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Hexose Kinases from the Plant Cytosolic Fraction of Soybean Nodules

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LES COPELAND* AND MATTHEW MORELL[1]
Department of Agricultural Chemistry, University of Sydney, New South Wales 2006, Australia

ABSTRACT

The enzymes responsible for the phosphorylation of hexoses in the plant cytosolic fraction of soybean (*Glycine max* L. Merr cv Williams) nodules have been studied and a hexokinase (ATP:D-hexose 6-phosphotransferase EC 2.7.1.1) and fructokinase (ATP:D-fructose 6-phosphotransferase EC 2.7.1.4) shown to be involved. The plant cytosolic hexokinase had optimum activity from pH 8.2 to 8.9 and the enzyme displayed typical Michaelis-Menten kinetics. Hexokinase had a higher affinity for glucose (K_m 0.075 millimolar) than fructose (K_m 2.5 millimolar) and is likely to phosphorylate mainly glucose *in vivo*. The plant cytosolic fructokinase had a pH optimum of 8.2 and required K^+ ions for maximum activity. The enzyme was specific for fructose (apparent K_m 0.077 millimolar) but concentrations of fructose greater than 0.4 millimolar were inhibitory. The native molecular weight of fructokinase was 84,000 ± 5,000. The roles of these enzymes in the metabolism of glucose and fructose in the host cytoplasm of soybean nodules are discussed.

Symbiotic nitrogen fixation in root nodules of legumes depends on the supply of carbohydrates from the host plant (13). The main carbohydrate translocated into the nodules is sucrose (2), which is broken down to provide nutrients for the bacteroids, energy for nitrogen fixation and carbon skeletons for the assimilation of fixed NH_4^+. The initial step in the breakdown of sucrose is cleavage by either invertase (β-D-fructofuranoside fructohydrolase EC 3.2.1.26) or sucrose synthase (UDPglucose:D-fructose 2-α-D-glucosyltransferase EC 2.4.1.13). Invertase has been found in the nodules of *Ornithopus sativus*, *Lupinus luteus* (8), *L. angustifolius* (15), and soybeans (11, 18) and the presence of sucrose synthase has been demonstrated in soybean nodules (11, 12). Essentially all of the invertase and sucrose synthase is in the plant cytosolic fraction (11, 15, 18), indicating that the cleavage of sucrose takes place in the host cytoplasm of the nodules.

The metabolism of the glucose and fructose produced from sucrose is likely to involve phosphorylation of the hexose by ATP as the first step. Plant tissues contain a number of enzymes which can phosphorylate hexoses. For example four forms of hexokinase (ATP:D-hexose 6-phosphotransferase EC 2.7.1.1) occur in wheat germ (9) and spinach leaves (3) and three isozymes have been separated from developing castor oil seeds (10). Pea seeds contain two hexokinases, thought to be primarily involved in the phosphorylation of glucose and mannose, and two fructokinases (ATP:D-fructose 6-phosphotransferase EC 2.7.1.4), which phosphorylate fructose (5, 20–22). Hexose phosphorylating activity has been detected in soybean nodules (11, 14) but the enzymes involved have not been studied. Phosphorylating activity with glucose was found in both the plant and bacteroid fractions of the nodules whereas fructose phosphorylating activity was predominantly in the plant cytosolic fraction (11, 14). In the present investigation the enzymes responsible for the phosphorylation of glucose and fructose in the plant cytosolic fraction of soybean nodules have been studied and a hexokinase and fructokinase shown to be involved.

MATERIALS AND METHODS

Materials. Biochemicals were obtained from Sigma Chemical Co. or Boehringer Mannheim GmbH. Fractogel TSK HW-55 (F) was from E. Merck, AGATP[2] (type 2) from P-L Biochemicals Inc., and Perlite from Australian Perlite Pty. Ltd., Sydney. Nodulaid inoculum (*Rhizobium japonicum* strain CB1809) was a generous gift from Agricultural Laboratories, Sefton, N.S.W. Nodulated soybeans (*Glycine max* L. Merr cv Williams) were grown as described previously (11).

Preparation of Enzymes. All steps were carried out at 4°C. Nodules (6 g) were harvested 40 d after planting and homogenized with a mortar and pestle in 15 ml of 20 mM Hepes-KOH buffer (pH 8) containing 1 mM EDTA and 5 mM 2-mercaptoethanol (buffer A). The homogenate was filtered through Miracloth, centrifuged at 30,000*g* for 15 min, the supernatant added to a suspension of insoluble PVP (6 g) in 10 ml of buffer A, and the mixture centrifuged at 30,000*g* for 15 min. The supernatant contained the plant cytosolic fraction of the nodules and was free of β-hydroxybutyrate dehydrogenase (11), which is a marker enzyme for the bacteroids (4).

