THE INFLUENCE OF SALTS ON PYRUVATE KINASE FROM TISSUES OF HIGHER PLANTS\textsuperscript{1,2}

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The enzyme pyruvate kinase which catalyzes the reversible transfer of phosphate from phosphoenolpyruvate (PEPA) to adenosine diphosphate (ADP) yielding pyruvate and adenosine triphosphate (ATP) is widely distributed in living organisms (2, 3, 4, 8, 9, 11, 15). Lohmann and Meyerhof (11) in 1934 demonstrated that the activity of pyruvate kinase from rat muscle was dependent on the presence of Mg\textsuperscript{2+} in the assay medium. Utter and Werkman (22) reported that extracts from Escherichia coli contained pyruvate kinase and that it was activated by Mg\textsuperscript{2+} or Mn\textsuperscript{2+}. In their experiments Ni\textsuperscript{2+}, Na\textsuperscript{+} and K\textsuperscript{+} were either without effect or were inhibitory.

The K\textsuperscript{+} activation of pyruvate kinase from rat muscle was shown initially by Boyer, Lardy and Phillips (3, 4) and later by Lardy and Ziegler (9). The complete dependence of the activity of the enzyme on univalent cations was demonstrated first by the work of Kachmar and Boyer (8). This requirement was in addition to that for divalent cations. K\textsuperscript{+}, NH\textsubscript{4}\textsuperscript{+} or Rb\textsuperscript{+} satisfied the univalent cation requirement but Na\textsuperscript{+}, Li\textsuperscript{+} or Ca\textsuperscript{2+} counteracted the activation. In the absence of K\textsuperscript{+}, Na\textsuperscript{+} additions resulted in a small but significant stimulation. Boyer (2) reported that extracts from eleven different vertebrate and invertebrate organisms contained pyruvate kinase and that the enzyme from all sources was strongly activated by K\textsuperscript{+}. The optimum concentrations of univalent cations in molarity were 0.15, 0.01 and 0.05 for activity of the enzyme from muses of rabbit, Anodonta and Limulus, respectively.

Introductory studies (14) on the fermentation of phosphoglyceric acid by dialyzed yeast extracts showed no univalent cation activation. Later, Seitz (18) reported that yeast extracts that had been thoroughly dialyzed failed to ferment phosphoglyceric acid unless K\textsuperscript{+} or NH\textsubscript{4}\textsuperscript{+} was supplied. In the absence of univalent cations the phosphoglyceric acid fermentation stopped at the phosphoenolpyruvate stage. With this system NH\textsubscript{4}\textsuperscript{+} was more effective than K\textsuperscript{+} as an activator which is in contrast to observations with the enzyme from other sources.

Stumpf (20) and Tewfik and Stumpf (21) have provided indirect evidence for the presence of pyruvate kinase in higher plants. Extracts of an acetone powder of pea seed showed no activity. When dialyzed extracts were used, the addition of adenine monophosphate (AMP) increased the rate of formation of pyruvate from phosphoglyceric acid. The effects of ADP or univalent cation additions to the system were not indicated in these studies.

It is apparent that pyruvate kinase is widely distributed in microorganisms and tissues of animals and indirect evidence suggests that this enzyme is present in higher plants. In extracts of animal tissues and yeast the activation of the enzyme by K\textsuperscript{+} and other univalent cations seems to be a general phenomenon representing a fundamental property of the enzyme. The experiments that have been conducted with higher plants have not been specifically designed to demonstrate cation requirements of the system. It was deemed necessary therefore, to conduct a systematic study of the influence of various salts on pyruvate kinase obtained from several higher plant species. In view of the limited available information on the role of univalent cations in the metabolism of higher plants, this study was considered especially appropriate.

