Effect of Potassium & Other Univalent Cations on Activity of Pyruvate Kinase in Pisum Sativum [1, 2]

Harold J. Evans

Department of Botany & Plant Pathology, Oregon State University, Corvallis, Oregon

Potassium is a major component of both plant and animal organisms, yet information on the role of this element in metabolism is limited. A compilation (1) of the inorganic composition of many plant species reveals that a major portion of normal leaves contain from 1 to 4 % K^+ on a dry weight basis. Potassium deficiency symptoms are noted when the K^+ contents of leaves range from 0.1 to 1.3 % of their dry matter. There are many general statements (3) to the effect that K^+, Na^+, and other ions that are present in biological materials in high concentrations function in the osmotic regulation of tissue fluids. Undoubtedly this is an important role of the so-called bulk ions, but relatively recent biochemical investigations (3) have provided convincing evidence that a series of enzyme systems require K^+, NH_4^+ or Rb^+ for activity and that the concentration of these ions needed for maximum reaction velocities is in the same range as the concentration of K^+ in normal biological materials.

Pyruvate kinase was one of the first enzyme systems shown to require a univalent cation. Initial experiments by Boyer, et al., (2) and further investigations by Lardy and Ziegler (6) and Kachmar and Boyer (5) provided evidence that either K^+, NH_4^+ or Rb^+ was essential for the activity of pyruvate kinase from animal sources. Sodium ions activated the enzyme slightly and Li^+ and Ca^{++} were inhibitory. Miller and Evans (9) demonstrated that pyruvate kinase from a series of higher plants also require K^+, NH_4^+ or Rb^+ and that a concentration near 0.04 N was necessary for maximum reaction velocity.

Several attempts have been made by the author to determine the effect of K^+ deficiency in higher plants on pyruvate kinase in tissue extracts. In all cases, crude extracts of the species investigated contained an active phosphatase which catalyzed the hydrolysis of phosphoenolpyruvate (PEP). The enzymic cleavage of the substrate for pyruvate kinase has prevented an accurate determination of the activity of this enzyme. McCollum, et al. (8) also have observed appreciable phosphatase activity in their investigations of the pyruvate kinase in higher plant species. A possible means of inhibiting certain plant phosphatases is suggested by the work of Spencer (10) who found that low concentrations of molybdate inhibited the hydrolysis of a series of phosphate esters by an acid phosphatase from tomato. Phosphoenolpyruvate was not used as a substrate for the phosphatase in Spencer's investigations.

The purpose of the experiments reported here was to develop procedures that will allow valid pyruvate kinase assays in crude extracts of higher plants and to determine the effect of a range of K^+ and other univalent cation concentrations in nutrient media on the pyruvate kinase activity of cell-free extracts of the pea plant.

Materials & Methods

Plant Materials. Garden peas (*Pisum sativum* 'Blue Bantam') were germinated in flats of "Perlite" and 5 days after planting, 4 seedlings were transferred to each of a series of polyethylene culture vessels. Each vessel contained 10 liters of nutrient solution and was protected from contamination by a polyethylene cover fitted with holes for insertion of seedlings. In all experiments where a composition of the nutrient solutions was varied, eight treatments were utilized and each was replicated three times. The vessels were arranged in a randomized block design in the greenhouse and the plants were supplied with about 500 ft-c of fluorescent light for 14 hours per day in addition to normal sunlight.

The complete nutrient solution (treatment No. 4 in table I) contained the following concentrations of macronutrient salts in mmoles per liter: $Mg(H_2PO_4)_2$, 1.0; $Ca(NO_3)_2 \cdot 4H_2O$, 6.0; $MgSO_4 \cdot 7H_2O$, 2.0; K_2SO_4, 6.0; $CaCl_2 \cdot 2H_2O$, 0.5. The concentrations of the essential micronutrient elements in μ moles per liter and the salts used to supply these for nutrient solutions were as follows: 4.6 B as H_3BO_3; 4.6 Mn as $MnSO_4 \cdot 4H_2O$; 0.8 Zn as $ZnSO_4 \cdot 7H_2O$; 0.3 Cu as $CuSO_4 \cdot 5H_2O$; 0.1 Mo as $Na_2SO_4 \cdot 2H_2O$ and 35.8 Fe as hydrogen ferric ethylenediamine di-*o*-hydroxyphenylacetate (Geigy Chemical Co., Yonkers, N. Y.). The complete solution (treatment No. 4 in table I) was altered to obtain the treatments listed in tables I through IV. The K_2SO_4 concentration was reduced and Na_2SO_4 was substituted at the appropriate concentrations (table I) to obtain treatments 5 and 6. The amount of K_2SO_4 was varied