The plant cytosolic fraction was applied directly to a DEAE-cellulose column (1.5 × 25 cm) which had been previously equilibrated with buffer A. The column was washed with buffer A until unbound protein was removed and eluted with a gradient produced by introducing 250 ml of 0.5 M KCl in buffer A into 250 ml of buffer A. Fractions of 5 ml were collected. Those fractions which contained hexokinase but no fructokinase were pooled and concentrated to approximately 4 ml by ultrafiltration in an Amicon apparatus (PM 10 membrane). Preparations of this type were used to study the properties of the hexokinase from the plant cytosolic fraction of soybean nodules.

The DEAE-cellulose column fractions which contained fructokinase but no hexokinase activity were pooled, concentrated by ultrafiltration to approximately 4 ml, and applied to a Fractogel TSK HW-55 column (1.6 × 72 cm) which had previously been equilibrated with 20 mM Tris-HCl buffer (pH 8.2) containing 50 mM KCl (buffer B). Fractions of 4 ml were collected and those which contained activity were pooled and $MgCl_2$ (0.5 M) added to a final concentration of 5 mM. The preparation was applied to an AGATP (type 2) column (1.5 × 2.8 cm) which had

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[2] Abbreviations: AGATP, agarose-hexane-adenosine-5′-triphosphate; U, unit; V, maximum velocity.

been previously equilibrated with buffer B containing 5 mM $MgCl_2$. The column was washed until the A_{280} was less than 0.01 and eluted with buffer B containing 5 mM $MgCl_2$ and 5 mM ATP. Active fractions were pooled and dialyzed against buffer B. The fructokinase preparation was free of 6-P-gluconate dehydrogenase and phosphatase activities and could be stored for at least 2 weeks at −15°C without loss of activity.

Assay of Enzyme Activity. All enzyme assays were carried out at 30°C. Hexokinase activity was measured by coupling the production of glucose-6-P to the reduction of NADP in the presence of excess glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase and following the change in A_{340}. Standard reaction mixtures contained, in a volume of 1 ml, 25 μmol Tris-HCl buffer (pH 8.2) 50 μmol KCl, 5 μmol glucose, 5 μmol ATP, 7 μmol $MgCl_2$, 0.33 μmol NADP, 0.6 μg glucose-6-P dehydrogenase, 8 μg 6-P-gluconate dehydrogenase, and an appropriate volume of enzyme.

Fructokinase activity was assayed by coupling the production of glucose-6-P (formed from fructose-6-P by the action of P-glucose isomerase) with the reduction of NADP in the presence of excess glucose-6-P dehydrogenase. Standard reaction mixtures contained, in a volume of 1 ml, 25 μmol Tris-HCl buffer (pH 8.2), 50 μmol KCl, 0.4 μmol fructose, 1 μmol ATP, 1.5 μmol $MgCl_2$, 0.33 μmol NADP, 0.6 μg glucose-6-P dehydrogenase, 3 μg P-glucose isomerase, and an appropriate volume of enzyme. For assays of crude extracts and DEAE-cellulose column fractions 6-P-gluconate dehydrogenase (8 μg) was added. When the phosphorylation of sugars other than fructose and glucose was tested, or when the effects of glucose-6-P and fructose-6-P were studied, the production of ADP was coupled to the oxidation of NADH in the presence of excess pyruvate kinase and lactate dehydrogenase. Reaction mixtures contained, in a volume of 1 ml, 25 μmol Tris-HCl, 50 μmol KCl, 1 μmol ATP, 1.5 μmol $MgCl_2$, 0.15 μmol NADH, 0.5 μmol P-enolpyruvate, 8 μg pyruvate kinase, 16 μg lactate dehydrogenase, substrate as indicated, and an appropriate volume of fructokinase.

Initial velocity data were first graphed to check the linearity of double reciprocal plots. Kinetic constants were calculated by fitting the data to the Michaelis-Menten equation as described by Duggleby (7). One U of activity is defined as the amount of enzyme which catalyzed the formation of 1 μmol product/min. Protein was determined by the Folin-Lowry method.

