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Pyruvate Kinase Activity of Wheat Plants Grown under Potassium Deficient Conditions[1]

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Abstract. **The activity of pyruvate kinase was determined in the first leaves of wheat plants grown under K^+-deficient conditions. An enhancement of the enzyme activity compared with the normal plants was found to start from eighth day of growth, and about 4-fold increase in the enzyme activity was observed in 14-day wheat leaves. The addition of K_2SO_4 to the nutrient solution given to the K^+-deficient plants at tenth day resulted in the restoration of the enzyme activity to the normal level after 3 days. The levels of K^+ as well as carbohydrates and chlorophyll were found to return normal over the same period. These findings are discussed in relation to the metabolic pattern of plants at the early stages of K^+-deficiency.**

Many biochemical studies have been carried out to elucidate the essentiality of K^+ in plant metabolism. Evans and Sorger (6) have critically reviewed the current status of our knowledge in the area. In investigating the relationship between the effect of different levels of nutrient K^+ on plant growth and on the activity of a K^+-requiring enzyme, Evans (5) noted that the activity of pyruvate kinase (ATP: pyruvate phosphotransferase) was appreciably decreased in the K^+-deficient pea plants. Recently Evans and his group (15) have demonstrated the requirement of K^+ as well as NH_4^+ and Rb^+ for the maintenance of the structural conformation of pyruvate kinase, using immunoelectrophoretic techniques. However, there is the possibility that some of the biochemical syndromes manifested in K^+-deficient plants during long-term experiments may be indirectly related to K^+-deficiency. The purpose of the present experiment is to examine the effect of K^+-deficiency on the activity of pyruvate kinase in wheat leaves, during the early stages of growth. In contrast to Evans' report we found that the enzyme activity was appreciably enhanced when plants were grown under K^+-deficient condition and the subsequent addition of K^+ to the nutrient medium caused the rapid restoration of the enzyme activity to the normal level.

Materials and Methods

Growing Plants. Wheat plants (*Triticum aestivum* L. var. Norin No. 61) were grown at 14° in sand beds supplemented with the standard culture medium (18) of the following composition (in mmoles per liter) which is the modification of Evans' procedure (5): $Ca(H_2PO_4)\cdot 4H_2O$, 1.0; $CaCl_2\cdot 2H_2O$, 0.25; $Ca(NO_3)_2\cdot 4H_2O$, 3.0; $MgSO_4$, 2.4; Fe-citrate, 0.018; K_2SO_4, 2.4; H_2BO_3, 2.3 × 10^{-3}; $MnSO_4\cdot 4H_2O$, 2.3 × 10^{-3}; $ZnSO_4\cdot 7H_2O$, 0.4 × 10^{-3}; $CuSO_4\cdot 5H_2O$, 0.15 × 10^{-3}; and $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, 0.05 × 10^{-3}. For growing K^+-deficient wheat plants, K_2SO_4 was omitted from the nutrient solution. Where the effects of different levels of K^+ were studied concentrations of K_2SO_4 were varied as indicated. When the first leaf started to emerge at about 2 days after germination, the nutrient solution was applied daily.

Preparation of Enzyme Solutions. Five grams of first leaves were harvested at each growth stage and were macerated with 15 ml of 0.05 M tris-HCl buffer (pH 7.5) in a prechilled mortar. The resulting homogenate was spun at 10,000 × *g* for 10 minutes after passing through several layers of cheesecloth. Three ml aliquots of the supernatant fluid were then applied to a column of Sephadex G-25 (1.8 × 20 cm), which was equilibrated with 0.05 M tris-HCl buffer (pH 7.5). An aliquot of the eluate collected quantitatively was used as an enzyme source. All the manipulations were carried out at or near 0°, and the enzyme assay was conducted within 30 minutes after collecting the enzyme solution.

