokinase is a Mg chelate of ATP, the role of the metal being to bind the nucleotide to the enzyme. Lowenstein (10) has proposed a model

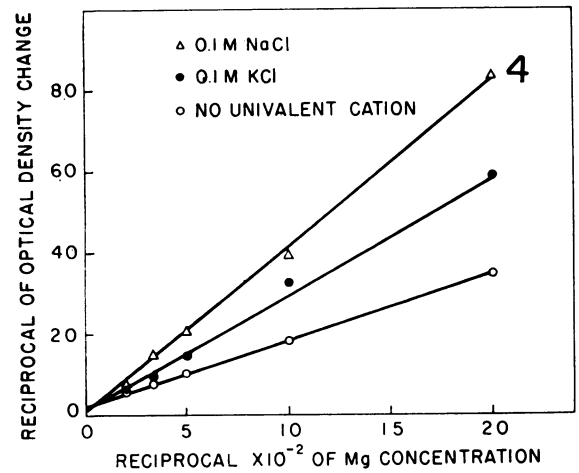
FIG. 1. Effect of adenyl acetate concentration on synthesis of ATP from adenyl acetate and PP_i. The dialyzed enzyme extract added to each reaction mixture contained 2 mg of protein.

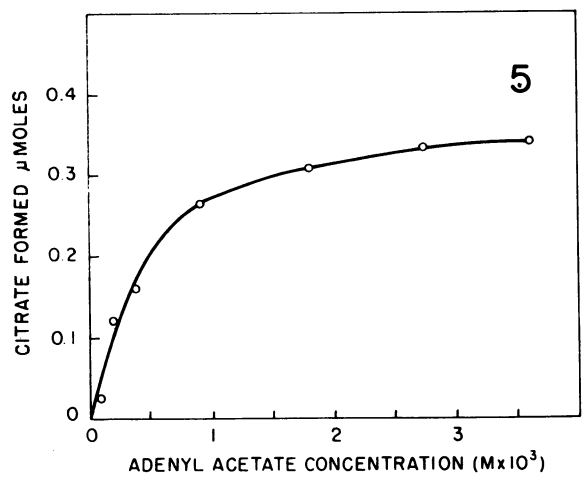
FIG. 2. Effect of Mg concentration on synthesis of ATP from adenyl acetate and PP_i. The dialyzed enzyme extract added to each reaction mixture contained 2 mg of protein.

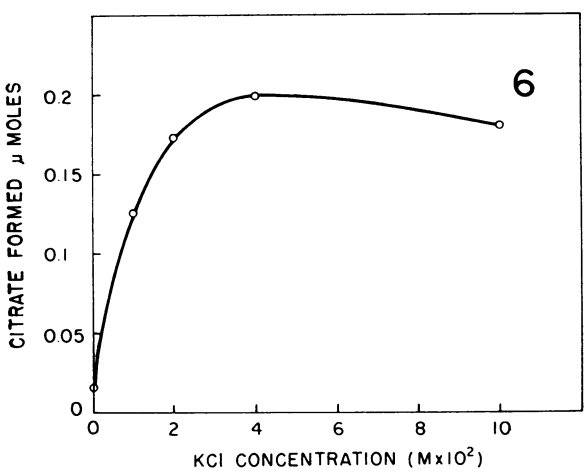
FIG. 3. Effect of KCl and NaCl concentration on synthesis of ATP from adenyl acetate and PP_i. The reaction mixture contained 2.5×10^{-3} M Mg and the dialyzed enzyme extract added to the reaction mixture contained 1.4 mg of protein.

by which ATP may be bound to the enzyme surface through chelation with Mg^{++} or Mn^{++}.

The synthesis of ATP from adenyl acetate and PP_i (equation I) was not stimulated by K^+ at any concentration. The acetylation of CoA (equation II), however, required the presence of K^+. This requirement was the same as that reported for the overall reaction (6). These results indicate that Mg^{++} and K^+, while being involved in the same system, act more or less independently in their catalytic role.







The inhibition of ATP synthesis from adenyl acetate and PP_i (equation I) by univalent cations was competitive with respect to Mg^{++}, suggesting that these ions were occupying sites normally occupied by Mg^{++} in the active complex. Several K^+ activated enzymes are inhibited by Na and Li ions (4, 5, 6, 8, 15, 16). There has been little detailed experimentation on the inhibitory action of Na^+ and Li^+, but it has often been assumed that these ions were interfering with K^+ activation of the enzyme. While this is undoubtedly true in some cases, the data presented in this paper suggest that Na and Li ion inhibition of some of the transphosphorylation reactions may be due to a Mg^{++}-univalent cation antagonism. This antagonism may be rather widespread in transphosphorylation reactions and yet go undetected since the effect of univalent cations on activity is not normally considered unless there is some indication of a K^+ stimulation of the reaction. Perhaps the inhibition of univalent cation-requiring enzyme reactions by concentrations of the activating ion greater than the optimum might also be explained on the basis of this antagonism.

The antagonism between univalent cations and Mg^{++} may be of significance in intact biological systems. Analysis of tobacco leaf tissues in our laboratory have shown that the tissue often contains sufficient K^+ to produce levels greater than 0.1 M in the cell sap. Perhaps excessive rates of potassium fertilization could lead to an interference of phosphorus metabolism.

Summary

The role of metals in the activation of acetic thiokinase from spinach (*Spinacia oleracea*) was studied. Adenyl acetate, an intermediate in the 2-step reaction, was synthesized and the individual steps were assayed by measuring the conversion of adenyl acetate to adenosine triphosphate and acetyl CoA, respectively. The reversible conversion of adenosine triphosphate and acetate to adenyl acetate and pyrophosphate exhibited an absolute requirement for Mg ions. Mg

FIG. 4. Reciprocal plots of reaction velocity versus Mg concentration with and without added univalent cations. Mg concentration was varied from 5 × 10^{-4} M to 5 × 10^{-3} M.

FIG. 5. Effect of adenyl acetate concentration on synthesis of acetyl CoA from adenyl acetate and CoA. The dialyzed enzyme extract added to each reaction mixture contained 4 mg of protein.

FIG. 6. Effect of K^+ concentration on synthesis of acetyl CoA from adenyl acetate and CoA. The dialyzed enzyme extract added to each reaction mixture contained 3 mg of protein.

ions were without effect on the subsequent conversion of adenyl acetate to acetyl CoA.

K ions did not stimulate the first step of the reaction. The synthesis of acetyl CoA from adenyl acetate and CoA, however, required the presence of univalent cations. K or Rb ions satisfied this requirement but Na ions were without effect on the reaction. The optimum concentration of K and Rb was 0.04 M.

Univalent cations inhibited the conversion of adenyl acetate and pyrophosphate to ATP. This inhibition was competitive with respect to Mg suggesting that univalent cations were occupying sites normally occupied by Mg ions in the active complex.

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