\textbf{Materials and Methods}

\textbf{Preparation of Extracts:} The enzyme extract used in most of the experiments reported herein was prepared from an acetone powder of Alaska pea seed (20). The seed were soaked in distilled water at room temperature for 5 hours then removed from the water and homogenized for 3 minutes in acetone at \(-10^\circ\text{C}\) by use of an Omni mixer (Ivan Sorvall Inc., Norwalk, Conn.). The acetone powder of this material was prepared as previously described (5). For the preparation of the extract, 4 gm of acetone powder were homogenized in 15 ml of 0.05 M tris (hydroxymethyl) aminomethane–hydrochloride buffer (TRIS) at pH 7.4 and the mixture centrifuged at 25,000 \(\times g\) for 10 minutes. The supernatant was dialyzed for 4 hours against 4 liters of 0.01 M TRIS buffer at pH 7.4, and used as a source of the enzyme. When extracts from pea seed were dialyzed for longer periods the activity was lost. The addition of glutathione or versene or both to the dialysis solution had no effect on enzyme stability. Since the enzyme activity of extracts declined rapidly, daily preparations were necessary. The extracts prepared by the described procedure contained 12 to 14 mg proteins per ml as determined by Folin’s phenol reagent (12).

Extracts from various tissues of other species were prepared for the experiments related to the occurrence of pyruvate kinase in higher plants. Five grams of the fresh tissue were homogenized in 15 ml of 0.05 M TRIS pH 7.4 and strained through cheesecloth. The extract was centrifuged for 10 minutes at 25,000 \(\times g\). The lability of the enzyme from the various plant species differed and accordingly the lengths of the

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dialysis periods varied with the different preparations (table II).

Since pyruvate kinase activity was low in extracts from certain plant materials, concentration of the enzyme was necessary. This was accomplished by precipitation of protein by the addition of 50 ml of acetone at \(-15^\circ C\) for each 20 ml of crude extract. The precipitate was collected by filtration rewashed with cold acetone and allowed to dry at room temperature. The dried precipitate from each 20 ml aliquot of original extract was taken up in 5 ml of 0.05 M TRIS buffer pH 7.4 and centrifuged at 25,000 \(x\) g for 10 minutes. The supernatant was used as a source of enzyme and is referred to as the acetone precipitated extract.

**Other Materials:** The TRIS salt of phospho- enolpyruvate acid (PEPA) was prepared from the silver-barium double salt (California Foundation for Biochemical Research of Los Angeles) by trituration in an ice bath with a 5 to 10% excess, on an equivalent basis, of \(N\) HCl (8). This was followed by the addition of a slight excess of \(M\) TRIS sulphate. The precipitate was removed by centrifugation and the clear solution was adjusted to pH 7.4 using TRIS buffer, then diluted to a concentration of 0.015 M. In later experiments the cyclohexylamine salt of PEPA was used and the results were identical with those obtained with TRIS PEPA.

The barium salts of ADP and AMP were purchased from the Sigma Chemical Company of St. Louis. TRIS ADP was prepared by dissolving the barium salt in 0.1 M HCl and precipitating Ba\(^{++}\) by addition of a calculated amount of TRIS sulphate. The solutions of TRIS ADP and TRIS AMP were adjusted to pH 7.4 using TRIS buffer and diluted to a concentration of 0.05 M. Buffer solutions were prepared as previously described (13).

**Inorganic Analyses:** Aliquots of the dialyzed extract from the acetone powder of pea seed were ashed in a muffle furnace at 600°C and analyzed for Na and K by use of a Perkin-Elmer flame photometer (24). NH\(_3\) was determined after distillation from alkaline extracts by Nesslerization (7).

**Standard Assay Procedure:** Enzyme activity was assayed by determination of the pyruvate formed from PEPA after incubation of the various reactants with the enzyme. In this procedure ADP was added directly to reaction mixtures instead of addition of the hexokinase system for the generation of ADP from ATP as reported by other workers (8). The omission of hexokinase was necessary since the work of Hersh (6) and unpublished experiments by the authors have shown that hexokinase activity is influenced by both univalent and divalent cations. Evidence for the validity of the standard procedure is presented under Results.

