Properties of Pyruvate Kinase from Soybean Nodule Cytosol[1]

JAY B. PETERSON AND HAROLD J. EVANS
Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon 97331

ABSTRACT

The properties of pyruvate kinase from soybean (*Glycine max* L.) nodule cytosol were examined to determine what influence the N_2 fixation process might have on this supposed key control enzyme. A crude enzyme preparation was prepared by chromatography of cytosol extract on a diethyl-aminoethyl-cellulose column. ATP and citrate at 5 mM concentrations inhibited pyruvate kinase 27 and 34%, respectively. Enzyme activation was hyperbolic with respect to both K^+ and NH_4^+ concentrations. In the presence of physiological concentrations of K^+ and high phosphoenolpyruvate (PEP) concentrations, NH_4^+ inhibited enzyme activity. Comparisons of kinetic parameters (V_{max} and apparent K_a) for NH_4^+ and K^+ with inhibition curves indicated that inhibition was very likely a result of competition of the ions for activation site(s) on the pyruvate kinase. In addition, apparent K_a (monovalent cation) and K_m (PEP) were influenced by PEP and monovalent cation concentrations, respectively. This effect may reflect a fundamental difference between plant and animal pyruvate kinases. It is concluded that control of cytosol pyruvate kinase may be closely related to reactions involved in the assimilation of NH_4^+.

Pyruvate kinase occupies a key position in the regulation of carbohydrate metabolism (4, 6, 29). Control of pyruvate kinase activity is often exerted through ADP levels and activators like AMP and fructose 1,6-diP and inhibitors such as organic acids and ATP. Posttranslational modification (phosphorylation) may also be important (14). In gluconeogenic animal tissues, the enzyme is thought to be critical in regulating the flow of carbohydrates between the degradative glycolytic and synthetic gluconeogenic pathways (4, 29). The same may be true for the plant enzyme (4). Regardless of whether gluconeogenesis is operative in the nodule, the availability of carbon skeletons and cofactors for ammonium assimilation could be influenced by pyruvate kinase activity. In addition, pyruvate kinases from the majority of plant and animal sources examined have been shown to have an absolute requirement for a monovalent cation for activity. Ammonium ion is a strong activator (10). As ammonia is the primary product of N_2 fixation, and because of the presumed role of pyruvate kinase in regulation of carbohydrate metabolism, regulatory properties of the soybean nodule cytosol enzyme were examined.

MATERIALS AND METHODS

Materials. Soybean (*Glycine max* L. var. Chippewa) root nodules were obtained from 25-day-old plants grown as described by Evans *et al.* (5). Commercial rhizobial inoculum was purchased from Nitragin (Milwaukee). Commercial enzymes, nucleotides, and phosphorylated sugars were obtained from Sigma Chemical

Co. (St. Louis, Mo.). Tetramethylammonium hydroxide was purchased from Matheson, Coleman and Bell (Norwood, Ohio). Imidazole (Sigma) was recrystallized from ethyl acetate. PVP (Polyclar AT) was obtained from GAF Corp. (New York) and washed with acid (15). All chemicals were reagent grade.

Enzyme Assays. Unless otherwise stated, pyruvate kinase assays contained 100 mM imidazole (pH 7.5, maleic acid), 50 mM $TMAH^2$-maleate, 100 mM KCl, 10 mM $MgCl_2$, 1 mM cyclohexylamine PEP, and 2 mM Tris ADP (prepared from Ba_2ADP [21]). Total maleate concentration was 60 mM. Assays coupled to lactate dehydrogenase also contained 0.16 mM NADH and 8 units of rabbit muscle lactate dehydrogenase. In some studies the 2,4-dinitrophenylhydrazine colorimetric assay (8, 21) was employed and reactions were terminated after 10 min. ADP and PEP concentrations were raised to 2.5 and 1.5 mM, respectively, for these assays. In both assays the reaction velocity was proportional to enzyme concentration over the range of concentrations used. Reactions were run at 30 C. In all cases, reaction mixtures without ADP were included in order to correct for PEP carboxylase activity. PEP carboxylase was assayed using a malate dehydrogenase enzyme couple. Assays contained 100 mM imidazole HCl (pH 7.5), 2 mM cyclohexylamine PEP, 5 mM $MgCl_2$, 0.16 mM NADH, 10 mM $KHCO_3$, and 20 units of beef heart malate dehydrogenase. Coupled assays were performed with a Cary 11 or Cary-118 spectrophotometer. The Cary 118 was used for colorimetric assays and spectral studies. One unit of enzyme activity is defined as 1 μmol of product/min at 30 C.