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in treatments 7 and 8 and $(NH_4)_2SO_4$ increased in acidity during the growth period, solid $CaCO_3$ was added to maintain the pH at the desired value. The original volume of nutrient solutions was maintained by addition of distilled water. All solutions were changed at 10 day intervals.

Experiment I from which results are reported in tables I, II, III, and IV was initiated on November 22, 1961 and terminated January 5, 1962. Experiment II (table IV) was initiated on June 17, 1962 and plants harvested on July 30. Six to ten days prior to the final harvest date, a 4 g sample of leaves was collected from each culture in the area of the sixth and seventh nodes above cotyledons. Also a 4 g sample of terminal roots was collected at random from each culture. Roots were washed thoroughly with cold distilled water and blotted with paper towels. Both roots and leaves were stored (for 1 hr or less) in crushed ice until they were assayed. The remainder of the plants were harvested after enzyme assays were completed, roots were washed with distilled water, and both roots and shoots dried in a forced draft oven at 70° and weighed. The dry material was ground to pass a 40 mesh screen and used in inorganic analyses.

Preparation of Extracts. Each sample of pea roots or leaves (4 g) was placed in a mortar containing 15 ml of 0.05 M Tris buffer at pH 7.5. The material was macerated first with a mortar and pestle and then macerated again for 3 minutes in a Ten Broeck homogenizer. The suspension was centrifuged at 15,000 × *g* for 10 minutes and the supernatant fluid used as a source of enzyme.

Extracts of cucumber seed (*Cucumis sativus* L., "Early White Spine") were prepared by macerating for 3 minutes in an Omni mixer (Ivan Sorvall, Inc., Norwalk, Conn.) 3 g of dry seed with 15 ml of 0.05 M Tris buffer at pH 7.5. The suspension was centrifuged at 17,000 × *g* for 15 minutes and the supernatant liquid used as a source of enzyme.

All preparatory operations were carried out at 0 to 4°. Extracts were stored in crushed ice and assayed for enzyme activity within 2 hours after preparation.

Enzyme Assays. Pyruvate kinase was assayed by the determination of the ADP-dependent formation of pyruvate from PEP in presence of enzyme. The procedure is an adaptation of that of Kachmar and Boyer (6) and, with the exception of the Tris molybdate addition, is the same as that described previously (10).

In the standard assay, the reaction mixture in a final volume of 1 ml contained the following constituents in μmoles: 50 of Tris buffer at pH 7.4; 1.5 of the cyclohexylammonium salt of PEP; 2.5 of Tris ADP (10); 10.0 of $MgSO_4$; 50 of KCl and 1.25 Tris molybdate. Sufficient enzyme extract was added to obtain 0.05 to 0.50 μ mole of pyruvate in 10 minutes. A reaction mixture lacking ADP served as a negative control for pyruvate kinase assays. An additional control containing either boiled or acid-treated enzyme was included with each test series. Reactions were terminated by the procedure described previously (9).

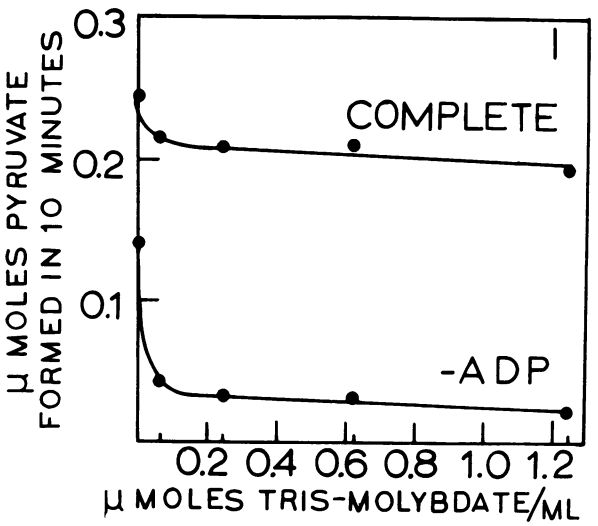
The phosphatase activity of extracts was measured in reaction mixtures and under conditions identical with those used for the pyruvate kinase assays except that ADP was not included in the mixtures. In these experiments, a complete mixture containing boiled extract instead of active extract served as a negative control.