The reaction mixture in a final volume of 1 ml contained the following constituents in micromoles: 50 of TRIS buffer at pH 7.4; 1.5 of TRIS or cyclohexylamine salt of PEPA; 2.5 of TRIS ADP; 8.0 of MgSO\(_4\); 50 of KCl and enzyme extract usually containing 0.6 to 0.7 mg protein. The mixtures were incubated at 37°C for 10 minutes and the reaction stopped by the addition of 1 ml of cold 0.0125 % 2,4-dinitrophenyl hydrazine (8). Pyruvate was estimated by the method described by Kaehmar and Boyer (8). Precipitates of protein or Mg(OH)\(_2\) in the reaction mixtures were removed by centrifugation in order to prevent interference with the colorimetric determination. In each assay the pyruvate formed under the desired experimental conditions was compared with a control reaction containing all reactants except ADP. This served to correct for pyruvate formation resulting from the action of phosphatases. No pyruvate was formed when the enzyme extracts were boiled and added to reaction mixtures with or without the various experimental additions. The various experiments were replicated at least three times and the results averaged. The error between determinations was less than 10%.

**RESULTS**

**Validity of the Standard Assay Procedure:** Since the literature contains no detailed studies of the properties of pyruvate kinase from higher plants it was essential to determine the concentrations of ADP and PEPA necessary for saturation of the enzyme. As shown in figure 1, a concentration of 1.4 \(\times\) \(10^{-3}\) M ADP was required for maximum activity. The \(K_M\) for ADP estimated from the saturation curve is 5.0 \(\times\) \(10^{-4}\). When AMP was used instead of ADP with dialyzed enzyme from pea seed no activity was observed. With the undialyzed enzyme extract, however, the addition of AMP resulted in weak activity.

The influence of various concentrations of PEPA on the enzymatic rate of pyruvate formation is shown in figure 2. The final concentration of PEPA required for the maximum rate was 1.3 \(\times\) \(10^{-3}\) M which is in good agreement with the figure reported for pyruvate kinase from rabbit muscle (8). The \(K_M\) for PEPA estimated from the curve in figure 2 is 3 \(\times\) \(10^{-4}\) M.

Under conditions of the standard assay the rate of pyruvate formation remained linear for at least the initial 15 minutes as shown in the major graph of figure 3. The inset in figure 3 provides evidence that the initial velocity (v) of the reaction was proportional to enzyme concentration. At the end of 15 minutes (major graph of figure 3) only 13% of the substrate had undergone reaction. Kaehmar and Boyer (8) reported the reaction velocity was linear with enzyme concentration and with time when less than 40% of the total PEPA present had reacted. In the various experiments reported in this study less than 40% of the PEPA was utilized during each assay.

The effect of pH on the activity of pyruvate kinase from pea seed was studied within the range 6.0 to 9.0 using phosphate and TRIS buffers. A broad optimum was observed between pH 7.0 and 9.0 and therefore pH 7.4 was arbitrarily chosen for the standard procedure.
Evidence that the concentrations of K⁺ and Mg⁺ used in the standard assay procedure were sufficient for maximum activation is presented in the following sections of this paper. Since the results to be presented indicate that cations such as K⁺ and Mg⁺ possess activating capacities for the enzyme and that anions such as Cl⁻ and SO₄²⁻ have no specific effect, the symbols for the cations are used in describing influences of cation salts on the system.

UNIVALENT CATION ACTIVATION OF THE ENZYME FROM PEA SEED: The curves in figure 4A illustrate the influence of univalent cations added as the chloride salts on enzyme activity. In figure 4B the data are plotted by the method of Lineweaver and Burk (10) and from these curves the Michaelis constants (Kₐ) for the activating univalent cations were calculated. When the data were plotted in figure 4B only those points on the curves in figure 4A that were free of apparent inhibition were utilized. In the absence of added salts, enzyme activity was approximately 10% of the maximum attainable when an optimum concentration of KCl was added. Since the enzyme was labile, prolonged dialysis was not possible and analyses indicated that the dialyzed extract from pea seed contained 8 × 10⁻⁴ M K⁺, no detectable NH₄⁺ and 2.2 × 10⁻³ M Na⁺. This was sufficient to account for the enzyme activity observed in the absence of added salts. Maximum enzyme activity with the chloride salts of either K⁺, Rb⁺ or Na⁺ was exhibited at a concentration of 0.05 M, and greater concentrations reduced the activity. Kₐ values for K⁺, Rb⁺ and Na⁺ were 2.4 × 10⁻³, 4.2 × 10⁻³ and 1.4 × 10⁻³ M, respectively. Maximum activation with NH₄⁺ was observed at a concentration of 0.035 M and greater concentrations caused a decline in activity. The Kₐ for NH₄⁺ was calculated to be 1.2 × 10⁻³ M. The addition of Na⁺ to reaction mixtures resulted in considerable stimulation of pyruvate kinase activity, but the

Fig. 1. The effect of ADP concentration on the rate of the enzyme reaction. The standard assay procedure was used with variation in concentration of ADP as indicated. The dialyzed enzyme extract from pea seed added to each reaction mixture contained 0.6 mg protein.

