

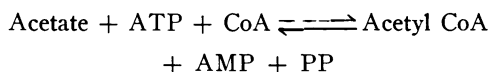
INFLUENCE OF CERTAIN CATIONS ON ACTIVITY OF ACETIC THIOKINASE FROM SPINACH LEAVES^{1, 2}

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The first evidence for a cofactor necessary in acetate metabolism was provided by Nachmansohn and Machado (13) who discovered a soluble enzyme system in extracts of brain tissue which catalyzed the acetylation of choline in the presence of adenosine triphosphate (ATP). Lipmann (8) and Kaplan and Lipmann (5) described an enzyme system in extracts of pigeon liver which catalyzed the ATP and coenzyme A (CoA) dependent acetylation of sulfanilamide. These investigators were the first to identify CoA as a necessary factor in acetate activation. Final proof of the constitution of active acetate was provided when Lynen and Reichert (10) isolated and identified acetyl CoA.

Enzyme extracts were obtained from pig heart and rabbit heart by Beinert et al (1) and pigeon liver and yeast by Jones et al (4) which catalyzed the reversible reaction of ATP, acetate, and CoA to yield acetyl CoA, adenosine monophosphate (AMP), and inorganic pyrophosphate (PP). The overall reaction is formulated in the following equation:

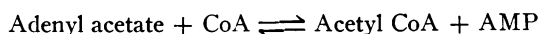
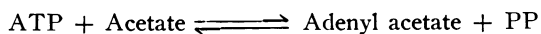


Acetic thiokinase was extracted from the tissues of higher plants by Millerd and Bonner (12). The enzyme was obtained by these authors from every tissue in which it was sought; however, the most active preparations were obtained from spinach leaves. The enzyme appeared to be associated with the mitochondria in both plant and animal organisms.

Acetic thiokinase has been shown to require Mg^{++} (1, 4, 12) for activity. In addition Jones et al (4) reported that the reaction rate appeared to be increased in a medium containing K^+ . Von Korff (15) made a study of the effects of alkali metals on the overall acetate activation system from animal tissues and concluded that the reaction was absolutely dependent upon univalent cations and that maximum activity was obtained with K^+ , Rb^+ , or NH_4^+ at concentrations of 0.04 M. Na^+ or Li^+ strongly inhibited the reaction. The inhibition by Na^+ , however, was not overcome by K^+ indicating that the inhibition was not due to a competition between Na^+ and K^+ . The alkali metal requirements of acetic thiokinase from higher plants has not been reported in the literature.

Berg (2, 3) has demonstrated that the reaction

catalyzed by acetic thiokinase proceeds in two steps. These are formulated in the following equations:



All attempts to demonstrate the involvement of more than one enzyme in the two reactions have been negative (3). According to Berg (3) Mg^{++} is required for the first step of the above sequence. This was demonstrated by measuring ATP formation in the presence of adenyl acetate, PP, and acetic thiokinase. Maximum activity was produced with Mg^{++} at a concentration of 1.5×10^{-3} M. In contrast to the Mg^{++} requirement for the conversion of adenyl acetate to ATP, the reaction of adenyl acetate and CoA yielding acetyl CoA was maximal in the absence of Mg^{++} . No study has been conducted to determine the possible univalent cation requirements of the individual reactions in the acetate activation sequence.

In view of the importance of acetate in plant metabolism and of the limited information concerning the role of univalent cations in plant metabolism it was considered that a detailed study of the requirements of univalent cations for acetate activation in plants was appropriate.

MATERIALS AND METHODS

PREPARATION OF ACETIC THIOKINASE: Acetic thiokinase was obtained from an acetone powder of the leaves of spinach (*Spinacea oleracea* L.). The acetone powder was prepared according to the procedure described by Millerd and Bonner (12). All reagents used and all operations involved in the preparation of the enzyme were maintained between 0 and 4° C. In preparing the crude extract the acetone powder was ground with mortar and pestle for 5 minutes with 20 weights of 0.02 M tris (hydroxymethyl)aminomethane (Tris) buffer at pH 7.4. The suspension was centrifuged for 10 minutes at a force of $15,000 \times G$ and the precipitate discarded. The concentration of nucleic acids in the supernatant solution was determined spectrophotometrically by the procedure described by Layne (6) and 0.025 ml of 2% protamine sulfate was added per milligram of nucleic acid in the extract. The suspension was centrifuged for 10 minutes at $15,000 \times G$ and the precipitate was discarded. To the supernatant solution was added with stirring, sufficient solid ammonium sulfate to bring the solution to 30% of saturation. After 10 minutes the precipitate was removed by centrifugation and the supernatant was brought to 50% of saturation with solid ammonium sulfate. The precipitate was collected by centrifugation and redissolved in a third the original volume of 0.02 M Tris