Analytical Method. The standard reaction mixture for the measurement of pyruvate kinase activity contained the following (in μmoles): tris-HCl buffer (pH 7.4), 50; phosphoenolpyruvate (PEP) (tricyclohexylammonium salt), 1.5; ADP (Na-salt), 2.5; $MgSO_4$, 10; KCl, 50; and enzyme solution, 0.5 ml (equivalent to about 1.0 mg protein) in a total volume of 1.0 ml. Whenever necessary 1.25 μmoles tris-molybdate was added to the reaction mixture to inhibit phosphatase activity in the crude enzyme preparation (5). In the control assays, ADP was omitted from the reaction mixture. The assay mixture was incubated at 37° for 10 minutes.

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At the end of this time, 1.0 ml of 0.0125 % 2,4-dinitrophenylhydrazine in 2 N HCl was added, and the amount of pyruvate formed was assayed spectrophotometrically in an alkaline condition (8).

Protein content was determined colorimetrically using Nessler's reagent after the digestion of materials by a micro-Kjeldahl procedure. For estimating chlorophyll content, 1 g of fresh first leaves was extracted with 80 % (v/v) aqueous acetone and the determination was made by the method of Arnon (1). For sugar analyses, leaves were first dried at 95° for 30 minutes, then at 45° overnight over $CaCl_2$ in a desiccator. Subsamples of the dried leaves were extracted repeatedly with 10 ml of 80 % (v/v) alcohol. To prevent acid hydrolysis of sugar components during extraction, a small amount of $CaCO_3$ was added to the ethanol solution. The extracts were deproteinized with lead-acetate and deionized with a mixed bed of IR-120 and IR-4B according to the method of Mori and Nakamura (10). Total and reducing sugars were determined by the phenol-H_2SO_4 (4) and Somogyi (14) methods respectively.

The content of K^+ was analysed by flamephotometry (12). All the determinations of enzymic activities as well as chemical composition were carried out in duplicate and average values are presented.

Reagents. ADP (Na-salt) and PEP (tricyclohexylammonium salt) were purchased from Sigma Chemical Company, U.S.A., and Boehringer, Germany, respectively.

Abbreviations Used. The terms of K(+) and K(−) denote conditions of growth in which the plants were given nutrient solutions with sufficient or deficient K^+ levels.

Results

The content of K^+ as well as of carbohydrates and chlorophyll was measured in the first leaves of K(+) and K(−) wheat plants (table I). There was a drastic decrease in the content of K^+ in K(−) plants. The K^+-content in normal plants looks abnormally high, about 8 % (dry wt basis). However, the use of young wheat plants in our study might have caused the high value. In fact Evans and Sorger (6) have cited 7 to 8 % K^+ content as the normal value in some plant tissues. The results also show that K^+-deficiency did not appreciably affect the content of either total or reducing sugars up to the sixth day. After the eighth day, however, the content of total or reducing sugars was higher in K(−) than in K(+) plants. The higher carbohydrate content in the K(−) plant leaves is consistent with a previous report (3, 13). In contrast, the chlorophyll content in K(−) plants was lower than in K(+) plants after 10 days of the treatment.

We next proceeded to study the pattern of activities of enzymes involved in carbohydrate metabolism. Our preliminary studies have shown that the activity of UDPG-sucrose transglucosylase (sucrose synthetase) was not markedly different in K(+) and K(−) plants. We then decided to investigate the pyruvate kinase, a typical K^+-requiring enzyme, under conditions of K^+-sufficiency or deficiency. To this end it was first necessary to develop a reliable assay method in which the potent phosphatase activity present in the crude wheat leaf extract would not interfere. Consistent with the result of Evans (5), we found that tris-molybdate exhibited a marked inhibitory effect

Table I. *Levels of K^+, Sugar, and Chlorophyll in the First Leaves of K^+-Sufficient and -Deficient Wheat Plants*

Growth stage of plants	K^+		Total sugar		Reducing sugar		Chlorophyll	
	K(+)	K(−)	K(+)	K(−)	K(+)	K(−)	K(+)	K(−)
Days	*mg/g dry wt*		*mg glucose/g dry wt*				*mg/g fr wt*	
6	26.6	10.6	227	229	185	187	0.75	0.73
8	41.5	5.0	111	163	103	159	0.94	0.94
10	42.8	4.2	136	144	116	139	1.24	0.95
12	78.3	2.5	125	142	116	140	1.36	1.02

Table II. *Inhibitory Effect of Tris-Molybdate on the Enzymic Formation of Pyruvate from Phosphoenolpyruvate by Crude Extract of Wheat Leaves*

First leaves of 10 day old wheat plants grown under K^+-sufficient or -deficient conditions as explained in text were the material for the enzyme preparation.