Plant Extracts. All isolation steps were performed at 0 to 4 C. In a typical extraction, 18 g of nodules were extracted by grinding with 3 volumes of grinding medium and 6 g of insoluble PVP in a mortar and pestle. Grinding medium consisted of a 3:1 (v/v) mixture of buffer (100 mM imidazole-phosphate [pH 7.5] and 1.33 mM DTT)-glycerol. The extracts were filtered through four layers of cheesecloth and centrifuged 15 min at 37,000g. The supernatant was removed and 30 ml applied to a column (3 × 4 cm) of DEAE-cellulose. The DEAE column was prepared by equilibrating with a 3:1 (v/v) mixture of buffer (10 mM imidazole-phosphate [pH 7.5] and 0.13 mM DTT)-glycerol (equilibrating medium). After addition of the enzyme, the column was washed with 2 column volumes of the equilibration medium, 0.075 M with respect to KCl. One column volume of equilibration medium, 0.175 M with respect to KCl, removed the single peak of pyruvate kinase activity (7.5–10 units) from the column. Column flow rate during the entire procedure was 60 ml/hr. The specific activity of pyruvate kinase was increased 3.5-fold (5.2-fold purification) over the crude extract when protein was estimated by the method of Lowry *et al.* (16) after trichloroacetic acid precipitation. The enzyme preparation was frozen at −79 C and retained full activity for at least 2 months. The preparation did not lose activity when thawed and kept at ice bath temperature for 1.5 hr. Carboxylase activity under standard pyruvate kinase assay conditions (with maleate) was 8 to

[2] Abbreviations: TMAH-maleate: maleic acid titrated to pH 7.5 with tetramethylammonium hydroxide; OAA: oxaloacetate; PEP: phosphoenolpyruvate; cytosol: plant cell cytoplasm.

14% of the total activity, depending on the preparation.

An assay for β-hydroxybutyrate dehydrogenase activity in the cytosol was made on crude extracts as proof that the pyruvate kinase was of plant and not bacterial origin (19). No measurable activity was detected whereas bacteroids sonicated in grinding medium had high activity.

For cation activation studies, K^+ was removed from the DEAE enzyme on a column (3 × 12 cm) of Bio-Gel P-6. In this case, the medium used in equilibrating the column and eluting proteins was 3:1 (v/v) 10 mM imidazole HCl (pH 7.5)-glycerol, all made to 10 mM MgSO₄ and 0.1 mM DTT. For studies with rabbit muscle pyruvate kinase, the desired amount of enzyme was mixed with the P-6 equilibration medium and frozen at −79 C until used.

Cation Determinations. Free ammonium in plant tissues was determined with an Orion model 95-10 ammonia electrode (Orion Research Corp., Cambridge, Mass.) calibrated as described in the instruction manual. In each determination, 1 g of fresh tissue was rapidly harvested and ground with 2 ml of distilled H₂O in a mortar and pestle at room temperature. Seven ml of water were added and the pH adjusted (from 6.3) to 11.1 with 1 N NaOH. Electrode potential was recorded directly as an indicator of free ammonia. Controls with added L-glutamine or L-asparagine showed no interference. Nodules frozen in liquid N₂ at harvesting gave very similar results.

Potassium levels were determined by atomic absorption analysis of perchloric acid extracts. Nodules were harvested and immediately frozen in liquid N₂. The frozen nodules were crushed in a mortar and pestle with 2 volumes of cold 0.25 M HClO₄, centrifuged, and the supernatant neutralized with NaOH and used for analysis.

PEP Determinations. Nodule PEP levels were determined in the HClO₄ extracts described above. The pyruvate kinase-lactate dehydrogenase-coupled assay was employed.

RESULTS

Control of Carboxylase Activity. In early experiments using a spectrophotometric method for detection of keto acids, enzyme assayed in the absence of ADP produced products at a rate which often was greater than 40% of activity in the complete reaction mixture. Since ADP is required for pyruvate kinase, activity without ADP could not have been due to this enzyme. Previous reports (3, 18) have assigned this interference to the activity of phosphatases. However, the colorimetric assay, which is dependent upon the formation of 2,4-dinitrophenylhydrazone derivatives of keto acids, is nonspecific. When spectra of terminated assay reactions which lacked ADP were recorded, they closely resembled a spectrum of OAA which had been incubated in the reaction mixture without enzyme for 10 min and treated with the 2,4-dinitrophenylhydrazine. Spectra of OAA incubated for longer periods of time before treating with this reagent confirmed that the OAA was decarboxylating to pyruvate. It was concluded from these data that most if not all of the activity without ADP was due to PEP carboxylase. Further studies under optimal conditions for this enzyme revealed that carboxylase specific activity was 2.4 times greater than pyruvate kinase specific activity in crude cytosol extracts. The presence of high malic dehydrogenase activity in the preparation meant that carboxylase would interfere with the coupled pyruvate kinase assay as well as the colorimetric assay. For testing the effects of metabolites, it was desirable to eliminate the contribution from carboxylase. Maleate was found to inhibit PEP carboxylase. The inhibition was approximately 70% under the standard assay conditions. Pyruvate kinase was inhibited approximately 9% under the same conditions. The inhibition of pyruvate kinase was not due to TMAH and probably not to high ionic strength, because replacement of KCl and TMAH-maleate with 50 mM dipotassium maleate did not alter the PEP and ADP substrate dependence curves. Therefore, TMAH-maleate was suitable for use in monovalent cation activation studies. PEP car-

boxykinase could not have interfered significantly because enzyme eluted from the P-6 column retained high "minus ADP" activity. This procedure would have eliminated contaminating nucleotide diphosphates. Similarly, a combination of phosphatase plus pyruvate carboxylase could not have interfered significantly. Replacement of PEP with pyruvate in the reaction mixture did not result in detectable OAA synthesis.