Other Determinations. Potassium and Na^+ were determined flame-photometrically by the procedure described by Johnson and Ulrich (4) and the protein content of extracts was determined by the Folin method (7).

Results

Inhibition of Interfering Phosphatase. Before it was possible to study the effect of univalent cation concentrations in nutrient solutions on the activity of pyruvate kinase in plant extracts, it was necessary to develop a procedure that would inhibit the phosphatase which cleaves PEP. The curves in figure 1 show that an $(NH_4)_2SO_4$ fraction of an extract of pea leaves catalyzed the formation of about 0.25 μ mole of pyruvate in 10 minutes when the reaction mixture contained all reactants except Tris molybdate. When ADP was omitted, approximately 0.14 μ mole of pyruvate was produced in the standard assay. This rapid formation of pyruvate in the absence of ADP was observed with extracts of leaves from all species tested and was not appreciably influenced by dialysis, $(NH_4)_2SO_4$ fractionation, or aging. The cleavage of PEP in absence of ADP apparently is due to a phosphatase whereas the ADP-dependent formation of pyruvate from PEP is due to pyruvate kinase. This conclusion is in agreement with that of McCollum et al. (8). The curves in figure 1 show clearly that the addition of 0.1 to 1.2 μ moles of Tris molybdate in absence of ADP decreased the rate of formation of pyruvate from 0.14 to approximately 0.04 μ mole in 10 minutes. In contrast, the effect of Tris molybdate on the rate of pyruvate formation in the complete reaction is relatively small. Since pyruvate formation in the complete reactions (fig 1) may represent a combination of pyruvate kinase and phosphatase activities, it appeared desirable to determine the effect of molybdate on pyruvate kinase in an extract that was devoid of an enzyme capable of catalyzing the hydrolysis of PEP.

The results are presented in figure 2 from an experiment similar to that shown in figure 1 except that an extract of cucumber seed instead of pea leaves was used as a source of enzyme. From the lower curve (fig 2) it is obvious that little or no pyruvate was formed when ADP was omitted from the reaction mixture; however, pyruvate formation was rapid in the complete mixtures. Since there is no evidence of appreciable phosphatase activity in the



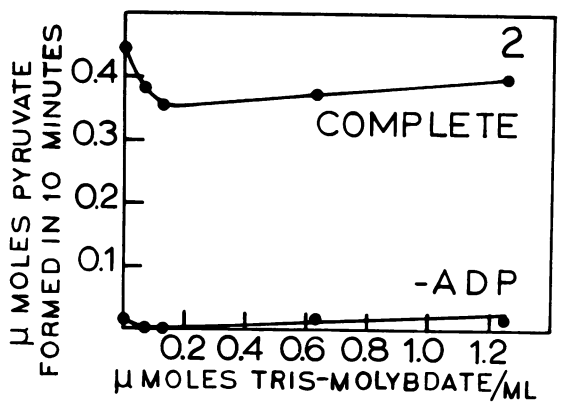


FIG. 1 (*upper*). The effect of molybdate on pyruvate kinase and phosphatase in an extract of pea leaves. The standard assay procedure was used with variation in ADP and Tris molybdate as indicated. The extracts in each reaction mixture contained 0.9 mg of protein. The extract was a 30 to 50 % $(NH_4)_2SO_4$ precipitate of a crude extract of normal pea leaves.

FIG. 2 (*lower*). The effect of molybdate on pyruvate kinase and phosphatase in extracts of cucumber seed. The procedure was the same as that described for the experiment in figure 1 except that a crude extract of cucumber seed was used as the enzyme. The extract in each reaction mixture contained 0.5 mg of protein.

cucumber seed extracts, it is possible to determine the effect of molybdate additions on pyruvate kinase per se. From the curve showing the complete reactions, it is apparent that Tris molybdate at low concentrations decreased the rate of pyruvate formation from 0.43 μ mole with no molybdate to 0.36 μ mole in 10 minutes where 0.14 μ mole of Tris molybdate was added. The data (fig 1 & 2) show clearly that Tris molybdate inhibits the phosphatase to a much greater extent than the kinase.