Tris molybdate M × 10^{-4}	K(+)		K(−)	
	−ADP	+ADP	−ADP	+ADP
	in assay mixture		in assay mixture	
	μmole pyruvate per 10 min per mg protein			
0	0.310	0.391	0.589	0.526
1.56	0.112	0.054	0.313	0.111
6.25	0.104	0.050	0.302	0.087
12.50	0.100	0.044	0.281	0.078

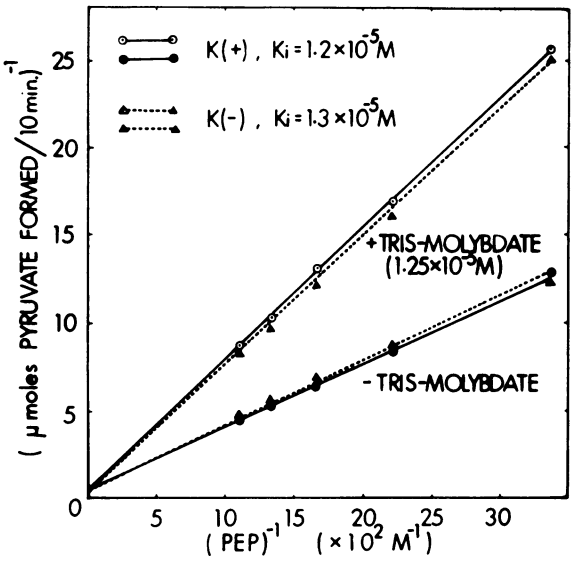

FIG. 1. Competitive inhibition of wheat leaves phosphatase by tris-molybdate. First leaves of both 14-day old K(+) and 12-day old K(—) wheat plants were used for the enzyme extraction as explained in text. Standard assay mixture containing with or without tris-molybdate (12.5 μM) was used for the enzyme experiment. In order to obtain the approximately equal initial reaction velocities, each of 1.0 mg of K(+) and 0.7 mg protein of K(—) plants was added to the reaction mixture.

on the enzymic cleavage of PEP by both K(+) and K(−) wheat leaf extracts (table II). It can be seen that tris-molybdate as low as 0.15 mM was sufficient to prevent pyruvate formation due to both the phosphatase and pyruvate kinase reactions. The results illustrated in figure 1 indicate the competitive type inhibition by tris-molybdate on the PEP-cleaving phosphatase activities of K(+) and K(−) wheat leaves. The Ki was determined to be 13 μM and 12 μM, respectively. The data in essence support the previous finding of Spencer on tomato acid phosphatase (16). It will be noted that the concentration of PEP included in the reaction mixture for the assay of pyruvate kinase activity was 0.125 mM, approximately 10 times higher than the Ki value for the inhibition of phosphatase by molybdate ion.

Table III. *Pyruvate Kinase Activity of Wheat Plants Grown at Various Levels of K⁺*

Same plants leaves as used in experiments shown in table II were the material for the enzyme preparation.

K^+ content in culture media	Pyruvate kinase activity in assay mixture	
	$-K^+$	$+K^+$
K_2SO_4, mmole/l	μmole pyruvate per 10 min per mg protein	
0	0.064	0.154
0.24	0.052	0.116
0.96	0.039	0.094
4.80	0.030	0.069

Using tris-molybdate as the phosphatase inhibitor, we determined the pattern of enzymic formation of pyruvate in both K(+) and K(−) wheat plants. The experimental results shown in table III and figure 2 demonstrate clearly the increase of pyruvate kinase activity in K(−) plants as compared with K(+) plants.