Enzyme Properties. Pyruvate kinase activity showed a typical hyperbolic dependence on both ADP and PEP concentrations. Calculated kinetic constants are presented in Table I. A pH curve (maleate and TMAH-maleate omitted) showed a maximum at pH 6.7 to 6.8, which is in agreement with values obtained with plant pyruvate kinases in recent studies (3, 23, 30). Activity without ADP increased markedly between pH 6.4 and 7.2, which would be expected for a carboxylase with bicarbonate as substrate.

Pyruvate kinase activity was dependent on the presence of both a divalent and a monovalent cation. Although effects of other divalent cations were not studied, the enzyme preparation had no activity in the absence of MgCl₂. Assays run with 5, 10, and 15 mM MgCl₂ had equal activities.

The first monovalent cation dependence curves were conducted in the presence of maleate and TMAH (Fig. 1) (K_a values, Table II). Relative activations by K^+, NH_4^+, and Li^+ were in agreement with the activations obtained with pyruvate kinase from other

Table I. Substrate kinetic constants for pyruvate kinase assayed under different conditions.

Substrate varied	*Assay method and conditions	KCl in assay (mM)	**Apparent K_m (mM)
	Nodule cytosol enzyme		
ADP	Standard, coupled enzyme (pH 7.5)	100	0.38
	Standard, coupled enzyme (pH 7.5) maleate and TMAH-maleate omitted	100	0.066
	Coupled enzyme, pH 6.4	50	0.25
	Coupled enzyme, pH 6.4	10	0.31
PEP	Standard, coupled enzyme (pH 7.5)	100	0.21
	Standard, coupled enzyme (pH 7.5) maleate and TMAH-maleate omitted	100	0.058
	Coupled enzyme, pH 6.4	50	0.058
	Coupled enzyme, pH 6.4	10	0.16
	Rabbit muscle enzyme		
PEP	Coupled enzyme, pH 6.4	100	0.043
	Coupled enzyme, pH 6.4	10	0.073

*Between 0.020 and 0.024 units enzyme/assay were used in the various experiments. Assays at pH 6.4 were buffered with 100 mM imidazole HCl and contained no TMAH-maleate. Other reagents were as in the standard assay (Materials and Methods) except where changes were made in KCl concentration.
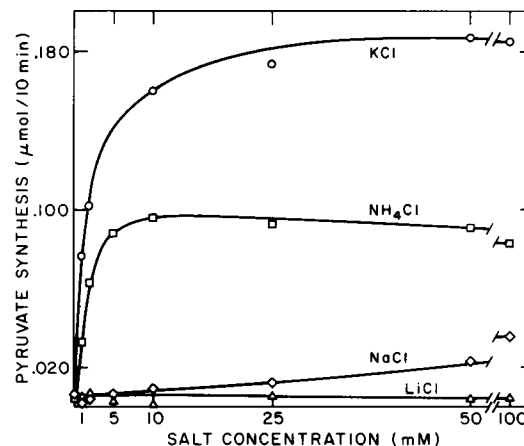**Calculated from least squares regression analyses of Hanes plots.

FIG. 1. Dependence of cytosol pyruvate kinase activity on concentrations of various monovalent cations. Reactions were assayed colorimetrically. Each 1-ml reaction mixture contained 0.022 unit of enzyme.

sources (10) although enzyme from adipose tissue is known to be more highly activated by NH_4^+ than K^+ (27). The results obtained with Na^+ activation were unexpected. With other plant pyruvate kinases, Na^+ activation over this range of concentrations was hyperbolic (17, 21).

To determine if NH_4^+ could serve in a regulatory capacity, NH_4^+ dependence curves were run in the presence of 10 and 50 mM KCl. Pyruvate kinase was inhibited over the entire NH_4^+ concentration range (Fig. 2). Greatest inhibitions were observed in the range from 1 to 10 mM NH_4^+. The magnitudes of these inhibitions were 23 and 13% for 10 and 50 mM KCl, respectively. To examine further the magnitude of the monovalent cation effect, assays were run at pH 6.4 to avoid the use of maleate. Activations by K^+ and NH_4^+ again appeared hyperbolic (Table II). The shape of the Na^+ curve was very similar to that obtained in the experiments conducted at pH 7.5 (Fig. 1). The V_{max} with K^+ was 1.9 times the V_{max} with NH_4^+. Again, NH_4^+ in the presence of 10 and 50 mM KCl inhibited the enzyme. In this case, inhibitions between 0.2 and 10 mM NH_4^+ were 27 and 17% for 10 and 50 mM K^+, respectively. The shapes of the inhibition curves closely resembled those in Figure 2. A similar experiment was performed with commercial rabbit muscle enzyme (Fig. 3). The enzyme was not inhibited as strongly by low concentrations of NH_4^+ as the soybean cytosol enzyme.

Inhibition of pyruvate kinase by NH_4^+ in the presence of K^+ may have been a result of direct competition for activation site(s) on the enzyme. K_a and V_{max} values for these ions indicate that the enzyme probably had a higher affinity for NH_4^+ than K^+. Assuming that these cations compete with each other, greatest decreases in activity would be expected at concentrations of NH_4^+ which are equal to those concentrations giving maximal increases in activity in the absence of K^+. Furthermore, it would be expected that the activities at high concentrations of NH_4^+ would approach those in reactions where no K^+ was added. Comparison of the data in Figure 1 with those in Figure 2 and corresponding data obtained at pH 6.4 showed this to be the case. Controls run with varying Li^+ (nonactivator) concentrations and 50 mM KCl (Fig. 2) indicated that high ionic strength did not cause the inhibition.