Fig. 2. The effect of concentration of PEPA on the rate of the enzyme reaction. The standard assay procedure was used with variation in concentration of PEPA as indicated. The dialyzed enzyme extract from pea seed added to each reaction mixture contained 0.6 mg protein.

Fig. 3. (Major graph) Proportionality of enzyme activity with time. The standard assay procedure was used with variation in the time as indicated. The enzyme extract from pea seed added to each reaction mixture contained 0.6 mg protein. (Inset) Proportionality of enzyme activity indicated as initial velocity (v), with enzyme concentration. The standard assay procedure was used with variation in quantity of enzyme extract added to each reaction mixture as indicated. The dialyzed extract from pea seed contained 13 mg protein per ml.
magnitude of the effect was much less than that obtained with the chloride salts of K\(^+\), Rb\(^+\) or NH\(_4\)^+; Li\(^+\) was completely ineffective as an activator for the system.

It has been reported that quantities of NH\(_4\)^+ greater than 0.5 micromole per ml interfere with the pyruvate kinase assay method (8) that included the hexokinase system. In the assay procedure reported here concentrations of NH\(_4\)^+ ranging from 1 to 100 micromoles per ml had no effect on the standard assay system. Unpublished experiments by the authors have demonstrated that NH\(_4\)^+ interferes with hexokinase assays determined by glucose disappearance. The hexokinase system was not included in the pyruvate kinase assay system used in these studies and perhaps this may account for the lack of interference under our conditions.

**Effect of Various Anions on Activity of Enzyme from Pea Seed:** Univalent cation salts differed in their influence on pyruvate kinase activity, and the effect was primarily independent of the anion present as shown in table I. The Cl\(^-\), NO\(_3\)^-, SO\(_4\)^2- and phosphate (mixture of HPO\(_4\)^2- and H\(_2\)PO\(_4\)^-) (13) salts of K\(^+\) gave similar results throughout the experimental range of concentrations. I\(^-\) and to a lesser extent the Br\(^-\) salts became inhibitory before the maximum activation of 0.05 M K\(^+\) was reached.

**Activation of Enzyme from Pea Seed by Divalent Cations:** The effect of various divalent cation

**Table I**

<table>
<thead>
<tr>
<th>SALT</th>
<th>CONC OF ADDED SALT (N)</th>
<th>Micromoles pyruvate formed in 10 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.00</td>
<td>0.001</td>
</tr>
<tr>
<td>KCl</td>
<td>0.015</td>
<td>0.082</td>
</tr>
<tr>
<td>KNO(_3)</td>
<td>0.020</td>
<td>0.078</td>
</tr>
<tr>
<td>KBr</td>
<td>0.018</td>
<td>0.065</td>
</tr>
<tr>
<td>KI</td>
<td>0.015</td>
<td>0.072</td>
</tr>
<tr>
<td>K(_2)SO(_4)</td>
<td>0.020</td>
<td>0.070</td>
</tr>
<tr>
<td>K(_2)HPO(_4)</td>
<td>0.016</td>
<td>0.069</td>
</tr>
</tbody>
</table>

*The standard assay procedure was used with variation of potassium salt as indicated. The dialyzed pea seed extract added to each reaction mixture contained 0.6 mg protein.*
Fig. 5. The activation of pyruvate kinase by various concentrations of divalent cation salts. The standard assay procedure was used with variation in concentration of divalent cation salts as indicated. The dialyzed enzyme extract from pea seed added to each reaction mixture contained 0.6 mg protein.