Table III shows the activities of pyruvate kinase in 10-day old wheat plants grown under various K^+-levels. It can be seen that the more severe the K^+-deficiency, the higher the specific activity of the enzyme. This trend is in fact more pronounced when K^+ was present in the assay mixture, although the same trend was observed even when K^+ was omitted from the assay. A proportionate increase in the production of pyruvate with an increase in the amount of crude enzyme preparation from K(−) and K(+) plants is seen in figure 2.

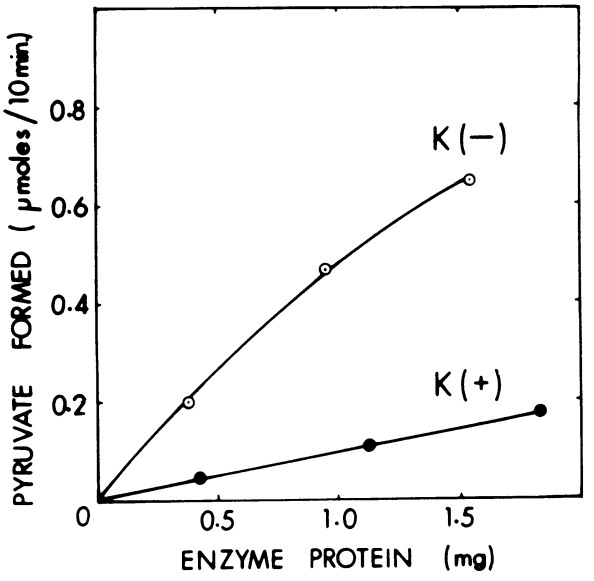

FIG. 2. Comparison of pyruvate kinase activity of K^+-sufficient and -deficient wheat leaf extracts. 14-day old K(+) and K(—) wheat plant leaves were used for extracting enzyme protein and the standard enzyme assay system was employed.

Enzyme activities were also determined in mixed extracts of K(−) and K(+) wheat leaves. The activities obtained were additive and recovery was complete. Thus, it seems that the higher enzyme activity is not caused by low molecular weight substances present in the crude extract of K^+-deficient plants. Furthermore, treatment of the extracts with Sephadex should minimize the presence of these substances in the enzyme preparations. An abnormally high content of some amino acids in the K(−) plants has been reported (15, 17). However, the addition of amino acids such as arginine and asparagine did not influence the activity of pyruvate kinase.

A typical result among serial studies of the

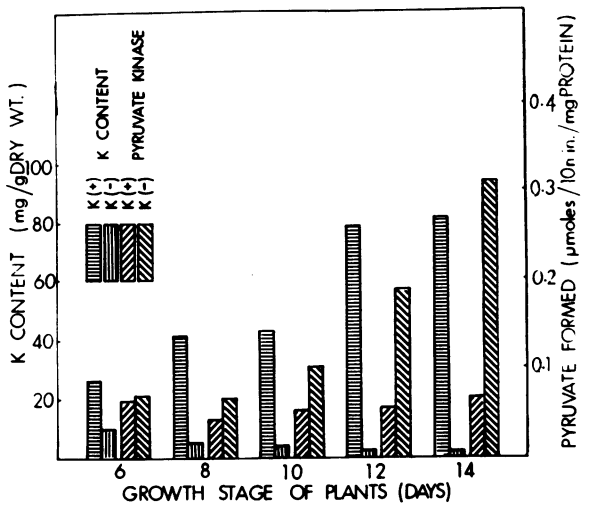

FIG. 3. Time-course analyses of pyruvate kinase activity and K^+ content in K^+-sufficient and -deficient wheat plants. Basic procedures of determining enzyme activity as well as K^+ content as described in text were employed.

time course for pyruvate kinase activity and K^+ content in both K(+) and K(−) wheat leaves is presented in figure 3. The enzyme activity in the K(−) plants began to increase after about the eighth day of growth. On the fourteenth day, the activity in K(−) plants was found to be nearly 4 times as high as that in K(+) plants. Conversely, the K^+ content in the K(−) wheat leaves was about 1.9 mg per g dry weight leaf, considerably lower than that of normal plants.