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buffer at pH 7.4. This extract contained a small amount of reduced diphosphopyridine nucleotide (DPNH) oxidase which was essentially removed by refractionation with ammonium sulfate. In this procedure the extract was brought to 33 % of saturation with ammonium sulfate and the precipitate was collected by centrifugation and discarded. Sufficient ammonium sulfate was added to the supernatant liquid to bring the concentration to 50 % of saturation. The precipitate was collected by centrifugation and re-dissolved in a sixth the original volume of 0.02 M Tris buffer, pH 7.4. This procedure produced a two-fold purification of the enzyme. The amount of DPNH oxidase activity present in this extract was negligible and therefore did not interfere with the acetic thiokinase assays. The extract was dialyzed for 3 hours against 0.001 M Tris buffer at pH 8.0. After this period the dialyzing solution was renewed and dialysis was continued for 3 additional hours. Extracts prepared by this procedure were used for the various experiments.

PREPARATION OF CONDENSING ENZYME: Partially purified condensing enzyme was prepared from pig hearts by a procedure modified from the method outlined by Ochoa (14). Two pig hearts were chilled in ice as rapidly as possible after removal from the animal. The fat and connective tissue were removed and the muscle tissue was ground in a food chopper. The resulting mince was stirred for 30 minutes with 0.02 M Tris buffer, pH 7.4 (2 ml/g of wet mince). The suspension was squeezed through four thicknesses of cheesecloth and the residue was re-extracted with Tris buffer at pH 7.4 as described above. The two extracts were combined and adjusted to pH 5.5 by slowly adding 0.5 M acetic acid with mechanical stirring. Calcium phosphate gel was then added in the proportion of 0.3 mg gel solids per milligram of protein. The suspension was stirred for 4 hours and allowed to settle overnight. The clear red supernatant solution was siphoned off, and the gel was collected by centrifugation. The supernatant fluid was discarded and the condensing enzyme was extracted from the gel by shaking it for 30 minutes with 50 ml of 0.1 M potassium phosphate buffer at pH 7.4. Several small glass beads were added to aid in breaking up the precipitate. After shaking, the suspension was centrifuged and the supernatant fluid was collected. The gel was re-extracted six times and the eluates were combined.

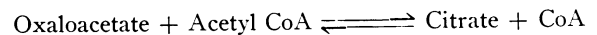
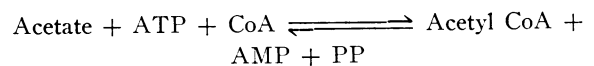
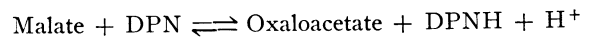
Further purification was accomplished by ammonium sulfate fractionation. To the combined eluates was added slowly, with stirring, sufficient solid ammonium sulfate to bring the solution to 40 % of saturation. The precipitate was removed by centrifugation and the supernatant solution was brought to 70 % of saturation with ammonium sulfate. The precipitate was collected by centrifugation and redissolved in a tenth the original volume of 0.02 M Tris buffer at pH 7.4. All operations were carried out at 0 to 4° C.

This procedure resulted in a ten-fold purification.

The purified extract was free of acetic thiokinase and DPNH oxidase activity. Before use in acetic thiokinase assays the extract was dialyzed 12 hours against 0.001 M Tris buffer at pH 7.4.