The Tris salt of molybdate was used as the inhibitor to avoid introduction of an alkali metal cation in reaction mixtures. Previous investigation (9) has shown that neither Tris nor chloride ions appreciably influence the activity of pyruvate kinase. Experiments conducted to compare the inhibitory effect of Na^+ or K^+ molybdate with that of Tris molybdate showed no consistent differences.

Effect of Treatments on Yields and Na^+ and K^+ Contents. As expected, the concentration of K^+ in the solutions markedly influenced the yield and appearance of pea plants (table I). Leaves of plants growing in culture solutions lacking K^+ were extremely necrotic and produced only 1.5 g of dry matter per culture. Undoubtedly the K^+ reserves in the seed were sufficient for the limited growth that was obtained. Treatment 2 (0.16 meq K) resulted in mild deficiency symptoms of plants whereas 1.00 and 6.00 meq of K^+ per liter respectively (treatments 3 & 4) produced plants that were normal in appearance. Substitution of Na^+ for K^+ (treatments 5 & 6) reduced to some extent the leaf necrosis that was characteristic of K^+ deficiency, but the yield increases from the Na^+ were not statistically significant. The addition of 6 meq of NH_4^+ to cultures lacking K^+ (treatment 7) resulted in a severe necrosis of leaves and death of plants within 2 weeks. The addition of 5.84 meq of NH_4^+ to cultures with 0.16 meq of K^+ (treatment 8) alleviated the mild deficiency symptoms that were apparent in plants receiving 0.16 meq of K^+ and no Na^+ or NH_4^+ but the yield of plants receiving this treatment was not significantly different from that of plants receiving 0.16 meq of K^+ alone (treatment 2).

Table I

Effect of K^+ & Other Univalent Cations in Nutrient Solution on Yield of Pea Plants

No.	Culture treatment (meq/liter)	Appearance	Dry wt (g/culture*)
1.	0.00 K^+	Severely deficient	1.5
2.	0.16 K^+	Slightly deficient	5.6
3.	1.00 K^+	Normal	4.5
4.	6.00 K^+	Normal	3.7
5.	0.00 K^+, 6.00 Na^+	Severely deficient	2.1
6.	0.16 K^+, 5.84 Na^+	Normal	5.1
7.	0.00 K^+, 6.00 NH_4^+	Severely deficient**	...
8.	0.16 K^+, 5.84 NH_4^+	Normal	3.2
LSD (5 %)			1.4

* Means of 3 replicate cultures each containing 5 plants.
** All plants died within 3 weeks.

As indicated in table II, the concentration of K^+ or Na^+ in the culture medium markedly affected the K^+ and Na^+ contents of the plant material. The K^+ and Na^+ in plants grown with solutions lacking

Table II

Effect of K^+ & Other Univalent Cation Concentrations in Nutrient Solutions on K & Na Contents of Pea Plants

No.	Treatment (meq/liter)	K in dry matter*		Na in dry matter*	
		Shoots %	Roots %	Shoots %	Roots %
1.	0.00 K^+	0.68	0.36	0.15	1.11
2.	0.16 K^+	1.73	0.55	0.07	0.62
3.	1.00 K^+	3.11	1.66	0.08	0.76
4.	6.00 K^+	3.24	4.04	0.18	0.04
5.	0.00 K^+, 6.00 Na^+	0.89	0.61	0.80	2.87
6.	0.16 K^+, 5.84 Na^+	1.85	0.55	0.15	3.29
7.	0.00 K^+, 6.00 NH_4^+ **	...	...	...	...
8.	0.16 K^+, 5.84 NH_4^+	1.86	1.71	0.05	0.80
LSD (5 %)		0.76	...	0.11	...

* Means of 3 replicate cultures each containing 5 plants except the K and Na contents of roots which were determined on a thoroughly washed composite sample. Initial samples of roots were contaminated with K and Na from the nutrient medium.

** All plants died within 2 weeks.

these elements very likely were derived from the seed or from impurities in salts and air. It is interesting to note that the Na^+ content of roots was markedly increased as the K^+ content of the nutrient medium was decreased.