The effect of applying K^+ to K(−) wheat plants on the pyruvate kinase activities as well as on the amounts of leaf constituents is illustrated in table IV. For this particular experiment, the K^+-containing nutrient solution was applied to the K(−) plants on the tenth day of growth and analyses were made 3 days later. The results clearly demonstrate the nearly complete restoration of enzyme activity to the normal level after 3 days of K^+-application. Both carbohydrate and chlorophyll contents were also found to approach the normal levels over the same period.

Discussion

The present study was conducted to examine whether or not different levels of K^+ influence the levels of pyruvate kinase activity in wheat leaf tissues. To this end, we specifically designed our experiments such that the lethal effects of K^+-deficiency did not occur in our wheat plants. According to Wall (19), and Evans and Sorger (6), the unique feature of K^+-deficiency at the initial stages lies in the rapid disappearance of the deficiency symptom by subsequent K^+-application with a concomitant restoration of normal growth. The present results are in sharp contrast to those reported by Evans (5). Evans observed a decrease in pyruvate kinase activity in pea plants grown for over a month under K^+-deficient conditions. The use of different plant tissues and differences in experimental conditions may account for the discrepancies between the results of these 2 studies. However, it is highly probable that wheat plants in the initial stages of mineral deficiency present a pattern of enzyme activity which is different from that displayed at later stages. The results presented in table IV show clearly that not only the activity of pyruvate kinase but also the amount of other leaf constituents, K^+, carbohydrates and chlorophyll, are concomitantly restored to normal levels after only 3 days of K^+-application. One of

Table IV. *Effect of K^+-Application to K^+-Deficient Wheat Plants on Pyruvate Kinase Activity and on the Levels of Other Leaf Constituents*

K^+ (9.6 mmoles K_2SO_4/l) was given to the K(—) plants on the tenth day, and both pyruvate kinase activity as well as other constituents were determined 3 days after.

	Growth stage of plants	K(—)	K(+)	K(—) → K(+)
	Days			
K^+ (mg/g dry wt)	10	4.6	32.4	...
	13	3.7	53.0	35.4
Pyruvate kinase activity	10	0.102	0.054	...
(μmole pyruvate	13	0.202	0.055	0.065
/10 min/mg protein)				
Total sugar	10	127.0	97.0	...
(mg glucose	13	122.0	93.0	98.0
/g dry wt)				
Reducing sugar	10	123.0	87.0	...
(mg glucose	13	121.0	87.0	92.0
/g dry wt)				
Chlorophyll	10	0.89	1.20	...
(mg/g fr wt)	13	0.90	1.25	1.08

the typical biochemical symptoms of K^+-deficiency is reported to be a relatively high content of carbohydrate as compared to normal plants (3, 13). Our results show this also. On the other hand, enhancement of pyruvate kinase activity appears to be a more characteristic feature of plant tissues at the initial stages of the K^+-deficiency, although it is not known whether the higher activity of the pyruvate kinase is directly related to the high carbohydrate content in K(−) plants. A more thorough investigation is certainly needed to reveal whether this phenomenon is uniformly observable in other plants grown under the same K^+-deficient conditions.

A very pertinent question is whether the increased enzyme activities reflect A) the synthesis of pyruvate kinase in K(−) wheat plants or B) the repression of pyruvate kinase in the presence of excess amounts of K^+ in normally grown plants. Regardless of these 2 alternatives, it should be noted that the content of K^+ in the K(−) wheat leaves (1.9 mg/g dry wt) is approximately equivalent to 5 mM per 1000 g fresh weight; this value is quite close to the Michaelis constant of pyruvate kinase of K^+ (K_A), 2.4 mM, reported by Evans (5). The fact that the values for these 2 terms are approximately equal leads us to speculate about the possible regulation of the activities as well as the amounts of pyruvate kinase by the concentration of K^+ in intact plant cells.