OTHER MATERIALS: Diphosphopyridine nucleotide (DPN) of 95 to 100 % purity and ATP of 99 to 100 % purity were obtained from the Sigma Chemical Co., St. Louis, Mo. CoA of 75 % purity, malic acid, and glutathione were obtained from Nutritional Biochemical Corp., Cleveland, Ohio. Other chemicals were of reagent grade and were obtained from commercial sources. The Tris salts of DPN and ATP were obtained by passing solutions and the sodium salts of these coenzymes through an exchange column containing Dowex 50 resin (Tris cycle). The Tris salts of acetate and malate were prepared by neutralizing the respective acids to pH 8.0 with Tris. Acetyl CoA was prepared from acetic anhydride and CoA according to the procedure outlined by Ochoa (14).

STANDARD ASSAY PROCEDURES: Acetic thiokinase was assayed by a method essentially the same as that described by Millerd and Bonner (12). Activity was followed spectrophotometrically by measuring the rate of reduction of DPN at a wave length of 340 m μ in a system involving the coupling of malic dehydrogenase, acetic thiokinase, and condensing enzyme. These reactions, in the proper sequence are as follows:



In this assay the rate of reduction of DPN is equal to the rate of citrate synthesis (14). The standard reaction mixture in a final volume of 1 ml contained the following constituents: 100 micromoles of Tris buffer adjusted to pH 8.0 with HCl; 10 micromoles of Tris acetate; 2.5 micromoles of MgCl₂; 50 micromoles of KCl; 5 micromoles of Tris malate; 10 micromoles of glutathione; 5 micromoles of Tris ATP; 0.67 micromole Tris DPN; 0.09 micromole of CoA; 20 to 30 units of condensing enzyme and acetic thiokinase extract usually containing between 0.6 and 1.0 mg of protein. The quantities, in micromoles, of ATP, CoA, acetate, and DPN required for maximum enzyme activity in a 1 ml reaction mixture were 2.5, 0.09, 5.0, and 0.4, respectively. Both the condensing enzyme extract and the acetic thiokinase extract contained large amounts of malic dehydrogenase. Sufficient malic dehydrogenase and condensing enzyme were present in the reaction mixture, therefore, to insure that acetic thiokinase was the rate-limiting step. The mixtures were incubated 30 minutes at 30° C and the reaction was stopped by adding 5 ml of 0.5 M sodium phosphate buffer at pH 7.2. DPNH formed under the desired experimental conditions was compared with a control reaction containing all the components except CoA. The reaction rate was proportional to acetic thiokinase concentration up to a con-

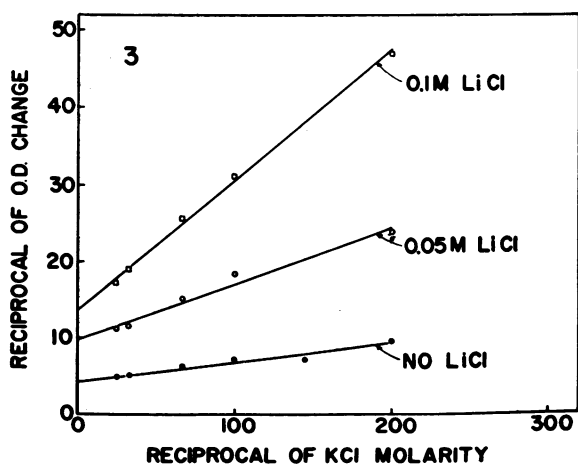
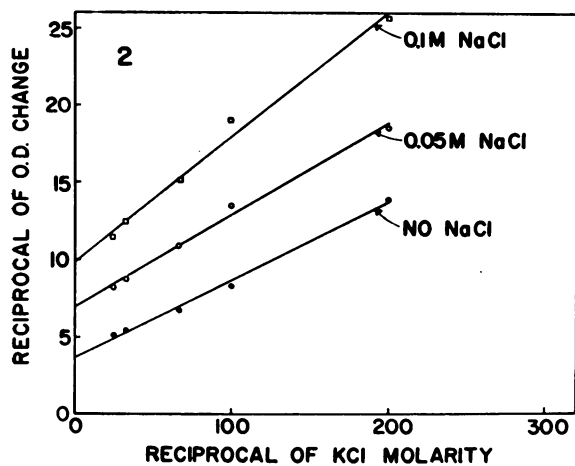
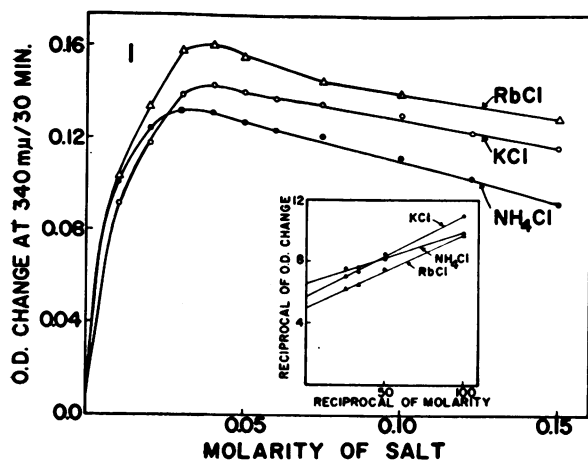