Effect of Treatments on Pyruvate Kinase. Samples of leaves were collected from cultures between 33 and 37 days after planting and assayed for pyruvate kinase both with and without KCl in the reaction mixtures (table III). The results of assays where K^+ was omitted in assay mixtures show that extracts from plants growing in a severely K^+-deficient medium (treatment 1) exhibited significantly less pyruvate kinase activity than extracts of plants grown with adequate K^+ (treatment 4) or with a low level of K^+ (0.16 meq K^+ & 5.84 meq of Na^+ or NH_4^+) (treatments 6 & 8). The addition of Na^+ or NH_4^+ to nutrient solution lacking K^+ (treatments 5 & 8) failed to significantly increase the pyruvate kinase activity of leaves.

Table III

Effect of K^+ & Other Univalent Cations on Pyruvate Kinase Activity of Pea Leaves

No.	Nutrient solution treatment (meq/liter)	Pyruvate kinase activity of leaves*	
		Without K^+ in assay	With 0.05M KCl in assay
		(μ moles pyruvate formed /10 min/mg protein)	
1.	0.00 K^+	0.29	0.36
2.	0.16 K^+	0.19	0.28
3.	1.00 K^+	0.28	0.37
4.	6.00 K^+	0.44	0.49
5.	0.00 K^+, 6.00 Na^+	0.26	0.37
6.	0.16 K^+, 5.84 Na^+	0.48	0.55
7.	0.00 K^+, 6.00 NH_4^+	...	...
8.	0.16 K^+, 5.84 NH_4^+	0.53	0.55
LSD (5 %)		0.14	N.S.

* All results are means of determinations on three replicate cultures each containing 5 plants.

When the extracts of leaves from cultures receiving the various treatments were assayed in reaction mixtures containing 0.05 M KCl, the pyruvate kinase activities of the various extracts were not significantly different at the 5 % level (table III). The effects of treatments on the enzyme activity of leaf extracts, however, resulted in trends similar to those obtained when K^+ was omitted in reaction mixtures.

Enzyme assays of leaf extracts from replicate cultures receiving the same treatment exhibited considerable variability. This is illustrated by the relatively large value required for a statistically significant difference between treatments.

The concentration of K^+ in nutrient solutions had a much greater effect on the pyruvate kinase activity of extracts of roots than of leaves (table IV). The data obtained in experiment I by using enzyme assay mixtures lacking added K^+, show that the increase in K content of nutrient solutions from 0.0 to 6.0 meq per liter (treatments 1 through 4) resulted in a progressive increase in the pyruvate kinase activity of root extracts. A comparison of the activities of root extracts (treatments 1 & 5) shows that the addition to nutrient solutions of Na^+ in place of K^+ appreciably increased the enzyme activity in roots. The addition of 6 meq of NH_4^+ to nutrient solution resulted in severe toxicity symptoms and death of plants.

Root extracts from all cultures, when assayed for pyruvate kinase with 0.05 M KCl in reaction mixtures, showed a decreased activity in extracts of the severely deficient plants (treatment 1). The difference in pyruvate kinase activities of extracts from roots receiving treatments other than No. 1 (0.0 meq of K^+ per liter) were not significant in experiment I, but were significant at the 10 % confidence value

Table IV

Effect of K^+ & Other Univalent Cations on Pyruvate Kinase Activity of Pea Roots

No.	Nutrient solution treatment (meq/liter)	Experiment I Pyruvate kinase activity*		Experiment II Pyruvate kinase activity*	
		Without K^+ in assay	With 0.05 M KCl in assay	Without K^+ in assay	With 0.05 M KCl in assay
		(μ moles pyruvate formed /10 min/mg protein)			
1.	0.00 K^+	0.19	0.34	0.04	0.12
2.	0.16 K^+	0.34	0.72	0.07	0.36
3.	1.00 K^+	0.52	0.72	0.12	0.54
4.	6.00 K^+	0.64	0.79	0.26	0.92
5.	0.00 K^+, 6.00 Na^+	0.43	0.81	0.22	0.37
6.	0.16 K^+, 5.84 Na^+	0.36	0.64	0.27	0.55
7.	0.00 K^+, 6.00 NH_4^+**	...	...	...	...
8.	0.16 K^+, 5.84 NH_4^+	0.34	0.62	0.26	0.79
LSD (5%)		0.19	0.28	0.16	NS
LSD (10%)					0.40

* All determinations are means of measurements on 3 replicate cultures each containing 5 plants.
** Plants died within 2 weeks.

in experiment II. In general, the addition of KCl to assay mixtures resulted in increased pyruvate kinase activities.