Recent investigations have demonstrated the inducible formation of enzymes in plant tissues, among which the induction of nitrate reductase in radish tissues should be most notable (2). On the other hand, there are very few instances cited in the literature on the repression of enzyme production in higher plant tissues. Price (11) has reported the phosphate-repressible synthesis of phosphatase in *Euglena*, in which the enzyme activity was found to disappear completely after addition of phosphate to the cultures. Similarly Hewitt and Tather (7) have reported the enhancement of phosphatase in tomato plants which are grown under phosphate-deficient conditions. Further studies on the regulation of pyruvate kinase activity at the molecular level might provide a clue in elucidating the role of K^+ in the metabolism of plant cells.

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Literature Cited

1. Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts polyphenol oxidase in *Beta vulgaris*. Plant Physiol. 24: 1–15.
2. Beevers, L., L. E. Schrader, D. Flesher, and R. H. Hageman. 1965. The role of light and nitrate in the induction of nitrate reductase in radish cotyledons and maize seedlings. Plant Physiol. 40: 691–98.
3. Cooil, B. J. and M. C. Slattery. 1948. Effects of potassium deficiency and excess upon certain carbohydrate and nitrogenous constituents in guayule. Plant Physiol. 23: 425–42.
4. Dubois, M. K., A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28: 350–56.
5. Evans, H. J. 1963. Effect of potassium and other univalent cations on activity of pyruvate kinase in *Pisum sativum*. Plant Physiol. 38: 397–402.
6. Evans, H. J. and G. J. Sorger. 1966. Role of mineral elements with emphasis on the univalent cations. Ann. Rev. Plant Physiol. 17: 47–76.
7. Hewitt, E. J. and P. Tatham. 1960. Interaction of mineral deficiency and nitrogen source on acid phosphatase activity in leaf extracts. J. Expt. Botany 11: 367–76.
8. Kachmar, J. F. and P. D. Boyer. 1953. Kinetic analysis of enzyme reactions. II. The potassium activation and calcium inhibition of pyruvic kinase. J. Biol. Chem. 200: 669–82.
9. Miller, G. and H. J. Evans. 1957. The influence of salts on pyruvate kinase from tissues of higher plants. Plant Physiol. 32: 346–54.
10. Mori, K. and N. Nakamura. 1959. Quantitative analysis of sugars in plant extracts by ion-exchange chromatography, with special reference to the examination of conditions for preparing the sample sugar solutions. Bull. Agr. Chem. Soc. Japan 23: 389–76.
11. Price, C. A. 1962. Repression of acid phosphatase synthesis in *Euglena gracilis*. Science 135: 46.
12. Scott, G. T. and H. R. Hayward. 1953. The influence of iodoacetate on the sodium and potassium content of *Ulva lactuca* and the prevention of its influence by light. Science 117: 719–21.
13. Sideris, C. P. and H. Y. Young. 1945. Effects of potassium on chlorophyll, acidity, ascorbic acid, and carbohydrate of *Ananas comosus* (L.) Merr. Plant Physiol. 23: 425–42.
14. Somogyi, M. 1952. Notes on sugar determination. J. Biol. Chem. 195: 19–23.
15. Sorger, G. J., R. E. Ford, and H. J. Evans. 1965. Effects of univalent cations on the immunoelectrophoretic behavior of pyruvic kinase. Proc. Natl. Acad. Sci. 54: 1614–21.
16. Spencer, D. 1954. The effect of molybdate on the activity of tomato acid phosphatases. Australian J. Biol. Sci. 7 (2): 151–60.
17. Steinberg, R. A., J. D. Bowling, and J. E. McMurtrey, Jr. 1950. Accumulation of free amino acids as a chemical basis for morphological symptoms in tobacco manifesting frenching and mineral deficiency symptoms. Plant Physiol. 25: 279–88.
18. Sugiyama, T. and Y. Goto. 1966. Enhanced activity of pyruvic kinase in the potassium-deficient wheat leaves. Agr. Biol. Chem. Tokyo 30: 621–22.
19. Wall, M. E. 1940. The role of potassium in plants. II. Effect of varying amounts of potassium on the growth status and metabolism of tomato plants. Soil Sci. 49: 315–31.