To test the physiological significance of NH_4^+ inhibition, substrate dependence curves were performed in the presence of KCl at 10 and 50 mM with NH_4Cl at 0 and 10 mM concentrations. Over the entire range of ADP concentrations, 10 mM NH_4^+ inhibited the enzyme at levels expected from the previous studies. However, in the 50 mM KCl experiment, differences at low ADP levels were not statistically significant. Therefore, only data from the 10 mM KCl experiment are presented (Fig. 4) because differences were significant at K_m (ADP) and higher concentrations.

When analogous studies were performed over a range of PEP concentrations, similar results were not observed. When KCl concentration was held at 50 mM, inhibition by 10 mM NH_4^+ was well below the expected 17% when concentrations of PEP were at or below 50 μM (Fig. 5). Furthermore, in experiments where 10 mM KCl was used, 10 mM NH_4^+ stimulated enzyme activity at low PEP concentrations (Fig. 6). Since these results seemed inconsistent with a competition hypothesis, monovalent cation (NH_4^+ and K^+) activation experiments were performed at two levels of PEP to see if relative activations changed. The results, presented as Hanes plots (Fig. 7), showed that apparent K_a values (Table II) for NH_4^+ and K^+ varied markedly with PEP concentration; a result contrary to that obtained by Kachmar and Boyer (8) who used rabbit muscle enzyme. At 0.05 mM PEP, activations by 10 mM concentrations of the two ions were nearly identical. Since neither K^+ nor NH_4^+ was present at a concentration sufficient for maximum velocities under these conditions, the ions would be expected to complement each other as activators.

The results with low PEP concentrations were interesting from an enzymological standpoint, as they represented a deviation from

Table II. Monovalent cation activation constants for nodule cytosol pyruvate kinase assayed under different conditions.

Activator varied	*Assay method and conditions	PEP in assay (mM)	**Apparent K_a (mM)
K^+	Standard, colorimetric (pH 7.5)	1.5	1.7
	Colorimetric, pH 6.4	1.5	0.89
	Coupled enzyme, pH 6.4	0.50	0.52
	Coupled enzyme, pH 6.4	0.050	5.4
NH_4^+	Standard, colorimetric (pH 7.5)	1.5	1.1
	Colorimetric, pH 6.4	1.5	0.32
	Coupled enzyme, pH 6.4	0.50	0.26
	Coupled enzyme, pH 6.4	0.050	1.4

*Between 0.020 and 0.024 units enzyme/assay were used in the various experiments. Assays at pH 6.4 were buffered with 100 mM imidazole HCl and contained no TMAH-maleate. NADH was in the cyclohexylamine form. Other reagents were as in the standard assay (Materials and Methods) except where changes were made in PEP concentration.
**Calculated from least squares regression analyses of Hanes plots. In some cases, 100 mM salt concentrations were inhibitory and these data were omitted from the regression analyses.
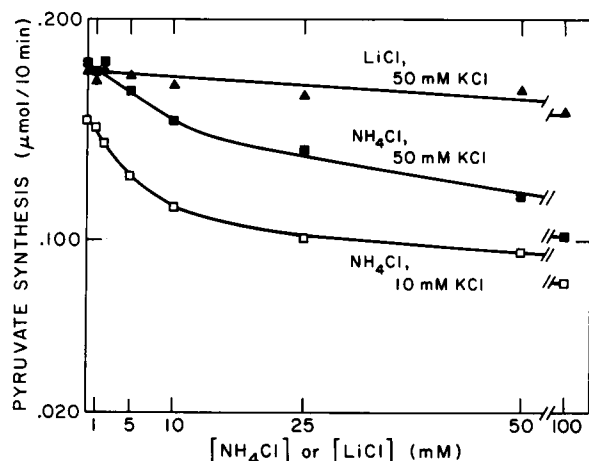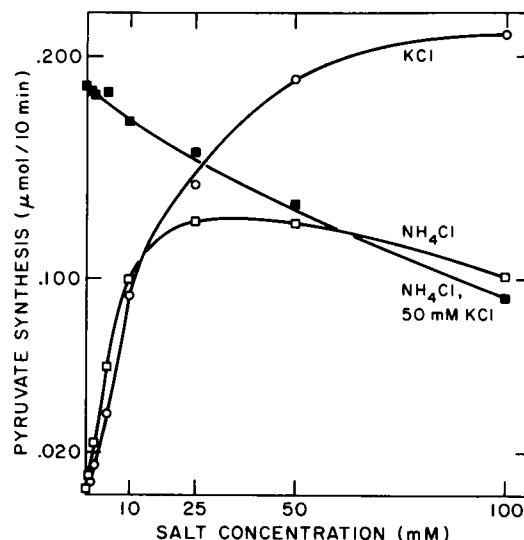

FIG. 2. Inhibition of cytosol pyruvate kinase by ammonium in the presence of KCl. NH_4Cl and LiCl concentrations were varied in the presence of indicated (constant) levels of KCl. Assays were conducted as described in Figure 1. Standard deviations of the means, expressed as nmol/10 min, were as follows: 0 NH_4^+, 10 mM KCl: 2.6; 10 mM NH_4Cl, 10 mM KCl: 1.5; 0 NH_4^+, 50 mM KCl: 5.7; 10 mM NH_4Cl, 50 mM KCl: 3.5.