The trends of the data presented for the two experiments are similar. Pyruvate kinase activity of root extracts from experiment II were generally lower than those of experiment I.

Discussion

Investigation of the properties of pyruvate kinase from higher plants (9) has provided conclusive evidence that the partially purified enzyme requires an univalent cation activator. The requirement is satisfied by K^+, NH_4^+, or Rb^+, and is not appreciably influenced by a series of different anions. It is obviously interesting, therefore, to attempt to determine the effects of K^+ deficiency and different concentrations of other univalent cations in nutrient solutions on the activity of pyruvate kinase in plant tissues. Experiments of this type are complicated because crude extracts of leaves or roots of the several species surveyed contain a phosphatase that rapidly hydrolyzes PEP which is the substrate for the pyruvate kinase reaction. It may be possible to free the extracts of phosphatase by purification but this approach is not considered to be feasible because the necessary purification procedures are usually involved and not sufficiently reproducible to be useful in the comparison of activities of extracts from several sources. The problem resulting from phosphatase in crude plant extracts has been simplified considerably by the addition of a low concentration of molybdate to pyruvate kinase assay mixtures. Molybdate salts strikingly inhibit the phosphatase and effect the activity of the pyruvate kinase only slightly. Although use of molybdate as an inhibitor permits valid pyruvate kinase assays of extracts that contain high phosphatase activity, pyruvate kinase measurements of extracts from replicate cultures of a particular treatment have varied considerably. Statistical procedures are necessary, therefore, to determine the magnitude of the difference between treatments that is meaningful.

Pyruvate kinase activities of extracts of roots and leaves of plants receiving various treatments show that the K^+ concentration in the nutrient solution has a marked effect on activity. The effects were greatest when the enzyme assays were carried out in absence of additional K^+ in the assay mixtures. Experiments with root extracts (table IV) provide some evidence that the addition of Na^+ or NH_4^+ to nutrient solutions containing insufficient K^+ caused an increased pyruvate kinase activity in extracts. These responses, although rather weak, are consistent with the finding that Na^+ is a weak and NH_4^+ a relatively good activator for purified pyruvate kinase from plant sources (9).

The greatest reduction in pyruvate kinase was obtained with extracts of plants that were severely deficient in K^+. Whether the diminished enzyme activity of the deficient plants was associated with K^+ deficiency per se obviously should be considered. Potassium was supplied to cultures of severely K^+ deficient plants and then tissues were assayed for pyruvate kinase 8 days after the K^+ addition. Some recovery in enzyme activity was observed; however, the activity did not reach the level of that in normal plants. The lack of complete recovery was attributed to the fact that plants were approaching senescence when the experiment was completed.

Summary

An investigation was conducted to determine the effect of K^+ and other univalent cation concentra-

tions in nutrient solutions on the activity of pyruvate kinase in extracts of leaves and roots of pea plants. Valid assays for pyruvate kinase in crude extracts could not be made until interfering phosphatase activity was reduced. Low concentrations of molybdate effectively reduced the phosphatase reaction and had little effect on the pyruvate kinase activity.

Extracts of roots and leaves of pea plants grown with different levels of K^+ in nutrient solutions were assayed for pyruvate kinase, first in a medium lacking exogenous univalent cations and second in a medium containing 0.05 N KCl. In the assays with no KCl added to the reaction mixtures, pyruvate kinase activities of both roots and leaves of severely K^+-deficient plants were considerably less than those of the extracts of normal roots and leaves. Extracts of plants grown in solutions in which either NH_4^+ or Na^+ was substituted completely or partially for K^+ exhibited greater pyruvate kinase activities than extracts of plants grown with insufficient K^+ but without Na^+ or NH_4^+. The addition of KCl to reaction mixtures resulted in increased enzyme activities of all extracts of plants regardless of the treatments.

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