FIG. 1 (major graph). The influence of various concentrations of univalent cations on the activity of acetic thiokinase. The standard assay procedure was used with variation in concentration of univalent cations as indicated. The dialyzed enzyme extract added to each reaction mixture contained 0.7 mg. of protein.

(Inset) Reciprocal plots of reaction velocities versus salt concentrations indicated in the major graph. Only those points were utilized in the major graph that were

TABLE I
ESSENTIALITY OF VARIOUS COMPONENTS OF STANDARD ASSAY MEDIUM FOR ACETIC THIOKINASE*

REACTION MIXTURE	ACTIVITY RELATIVE TO COMPLETE MIXTURE
Complete	100
No ATP	0
No CoA	0
No Acetate	15
No MgCl ₂	0
No KCl	0
No DPN	0
No malate	0
No condensing enzyme	0

* The complete reaction mixture in a volume of 1 ml contained the following constituents in micromoles: Tris buffer, pH 8.0, 100; Tris acetate, 10; MgCl₂, 2.5; Glutathione, 10; Tris ATP, 5; Tris DPN, 0.67; CoA, 0.09; KCl, 50; Tris malate, 5; condensing enzyme (20–30 units) and acetic thiokinase (1.0 mg protein.).

centration of 2.0 mg of protein in each assay vessel. No activity was obtained when the active enzyme was replaced in the standard mixture by a boiled enzyme preparation. Evidence for the dependence of enzyme activity on the various constituents of the assay medium is presented in table I.

In addition to the procedure involving coupling of the acetic thiokinase reaction with the condensing enzyme and malic dehydrogenase reactions, incubation mixtures containing hydroxylamine prepared as described by Millerd and Bonner (12) exhibited positive dependent hydroxamic acid reactions.

Condensing enzyme was assayed according to the optical procedure outlined by Ochoa (14). One unit

free of apparent inhibition. Calculation of regression lines by the method of least squares gave the following regression equations: K⁺, $Y = 5.65 + 0.054 X$; NH₄⁺, $Y = 6.56 + 0.033 X$; Rb⁺, $Y = 4.98 + 0.048 X$.

FIG. 2. Reciprocal plots of reaction velocities versus KCl concentration at different NaCl concentrations. The standard assay procedure was used with variation in salts as indicated. The dialyzed enzyme extract added to each reaction mixture contained 1.0 mg of protein. Calculation of regression lines by the method of least squares gave the following regression equations: No NaCl, $Y = 3.69 + 0.050 X$; 0.05 M NaCl, $Y = 6.98 + 0.059 X$; 0.1 M NaCl, $Y = 9.77 + 0.082 X$.

FIG. 3. Reciprocal plots of reaction velocities versus KCl concentration at different levels of LiCl concentration. The dialyzed enzyme extract added to each reaction mixture contained 1.0 mg of protein. The standard assay procedure was used with variation in salt as indicated. Calculation of regression lines by the method of least squares gave the following regression equations: No LiCl, $Y = 4.27 + 0.26 X$; 0.05 M LiCl, $Y = 9.6 + 0.074 X$; 0.1 M LiCl, $Y = 13.45 + 0.17 X$.

of condensing enzyme is defined as that amount which gives an optical density change of 0.01 per minute under the standard assay conditions. Protein was determined by the Folin-phenol method of Lowry et al (9) using bovine albumin as the standard.