FIG. 3. Dependence of rabbit muscle pyruvate kinase activity on concentrations of KCl and NH_4Cl and inhibition by NH_4^+ in the presence of KCl. Reactions were assayed colorimetrically. Each 1-ml reaction mixture contained 0.026 unit of enzyme.
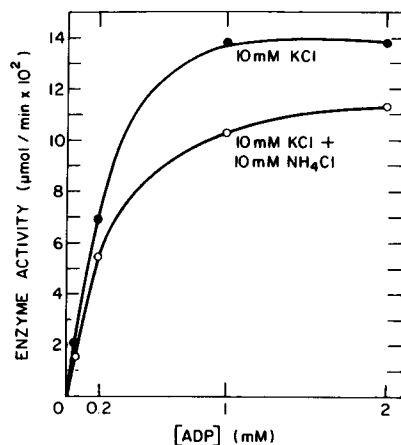
FIG. 4. Effect of 10 mM KCl with or without NH₄Cl on cytosol pyruvate kinase over a range of ADP concentrations. Each assay contained 0.020 unit of enzyme in 2 ml of reaction volume. The coupled enzyme assay was used ("Materials and Methods") except assays were run at pH 6.4 and the cyclohexylamine form of NADH was used. Corresponding points at 0.2 mM ADP and above differ by more than 2 s.D. of their means.
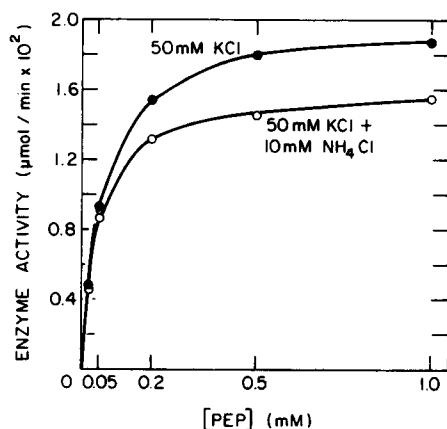

FIG. 5. Effect of 50 mM KCl with or without NH₄Cl on cytosol pyruvate kinase over a range of PEP concentrations. Conditions were as described in Figure 4. Differences between corresponding points at 0.2 mM PEP and above are greater than 2 s.D. of their means.
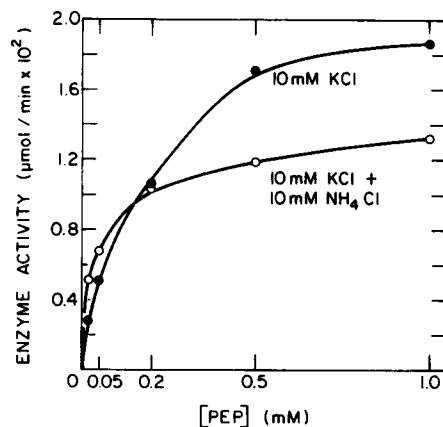
FIG. 6. Effect of 10 mM KCl with or without NH₄Cl on cytosol pyruvate kinase over a range of PEP concentrations. Conditions were as described in Figure 4. Differences between corresponding points at 0.02, 0.05, 0.5 and 1 mM PEP are greater than 2 s.D. of their means.
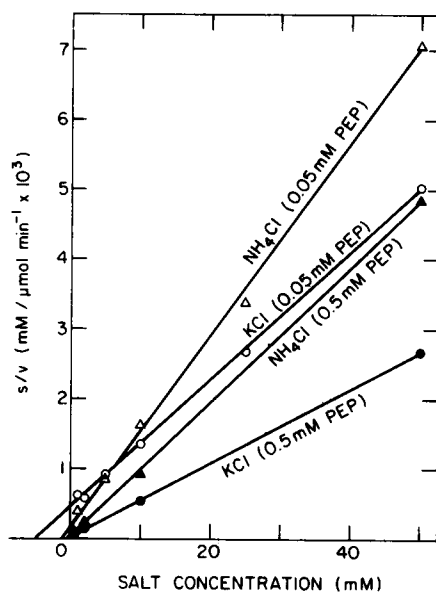

FIG. 7. Activation of cytosol pyruvate kinase by KCl and NH₄Cl at two concentrations of PEP. Reaction mixture contained 0.021 unit of enzyme. Assay conditions were as described in Figure 4.

the accepted kinetics of the rabbit muscle enzyme (8). A further deviation was noted when the PEP dependence curves of Figures 5 and 6 with K⁺ alone were replotted as Hanes plots. The decrease in K⁺ concentration from 50 to 10 mM caused a 3-fold increase in apparent K_m (PEP) as calculated from the Hanes plots (Table I). The ADP dependence data showed only a 20% difference in apparent K_m values between 50 and 10 mM potassium experiments (Table I). The possibility was considered that these kinetic data were due to the pH used in assaying the enzyme. However, an experiment analogous to that in Figure 6 with the exception that standard assay conditions (pH 7.5) were used produced similar results. In addition, studies with commercial rabbit muscle pyruvate kinase showed very little effect of monovalent cation or PEP on reciprocal activations. The 10-fold decrease in K⁺ concentration caused only a 41% increase in apparent K_m (PEP) (Table I). Monovalent cation activation studies were run at 0.05 and 0.5 mM PEP and no large variation in activation was observed. These curves displayed slight cooperativity (e.g. Fig. 3) and K_m values were therefore not calculated.