Fig. 6. The inhibition of pyruvate kinase by CaCl₂ at various concentrations of KCl. The standard assay pro-
chlorides on pyruvate kinase activity is illustrated by the curves in figure 5. In these experiments the standard assay procedure was used with the exception of the variation in divergent cation salt as indicated (fig 3). The activating capacities of Mg\(^{2+}\) and Mn\(^{2+}\) were approximately equal at all concentrations tested and the maximum enzyme activity was observed at a concentration of 5 × 10\(^{-3}\) M. The K\(_A\) calculated for either Mg\(^{2+}\) or Mn\(^{2+}\) was 9 × 10\(^{-4}\) M. Co\(^{2+}\) was an effective divalent cation activator at concentration up to 5 × 10\(^{-3}\) M. This concentration was optimum and resulted in approximately 87% of the activity obtained with equal concentrations of either Mg\(^{2+}\) or Mn\(^{2+}\). Concentrations of Co\(^{2+}\) greater than 5 × 10\(^{-3}\) M caused a sharp decline in activity. A slight activation with Be\(^{2+}\) was observed at concentrations near 10\(^{-3}\) M, but concentrations greater than 4 × 10\(^{-3}\) M were completely ineffective. Ni\(^{2+}\) and Ca\(^{2+}\) salts exhibited no activating capacity at concentrations ranging up to 0.2 M.

**Interaction of Cations on the Enzyme from Pea Seed:** The inhibitory action of Ca\(^{2+}\) at various concentrations of K\(^{+}\) is illustrated by the curves in figure 6. When the reaction mixtures contained K\(^{+}\) at a concentration of 0.01 M, a concentration of 10\(^{-3}\) M Ca\(^{2+}\) inhibited the activity approximately 50%. An increase in the K\(^{+}\) concentration up to 0.07 M resulted in a decline in the inhibition to 30%. Greater concentrations of K\(^{+}\) resulted in no further reversal of the Ca\(^{2+}\) inhibition. With K\(^{+}\) at 0.01 M and Ca\(^{2+}\) at 2.5 × 10\(^{-4}\) M in the assay medium approximately 13% inhibition of pyruvate kinase was manifested. Greater concentrations of K\(^{+}\) did not reverse this inhibition to any appreciable extent. The data in figure 6 were plotted by the reciprocal method (10) and it was concluded that the inhibition by Ca\(^{2+}\) was primarily but not completely, non-competitive with respect to K\(^{+}\).

Ca\(^{2+}\) inhibition of pyruvate kinase at various Mg\(^{2+}\) concentrations is illustrated by the curves in figure 7 A. When the Mg\(^{2+}\) concentration was 10\(^{-3}\) M and Ca\(^{2+}\) added at concentrations of 0.01 and 5 × 10\(^{-3}\) M, inhibitions of 92 and 70%, respectively, were obtained. Increasing the Mg\(^{2+}\) concentration of 0.01 M partially reversed the inhibition, resulting in 46 and 28% inhibition from Ca\(^{2+}\) concentrations of 10\(^{-3}\) M and 5 × 10\(^{-3}\) M, respectively. Further reversal of the inhibition by Ca\(^{2+}\) at these two concentrations was not possible by increasing the Mg\(^{2+}\) concentration in assays. The curves plotted by the Lineweaver and Burk method (10) (fig 7 B) and obtained from data in figure 7 A, suggests that Ca\(^{2+}\) inhibition of pyruvate kinase is complex and probably consists of a combination of both competitive and non-competitive types of inhibition.

Pyruvate kinase activity of pea seed acetone powder determined with the concentrations of K\(^{+}\) and Mg\(^{2+}\) used in the standard assay procedure was not influenced by the addition of Li\(^+\) or Na\(^+\) at concentrations up to 0.2 M. These results are not in accord with those reported for the enzyme from rabbit muscle (8).

K\(^{+}\) Activation of Enzyme from Other Species: Pyruvate kinase activity was found to be present in extracts of a variety of plant species. In general seeds were the best sources of the enzyme, but considerably was used with variation in concentrations of KCl and CaCl\(_2\) as indicated. The dialyzed enzyme extract from pea seed added to each reaction mixture contained 0.6 mg protein.