Molecular Weight Determination by Gel Filtration. The mol wt of fructokinase was determined on a Sephadex G-200 column according to the method of Andrews (1). Ferritin, catalase, aldolase, BSA, and Cyt *c* were used as calibration proteins.

RESULTS

When the plant cytosolic fraction of a soybean nodule extract was chromatographed on DEAE-cellulose two peaks of hexose phosphorylating activity were separated (Fig. 1). The first peak which was eluted contained a hexokinase while the second and major peak contained a fructokinase. The nomenclature which has been adopted for these enzymes is based on that used previously (21). Because of the small amount of hexokinase in the plant cytosolic fraction this enzyme was studied without further purification. Fructokinase was purified 75-fold using the procedure summarized in Table I. The mol wt of native fructokinase was determined by gel filtration to be 84,000 ± 5,000.

Effect of Hexose Concentration. The plant cytosolic hexokinase of soybean nodules followed typical Michaelis-Menten kinetics when glucose or fructose were the varied substrates. The enzyme had a much lower K_m for glucose than fructose although the V with fructose was greater than with glucose (Table II). Mannose (final concentration 5 mM) was phosphorylated at 0.5% of the maximum rate observed with glucose.

The activity of the plant cytosolic fructokinase at pH 8.2 increased as the concentration of fructose was increased to 0.4 mM (Fig. 2). Higher fructose concentrations were inhibitory such that at 1 mM fructose the activity was 77% of the maximum (Fig. 2). When the concentration of fructose in the assay mixtures was increased to 5 mM (not shown) fructokinase gave 44% of the maximum activity. The apparent K_m for fructose at pH 8.2, calculated from the initial rate at noninhibitory concentrations, was 0.077 ± 0.009 mM. At pH 6.6 substrate inhibition was not observed (Fig. 2) and the K_m for fructose was 0.055 ± 0.003 mM. The following sugars (final concentration 5 mM) were not phosphorylated by fructokinase: D-glucose, D-mannose, D-galactose, D-tagatose, L-sorbose, D-xylose, D-mannoheptulose, D-glucosamine, and 2-deoxy-D-glucose.

Effect of the Concentration of Mg^{2+} and MgATP. In reaction mixtures which contained 5 mM ATP, activity of the plant

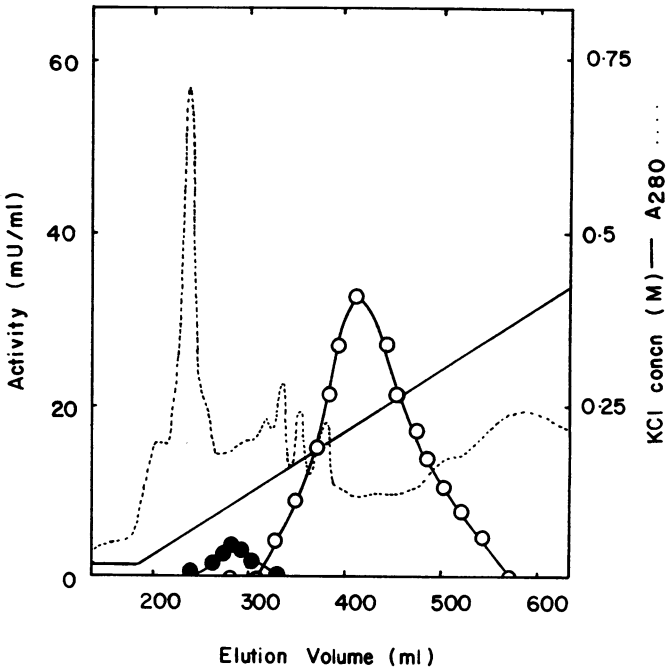

FIG. 1. DEAE-cellulose chromatography of the plant cytosolic fraction of soybean nodules. The extract from 6 g of soybean nodules was applied to a DEAE-cellulose column. (●), Hexokinase; (○), fructokinase.