To analyze the possibility of a physiological role of NH₄⁺ in control of cytosol pyruvate kinase, levels of NH₄⁺, K⁺, and PEP were measured. Concentrations between 4.4 and 6.6 mM (mean 5.5 mM [19 samples]) free NH₄⁺ were measured in nodules, which is in close agreement with values obtained by a microdiffusion

technique (1). These values very likely are conservative because a fluid volume/fresh wt value of 1 ml/g was assumed during extraction. In addition, the volume of bacteroids should be subtracted as they probably excrete NH₄⁺ (24). In contrast to these results the concentration in root tissues was calculated at 0.14 mM. Potassium concentrations in fresh nodules (five determinations) ranged between 37 and 45 mM with a mean of 41 mM. In two nodule extractions, PEP levels (corrected for recovery) were measured at 21 and 25 µM.

Effects of Various Metabolites on Enzyme Activity. Several metabolites at concentrations of 5 mM were tested for effects on pyruvate kinase activity. Compounds tested were glycine, L-glutamate, L-glutamine, L-aspartate, L-asparagine, L-alanine, citrate, α-ketoglutarate, succinate, fumarate, malate, β-hydroxybutyrate, ATP, AMP, fructose-1,6-diP, and glucose-6-P. ATP, citrate, fumarate, malate, and β-hydroxybutyrate were also tested in the absence of maleate and no qualitative differences in results were observed. ATP and citrate were observed to inhibit the enzyme. As expected from the previously discussed effect of Mg²⁺ concentration on enzyme activity, the inhibition could not be overcome by supplementing the reaction mixture with an additional 5 mM

MgCl₂. Mg^{2+} chelation therefore did not appear to cause the inhibition. Effects of ATP and citrate were examined over a range of concentrations. Under standard assay conditions, inhibition at 5 mM was 27% with ATP and 34% with citrate. At 1 mM, inhibitions were 8 and 11%, respectively. Inhibition by these compounds has been observed with pyruvate kinase from other sources including plants (3, 4, 30). However, there are exceptions (23). AMP stimulates pyruvate kinase from cotton seeds (3, 4), but does not increase activity of the enzyme from pea seed, carrot (30), or castor bean endosperm (23). The possibility that chloride ion interfered with AMP activation of pyruvate kinase (3) was investigated by replacing MgCl₂ and KCl with the sulfate salts. No activation by AMP was observed.

DISCUSSION

Our studies indicate that pyruvate kinase from the plant cells of soybean nodules is regulated in a way that contributes to efficient nitrogen fixation. The product of fixation, ammonium ion, may be a weak inhibitor along with ATP and citrate. The single most important factor is probably the ADP level, which may in turn be influenced by the routes of NH_4^+ assimilation. Such regulation would aid in providing proper quantities of PEP for PEP carboxylase and the subsequent synthesis of aspartate and asparagine during nitrogen fixation. Control of pyruvate kinase in relation to the presumed assimilatory reactions (19, 20, 28) is summarized in Figure 8.

The activation of various pyruvate kinases by monovalent cations has been a subject of considerable interest (10). However, with the exception of an NH_4^+ stimulation of pyruvate kinase detected in vivo (9, 26), no physiological significance of this phenomenon has been elucidated. The enzyme from soybean nodule cytosol, like that from pea seed (21) and cucumber (17), has a much lower K_a for both K^+ and NH_4^+ (Table II) than the yeast (7) and mammalian (8, 27; Fig. 3) enzymes. The effect of NH_4^+ on enzyme activity was studied in the presence of various levels of K^+, PEP, and ADP. Ammonium ion acted as a inhibitor, activator, or had no significant effect, depending on K^+ and PEP concentrations. From a consideration of all these kinetic data, it is likely that the effects of NH_4^+ in the presence of K^+ were due to activation at the same site(s) on the enzyme. However, other

interpretations cannot be ruled out. To determine what role NH_4^+ might play in the regulation of pyruvate kinase, levels of NH_4^+, K^+, and PEP were measured in the nodules. The values obtained indicate that NH_4^+ has no effect or is a weak inhibitor under physiological conditions. However, until more is known about subcellular levels of NH_4^+, K^+, and PEP, this conclusion can only be tentative. It is interesting that in vivo studies detected a stimulation of pyruvate kinase in alfalfa leaf discs (26) and Chlorella (9) with NH_4^+ addition. Our experiments have shown an almost 2-fold stimulation by NH_4^+ under certain conditions (Fig. 6).

The significance of citrate as a regulator of cytosol pyruvate kinase is in doubt. Although this metabolite was shown to be an inhibitor of the enzyme, researchers (11, 31) could not detect citrate in nodules of several plant species which include soybeans (31).