**Table II**

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>PLANT PORTION</th>
<th>KCl ADDED TO ASSAY MEDIUM (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.00</td>
</tr>
<tr>
<td><em>Avena sativa</em> Germ (embryo)</td>
<td>0.026</td>
<td>0.156</td>
</tr>
<tr>
<td><strong>Nicotiana Tobacum</strong> Leaf</td>
<td>0.026</td>
<td>0.038</td>
</tr>
<tr>
<td><em>Gossypium barbadense</em> Seed</td>
<td>0.0</td>
<td>0.030</td>
</tr>
<tr>
<td><em>Avena sativa</em> Seed</td>
<td>0.130</td>
<td>0.260</td>
</tr>
<tr>
<td>Avena sativa</td>
<td>0.0</td>
<td>0.117</td>
</tr>
<tr>
<td><strong>Zea Mays</strong> Seed</td>
<td>0.0</td>
<td>0.117</td>
</tr>
<tr>
<td><strong>Pisum sativum</strong> Seed</td>
<td>0.081</td>
<td>0.172</td>
</tr>
<tr>
<td>Pisum sativum</td>
<td>0.110</td>
<td>0.120</td>
</tr>
<tr>
<td><em>Apium graveolens</em> Petiole</td>
<td>0.026</td>
<td>0.035</td>
</tr>
<tr>
<td>Beta vulgaris</td>
<td>0.035</td>
<td>0.090</td>
</tr>
</tbody>
</table>

*Crude extract dialyzed 4 hrs against 0.01 M TRIS buffer, pH 7.4.
**Acetone precipitate of the crude extract.
††Crude undialyzed extract.

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**Fig. 7 A.** The inhibition of pyruvate kinase by CaCl\(_2\) at various concentrations of MgCl\(_2\). The standard assay procedure was used with variation in concentration of MgCl\(_2\) as indicated. The dialyzed enzyme extract from pea seed added to each reaction mixture contained 0.6 mg protein.

**Fig. 7 B.** The data of figure 7 A plotted by the method of Lineweaver and Burk.
erable activity was found in leaves and other tissues as indicated in table II. Little or no activity was found in extracts of roots from the various plants. An absolute dependence of K+ for activity was demonstrated for dialyzed extracts of cotton, oat and corn seeds. This evidence strongly supports the conclusion that K+ or some other univalent cation serves as indispensable activator for pyruvate kinase of higher plants. It is apparent from the data of table II that the addition of KCl resulted in a striking increase in the activity of the enzyme from all the sources studied. All enzyme extracts except those from cotton seed were maximally activated with a K+ concentration of 0.05 M. With the extract from cotton seed, however, 0.01 M K+ was sufficient for the maximum rate. This lower cation requirement was observed consistently in several experiments. It was considered desirable, therefore, to carry out further studies with the enzyme from this source.

An extract was prepared from cotton seed and dialyzed for 20 hours as described under Materials and Methods. In contrast to extracts from other sources the activity of these preparations remained active after this relatively long dialysis period. The influence of various concentrations of the chlorides of K+, Rb+, NH4+ and Na+ on the enzyme activity is presented graphically in figure 8 A. The data plotted by the Lineweaver and Burk method (10) is presented in figure 8 B. Maximum activation was observed with K+ at 0.01 M and increased concentrations resulted in a slight decrease in activity. A concentration of 0.03 M NH4Cl resulted in maximum activity with this salt and greater concentrations were less effective. The maximum activity of the enzyme was obtained with both Rb+ and Na+ at a concentration of 0.05 M, however, as observed with extracts from peas, the effectiveness of Na+ as an activator was much less than that of K+, NH4+ or Rb+.

The results of these experiments provide direct evidence that pyruvate kinase is widely distributed in the tissues of higher plants. The detailed studies of dialyzed extracts of pea seed indicate that pyruvate kinase from this material possesses properties

![Graph](image)

**Fig. 8 A.** The influence of various concentrations of univalent cation salts on the activity of pyruvate kinase from cottonseed extract. The standard assay procedure was used with variations in univalent cation salts as indicated. The enzyme extract (dialyzed for 20 hours) added to each reaction mixture contained 0.80 mg protein.