RESULTS

UNIVALENT CATION ACTIVATION: The curves in figure 1 (major graph) illustrate the influence of chloride salts of K^+ , NH_4^+ , and Rb^+ on the activity of acetic thiokinase. In these experiments an extract was utilized that had been dialyzed for 6 hours against 0.001 M Tris buffer at pH 8.0. As indicated by these curves the enzyme activity was absolutely dependent upon univalent cations. Periods of dialysis less than 6 hours were not sufficient to remove all endogenous univalent cations. Maximum enzyme activity was exhibited with chloride salts of K^+ , NH_4^+ , or Rb^+ at a concentration of 0.04 M. The addition of these salts at levels higher than 0.04 M resulted in inhibition. Using the points on the curve which were free of apparent inhibition, plots were constructed (inset graph, fig 1) according to the method of Lineweaver and Burk (7) and the Michaelis constants (K_A) for the activating cations were calculated. K_A values for K^+ , NH_4^+ , and Rb^+ were, respectively: 9×10^{-3} M; 5×10^{-3} M, and 9×10^{-3} M. These values are only approximations, however, because concentrations of salts greater than 0.04 M resulted in inhibition. This was most apparent with NH_4Cl .

The enzyme was not activated by Na^+ or Li^+ and inhibition was produced by low concentrations of these cations. When a concentration of 0.05 M Na^+ or Li^+ was included in the standard assay mixture, enzyme activity with the two ions was inhibited 38% and 56%, respectively. To determine whether

or not the inhibition produced by Na^+ or Li^+ was due to competition of these cations with the activating univalent cations, KCl saturation curves were determined at various concentrations of Na^+ or Li^+ . Lineweaver-Burk plots of these data are presented in figures 2 and 3. Failure of the projected curves to intersect at infinite KCl concentration demonstrates that the inhibition by Na^+ or Li^+ is not competitive with respect to K^+ .

EFFECT OF VARIOUS ANIONS OF ENZYME ACTIVITY: As indicated by the curves in figure 4, the influence of univalent cation salts on enzyme activity was not independent of the balancing anion. Although the optimum concentration varied slightly with potassium salts of different anions, maximum enzyme activity varied markedly with different potassium salts. Enzyme activity in the presence of the nitrate salt of potassium was 55% of the activity in the presence of chloride. The activity obtained with potassium sulfate was intermediate between the activities obtained with potassium fluoride and potassium nitrate.

DIVALENT CATION ACTIVATION OF ACETIC THIOKINASE: In addition to the requirement for univalent cations for activity, acetic thiokinase from spinach exhibits an absolute requirement for divalent cations. The effect of various divalent cation chlorides on the enzyme activity is illustrated by the curves in figure 5. In these experiments the standard assay procedure was used with the exception that divalent cation salts were included in the assay mixture as indicated. The activating capacities of Mg^{++} and Mn^{++} were approximately equal at optimal concentrations. Ca^{++} was 77% as effective as Mg^{++} or Mn^{++} , and Ba^{++} failed to activate the enzyme at any concentration tested. Maximum enzyme activity was observed at

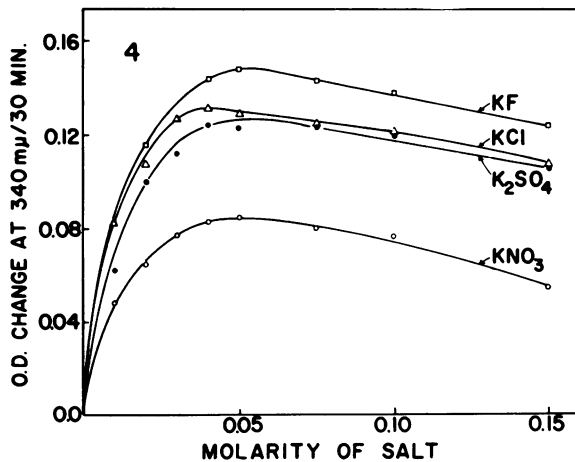


FIG. 4. The influence of various concentrations of potassium salts of several anions on the activity of acetic thiokinase. The standard assay procedure was used with variation in anions as indicated. The dialyzed enzyme extract added to the reaction mixture contained 0.7 mg of protein.