Substrate dependence curves have shown the cytosol pyruvate kinase to be extremely responsive to changes in ADP levels near K_m concentration of this substrate. The enzyme was also inhibited somewhat by ATP. For regulation of pyruvate kinase by ADP and ATP to be important, levels of these metabolites must be influenced by reactions involved in N₂ fixation or assimilation of ammonia. Figure 8 outlines the possible roles of adenine nucleotides and NH_4^+ assimilatory reactions in the control of cytosol pyruvate kinase. The synthesis of 1 mol of glutamate via glutamine α-ketoglutarate aminotransferase (GOGAT) would liberate 0.5 mol of ADP (from glutamine synthesis). The synthesis of each glutamine from glutamate produces 1 ADP for a total of 1.5 mol of ADP/mol of glutamine via this route. However, the synthesis of 1 mol of aspartate presumably would result in the generation of 2.5 mol of ADP (assuming the presence of an ADP-regenerating adenylate kinase and from the glutamate requirement). If asparagine synthetase uses glutamine as a source of amide nitrogen, the total number of ADP molecules generated for each asparagine synthesized from OAA would be six. It is also possible that amide nitrogen arises directly from NH_4^+ (28). In this case, the number would be 4.5. In relation to the assimilatory reactions, therefore, a relatively high rate of ADP generation during asparagine synthesis would tend to stimulate pyruvate kinase. This would direct the flow of carbohydrate in favor of the citric acid cycle, the synthesis of ATP, and α-ketoglutarate for assimilation to glutamine. A similar argument can be made for ATP shifting the balance of carbohydrate flow through PEP carboxylase and the aspartate and asparagine route.

Regulation of cytosol pyruvate kinase may be closely linked to reactions involved in nitrogen fixation. The work of Lawrie and Wheeler (13) has shown that PEP carboxylase, which competes with pyruvate kinase for substrate (PEP), is very likely the source of carbon for aspartate synthesis in nodules. In addition, PEP carboxylase from lupin nodules was recently examined (2) and it was found that activity paralleled increasing acetylene reduction activity during nodule development. Fifteen per cent of total leaf carbon fixed eventually returns to apical plant parts as amino transport compounds (22), primarily as asparagine (25) in peas. Therefore, the need for a controlled flow of carbohydrate between OAA synthesis and the terminal steps of glycolysis is obvious.

While these studies have shown how monovalent cation activation may be involved in the control of plant pyruvate kinases, they were also interesting from an enzymological point of view. The plant and animal enzymes appear to differ in several respects. In the classical studies of Kachmar and Boyer (8) who used rabbit muscle enzyme, no effect of PEP concentration on the apparent K_a for K^+ nor an effect of K^+ concentration on the apparent K_m for PEP was detected. This report is the first demonstration of this type. It is possible that this represents a fundamental difference in the mechanisms of the animal and legume nodule enzymes. However, analogous experiments with rabbit muscle enzyme were run and small effects were noted. Therefore, the observed effects on cytosol enzyme may in some manner reflect the fact that plant enzymes are more highly activated at low concentrations of mon-
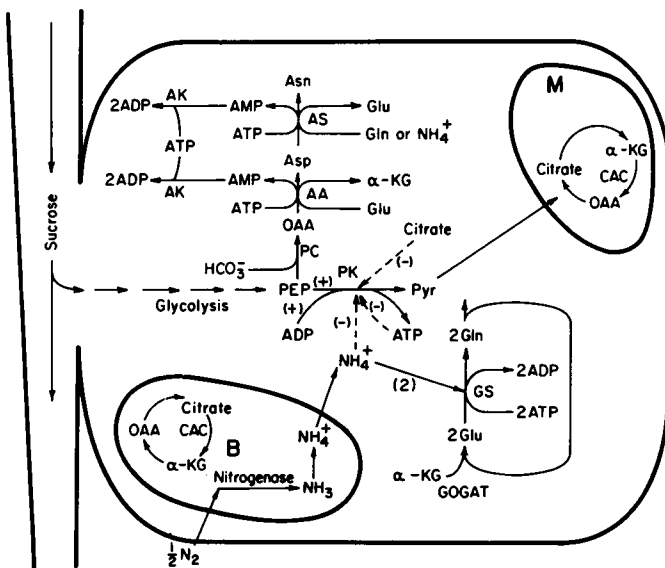

FIG. 8. Possible relationships between pyruvate kinase and assimilatory reactions in a legume nodule cell. B: bacteroid; CAC: citric acid cycle; M: mitochondrion; AA: aspartate aminotransferase; AK: adenylate kinase; AS: asparagine synthetase; GOGAT: glutamine α-ketoglutarate aminotransferase; PC: PEP carboxylase; PK: pyruvate kinase. Factors increasing (+) and decreasing (−) pyruvate kinase activity are indicated.

ovalent cations than the rabbit muscle enzyme. In addition, rabbit muscle enzyme displayed a slightly cooperative response to monovalent cation concentration. The cytosol enzyme kinetics appeared hyperbolic. There is obviously a need for further studies with more highly purified plant enzyme.