**Fig. 8 B.** The data of figure 8 A plotted by the method of Lineweaver and Burk. Calculation of regression lines by the method of least squares for the various chloride salts gave the following regression equations: Na+, \( Y = 0.032 + 22.5 X \); NH4+, \( Y = 0.011 + 7.1 X \); Rb+, \( Y = 0.032 + 6.1 X \); K+, \( Y = 0.009 + 6.1 X \).
that are very similar to those reported for the enzyme from animals and yeast. An absolute requirement for divalent cations was established and the maximum activity was obtained with either Mg\(^{2+}\) or Mn\(^{2+}\) salts at a concentration of approximately 4 x 10\(^{-3}\) M. These findings correspond closely to those reported for the enzyme from _Escherichia coli_ (22) and animal tissues (11). Co\(^{2+}\) and to a small extent Be\(^{2+}\) served as divalent cation activators. As far as the writers are aware the effects of these two cations on the enzyme from other sources has not been determined.

The system from all the plant sources investigated exhibited a striking requirement for univalent cations. The activity of the enzyme was essentially independent of the anion present with the exception of I\(^-\) and Br\(^-\), which were slightly inhibitory at concentrations greater than 0.05 N. With the extracts from either pea or cotton seed, K\(^+\), NH\(_4\)^+ or Rb\(^+\) were effective activators and presumably NH\(_4\)^+ or Rb\(^+\) would have substituted for K\(^+\) as an activator for the enzyme from wheat, tobacco, oat, corn, celery and red beet which were the other species surveyed. The work of Kaehm and Boyer (8) on the rabbit muscle enzyme indicated that K\(_A\) values for K\(^+\), NH\(_4\)^+ or Rb\(^+\) were all near 0.011 M. Our calculations (fig 4 B) with the pea seed enzyme revealed K\(_A\) values of 2.4 x 10\(^{-3}\), 1.2 x 10\(^{-3}\), 4.2 x 10\(^{-3}\) and 1.4 x 10\(^{-3}\) M for K\(^+\), NH\(_4\)^+, Rb\(^+\) and Na\(^+\), respectively, which are roughly one-fifth the value reported for the enzyme from rabbit muscle. The K\(_A\) values for these cations determined with the enzyme from cotton seed were of the same order of magnitude with exception of the value for K\(^+\) which was 1.5 x 10\(^{-3}\) M. Even though the extracts from pea or cotton seed were not purified it seems apparent that the enzyme from these sources has a greater affinity for K\(^+\), NH\(_4\)^+ or Rb\(^+\) than does the enzyme from rabbit muscle. Boyer (2) has pointed out that the K\(_A\) for K\(^+\) with the rabbit muscle enzyme is 37 times greater than that for the enzyme from the muscle of Anodonta. If one compares the velocities of the pea seed enzyme reactions at points where the maximum velocities were observed with the various cations relative values for: K\(^+\), Rb\(^+\), NH\(_4\)^+, Na\(^+\), Li\(^+\) are 100, 85, 76, 31, 0, respectively, and the comparable values for the cotton seed enzyme are almost the same. Certain of these relative rates may be compared with those obtained with rabbit muscle (8) which are: K\(^+\), Rb\(^+\), NH\(_4\)^+ =100, 84, 72, respectively. In a comparison of the effects of univalent cations of the enzyme from pea seed (fig 4 A and B) with those of the enzyme from cotton seed (fig 8 A and B) it is apparent that the maximum velocity of the former was obtained at a concentration of 0.05 N, whereas the maximum velocity with the latter enzyme was observed at a KCl concentration of 0.01 N. A decision on the possible significance of this difference perhaps should be delayed until studies have been conducted with enzymes that have been highly purified.