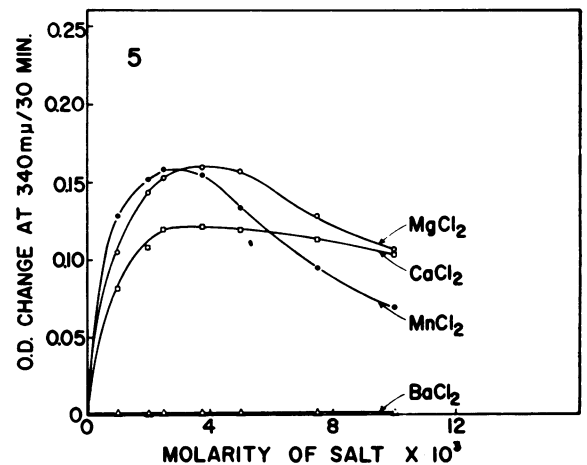


FIG. 5. The influence of various divalent cations on the activity of acetic thiokinase. The standard assay procedure was used with variation in divalent cations as indicated. The dialyzed enzyme extract added to the reaction mixture contained 0.8 mg of protein.

a Mg^{++} , Mn^{++} , or Ca^{++} concentration of approximately $3 \times 10^{-3} M$. Activity was markedly inhibited at concentrations higher than optimal.

DISCUSSION

Acetic thiokinase from spinach exhibits an absolute requirement for univalent cations. Maximum enzyme activity was produced by K^+ , NH_4^+ , or Rb^+ at a concentration of 0.04 M. A similar concentration of these cations produced maximum activity of the enzyme from animal tissues (15). Enzyme activity was inhibited by Na^+ or Li^+ and the inhibition was not competitive with respect to the activating univalent cations. The influence of these ions on acetic thiokinase from animals (15) was similar to that observed for the plant enzyme.

The failure of Na^+ to replace K^+ in the activation of acetic thiokinase is in contrast with the capacity of this cation to function as an activator for pyruvate kinase from higher plants (11). One might postulate that the acetic thiokinase reaction may represent an important site of physiological antagonism between K^+ and Na^+ in higher plant metabolism.

The reason for the differences in maximum enzyme activity obtained when different anions were used in conjunction with a common cation is not readily apparent. Perhaps there is a total salt or anionic inhibition which partially overcomes the activating effect of the univalent cation. Further investigation is necessary to clarify this observation.

In addition to its requirement for univalent cations the activity of acetic thiokinase also is dependent upon divalent cations. This requirement was satisfied by Mg^{++} , Mn^{++} , or Ca^{++} ; however, the maximum activity with the latter cation was only 77% of that with either Mg^{++} or Mn^{++} . Further experimentation would be necessary in order to postulate reasons for the variability in activating capacities of different divalent cations.

In regard to the physiological importance of various univalent cation activators of this system it is concluded that the concentrations of either NH_4^+ or Rb^+ normally present in fresh plant material are insufficient to play an important role in activating this enzyme. In view of this conclusion and of the toxicity of high concentrations of either NH_4^+ or Rb^+ to plant tissues, the most probable physiologically active univalent cation for this enzyme is K^+ . In this regard fresh plant material normally contains about 0.2% of K^+ . This concentration is very near to the 0.04 M concentration required for maximum activity of the enzyme *in vitro*.

Experiments are presently being conducted to determine the possible role of univalent cations in the individual reactions of the acetate activation sequence. Preliminary experiments have indicated that the conversion of adenylyl acetate and CoA to acetyl CoA and AMP is stimulated by potassium ions.

SUMMARY

Acetic thiokinase was obtained from an acetone powder of the leaves of Spinach (*Spinacea oleracea* L.). Dialyzed extracts of this enzyme required both

univalent and divalent cations for activity. The univalent cation requirement was satisfied by K^+ , NH_4^+ , or Rb^+ and maximum activity was obtained in the presence of 0.04 M concentration of these cations. Enzyme activity was inhibited when Na^+ or Li^+ was added to the standard assay mixture. This inhibition apparently was not due to a competition of these cations with the activating univalent cations.

The divalent cation requirement for enzyme activity was satisfied by Mg^{++} , Mn^{++} , or Ca^{++} . Ba^{++} was ineffective at all concentrations tested.

In general it is concluded that the cation requirements for acetic thiokinase from spinach leaves are very similar to those that have been established for this enzyme from animal sources.

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