LITERATURE CITED

1. BISHOP PE, JG GUEVARA, JA ENGELKE, HJ EVANS 1976 Relation between glutamine synthetase and nitrogenase activities in the symbiotic association between *Rhizobium japonicum* and *Glycine max*. Plant Physiol 57: 542–546
2. CHRISTELLER JT, WA LAING, WD SUTTON 1977 Carbon dioxide fixation by lupin root nodules. Plant Physiol 60: 47–50
3. DUGGLEBY RG, DT DENNIS 1973 Pyruvate kinase, a possible regulatory enzyme in higher plants. Plant Physiol 52: 312–317
4. DUGGLEBY RG, DT DENNIS 1973 The characterization and regulatory properties of pyruvate kinase from cotton seeds. Arch Biochem Biophys 155: 270–277
5. EVANS HJ, JB KOCH, R KLUCAS 1972 Preparation of nitrogenase from nodules and separation into components. Methods Enzymol 24: 470–476
6. GIVAN CV 1974 Regulation of glycolysis by adenosine diphosphate in *Pisum sativum*. Phytochemistry 13: 1741–1745
7. HUNSLEY JR, CH SUELTER 1969 Yeast pyruvate kinase. II. Kinetic properties. J Biol Chem 244: 4819–4822
8. KACHMAR JR, PD BOYER 1953 Kinetic analysis of enzyme reactions. II. The potassium activation and calcium inhibition of pyruvic phosphoferase. J Biol Chem 200: 669–682
9. KANAZAWA T, MR KIRK, JA BASSHAM 1970 Regulatory effects of ammonia on carbon metabolism in photosynthesizing *Chlorella* pyrenoidosa. Biochim Biophys Acta 205: 401–408
10. KAYNE FJ 1973 Pyruvate kinase. *In* PD Boyer, ed, The Enzymes, Ed 3 Vol 8. Academic Press, New York, pp 353–382
11. KIDBY DK 1966 Activation of a plant invertase by inorganic phosphate. Plant Physiol 41: 1139–1144
12. KURZ WGW, DA ROKOSH, TA LARUE 1975 Enzymes of ammonia assimilation in *Rhizobium leguminosarum* bacteroids. Can J Microbiol 21: 1009–1012
13. LAWRIE AC, CT WHEELER 1975 Nitrogen fixation in the root nodules of *Vicia faba* L. in relation to the assimilation of carbon dioxide. New Phytol 74: 437–445
14. LJUNGSTROM O, G HJELMQUIST, L ENGSTROM 1974 Phosphorylation of purified rat liver pyruvate kinase by cyclic 3',5'-AMP-stimulated protein kinase. Biochim Biophys Acta 358: 289–298
15. LOOMIS WD 1974 Overcoming problems of phenolics and quinones in the isolation of plant enzymes. Methods Enzymol 31: 528
16. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
17. McCOLLUM RE, RH HAGEMAN, EH TYNER 1958 Influence of potassium on pyruvic kinase from plant tissue. Soil Sci 86: 324–331
18. McCOLLUM RE, RH HAGEMAN, EH TYNER 1960 Occurrence of pyruvic kinase and phosphoenolpyruvate phosphatases in seeds of higher plants. Soil Sci 89: 49–52
19. McPARLAND RH, JG GUEVARA, RR BECKER, HJ EVANS 1976 The purification and properties of the glutamine synthetase from the cytosol of soya-bean root nodules. Biochem J 153: 597–606
20. MIFLIN BJ, PJ LEA 1976 The pathway of nitrogen assimilation in plants. Phytochemistry 15: 873–885
21. MILLER G, HJ EVANS 1957 The influence of salts on pyruvate kinase from tissues of higher plants. Plant Physiol 32: 246–254
22. MINCHIN FR, JS PATE 1973 The carbon balance of a legume and the functional economy of its root nodules. J Exp Bot 24: 259–271
23. NAKAYAMA H, M FUJII, K MIURA 1976 Partial purification and some regulatory properties of pyruvate kinase from germinating castor bean endosperm. Plant Cell Physiol 17: 653–660
24. O'GARA F, KT SHANMUGAM 1976 Regulation of nitrogen fixation by rhizobia: export of fixed N₂ as NH₄⁺. Biochim Biophys Acta 437: 313–321
25. PATE JS 1962 Root-exudation studies on the exchange of C¹⁴-labelled organic substances between the roots and shoot of the nodulated legume. Plant Soil 17: 333–356
26. PLATT SG, Z PLAUT, JA BASSHAM 1977 Ammonia regulation of carbon metabolism in photosynthesizing leaf discs. Plant Physiol 60: 739–742
27. POGSON CI 1968 Adipose-tissue pyruvate kinase. Properties and interconversion of two active forms. Biochem J 110: 67–77
28. SCOTT DB, KJF FARNDEN, JG ROBERTSON 1976 Ammonia assimilation in lupin nodules. Nature 263: 703–705
29. SCRUTTON MC, MF UTTER 1968 The regulation of glycolysis and gluconeogenesis in animal tissue. Annu Rev Biochem 37: 249–302
30. TOMLINSON JD, JF TURNER 1973 Pyruvate kinase of higher plants. Biochim Biophys Acta 329: 128–139
31. TUZIMURA K 1962 Organic acids and sugars in soybean nodules. J Soil Sci Manure Japan 33: 320. As cited in ref 11