Some of the observations concerning the capacities of various univalent cations to activate pyruvate kinase from plants may have a bearing on physiological problems that involve these elements. In the absence of other univalent cations (fig 4 A) Na\(^+\) was a weak activator but Li\(^+\) exhibited no activating capacity. In contrast to the results reported with pyruvate kinase from muscle (8) neither Na\(^+\) nor Li\(^+\) at concentrations up to 0.2 N counteracted the activating influence of K\(^+\) at a concentration of 0.05 N. On the basis of these observations it would seem logical to conclude that pyruvate kinase in the animal organism may represent a locus of physiological antagonism between K\(^+\) and Na\(^+\). On the other hand, the stimulatory effects of low concentrations of these two cations on pyruvate kinase in plants would be expected to be additive. This point may be related to the well known capacity (23) of Na\(^+\) salts to stimulate plant growth under conditions where the K\(^+\) supply is limited. Since both Rb\(^+\) and NH\(_4\)^+ are relatively efficient activators of pyruvate kinase and other enzymes such as phosphotransacetylase (19) and aldehyde dehydrogenase (1) it is necessary to consider the possible physiological role of these cations as activators for these enzymes in living plants. One would expect that either NH\(_4\)^+ or Rb\(^+\) would substitute to some degree for K\(^+\) as a univalent cation in plant metabolism. In this regard Richards (17) has reported that the addition of Rb\(^+\) to cultures of barley decreased the K\(^+\) requirements for growth. In considering the possible capacity of certain cations to substitute for others in specific enzymes of an organism, one must keep in mind the capacity of the organism, in general, to tolerate the concentrations of the particular cations that are required for activation of the specific systems.

The evidence presented in figures 6, 7 A and 7 B indicates that Ca\(^{2+}\) is a relatively strong inhibitor of pyruvate kinase and that the inhibition is partially competitive with respect to both K\(^+\) and Mg\(^{2+}\). This is interesting in view of the well known antagonistic effects of Ca\(^{2+}\) toward both K\(^+\) and Mg\(^{2+}\) (16). The mechanism of the cation activation or inhibition of pyruvate kinase is not known, but from the work of Kaehm and Boyer (8) and Hers (6) one might speculate that Mg-ADP is the active cofactor and that Ca-ADP is inactive and competes to some extent for an active enzyme site. Ca\(^{2+}\) also may render the enzyme inactive by combining with a site requiring K\(^+\). The elucidation of the mechanism of activation and interaction of cations on this enzyme must await further investigation.

**Summary**

Experiments have been conducted to determine the properties and specifically the cation requirements of pyruvate kinase from certain species of higher plants. The concentration of both ADP and PEPA required for saturation of the enzyme from a dialyzed extract of pea seed were determined and from these data the appropriate Michaelis constants were estimated. After the cation requirements were established, evidence was obtained indicating that enzyme activity
was proportional to both enzyme concentration and to reaction time under the specified conditions.

Detailed studies with the enzyme from pea seed demonstrated an absolute divalent cation requirement that was satisfied by salts of Mg"² or Mn"². The optimum concentration of these cations was \(5 \times 10^{-3}\) M. Co"² at this concentration was approximately 80 % as effective as the other two cations. The chloride salts of Ni"² or Ca"² exhibited no activating capacity and Be"² was only slightly stimulatory.

The enzyme from pea seed also required a univalent cation salt for maximum activity and this requirement was satisfied by the chloride salts of K"⁺, Rb"⁺ or NH₄"⁺. A concentration of 0.05 M resulted in maximum activity. NaCl at a concentration of 0.05 M, which was optimum, resulted in approximately 20 % of the activity obtained with a comparable concentration of K⁺. The enzyme activity was essentially independent of the anion present with the exception of Br⁻ and I⁻ which were slightly inhibitory at concentrations greater than 0.05 M. An absolute requirement of univalent cations for the enzyme from pea seed was not established, however, the low activity of the preparation in absence of added univalent cations was accounted for by the content of K⁺ and Na⁺ in the extracts. The Michaelis constants (Kₐ) for the various univalent cation activators were approximately one-fifth the value reported for pyruvate kinase from rabbit muscle.

Pyruvate kinase activity was demonstrated in extracts of the seeds of wheat, cotton, oat, corn and beet; in the leaves of tobacco and peas and in the petioles of celery. The enzyme activity of extracts from all these sources was strikingly stimulated by KCl. An absolute requirement for univalent cations was demonstrated with extracts of cotton, oat and corn seed. The pyruvate kinase in those extracts, that did not show an absolute dependence on univalent cations, would not withstand dialysis periods of sufficient length to remove all endogenous univalent cations.

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**LITERATURE CITED**

17. Richards, F. J. Physiological studies in plant nutrition. XI. The effect on growth of rubidium with low potassium supply and modification of this effect by other nutrients. Annals Bot. 5: 263-296. 1941.