Table I. *Purification of Hexose Kinases from the Plant Cytosolic Fraction of Soybean Nodules*

Stage	Protein Content	Activity	Specific Activity
	mg	*U*	*U/mg protein*
Hexokinase			
Crude extract	67.7	0.74	0.011
DEAE-cellulose	4.0	0.098	0.024
Fructokinase			
Crude extract	67.7	10.1	0.15
DEAE-cellulose	13.2	3.30	0.25
Fractogel	5.0	2.04	0.41
AGATP	0.12	1.34	11.2

Table II. *Kinetic Constants of Hexokinase from the Plant Cytosolic Fraction of Soybean Nodules*

Substrate	K_m	V	V/K_m
	mM	*U/mg protein*	
D-Glucose	0.075 ± 0.005	0.032 ± 0.001	0.43
D-Fructose	2.5 ± 0.1	0.057 ± 0.002	0.023

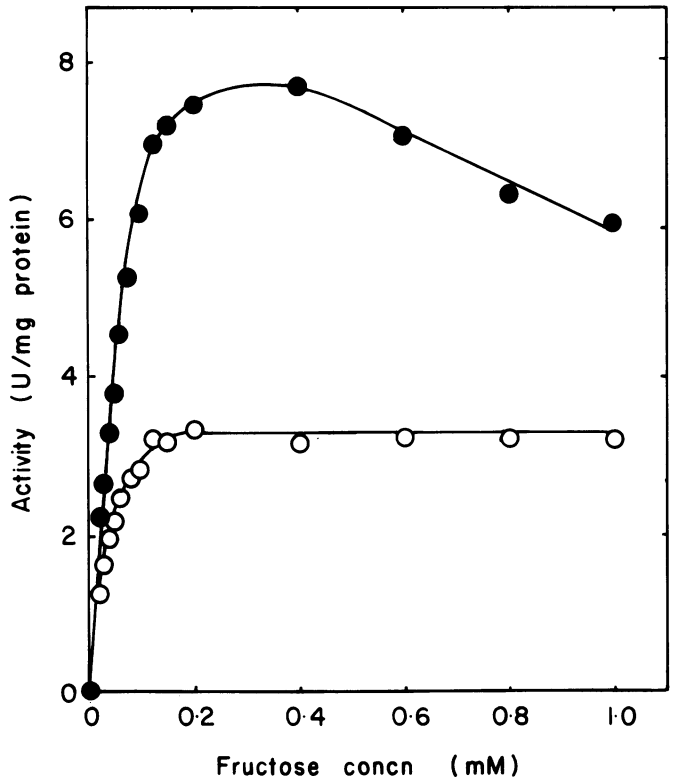

FIG. 2. Effect of fructose concentration on fructokinase from the plant cytosolic fraction of soybean nodules. Reaction mixtures at pH 6.6 contained 20 μmol Mes. KOH buffer. pH 8.2 (●); pH 6.6 (○).

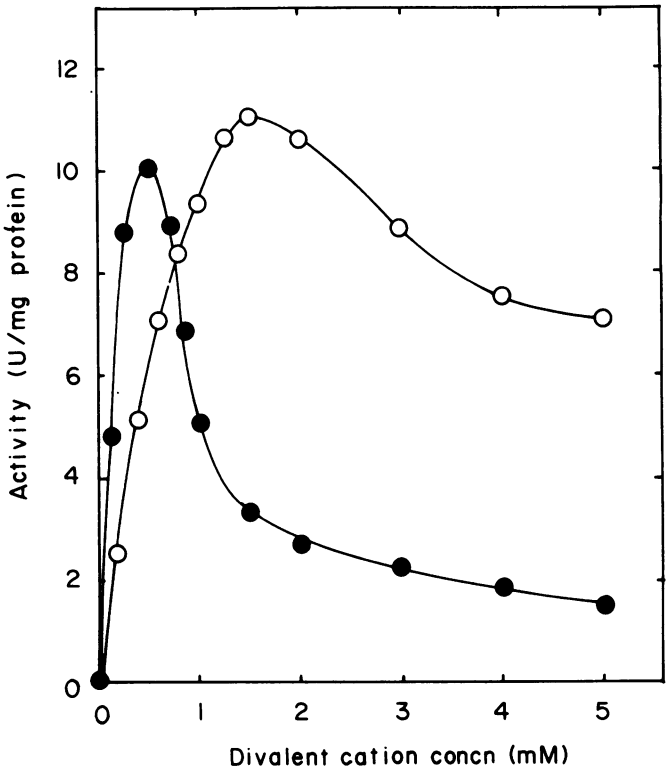

FIG. 3. Effect of varying the concentration of Mg^{2+} and Mn^{2+} on fructokinase from the plant cytosolic fraction of soybean nodules. Mg^{2+} (○); Mn^{2+} (●).

cytosolic hexokinase was maximum when the concentration of $MgCl_2$ was 7 mM. Higher concentrations of $MgCl_2$ were not inhibitory. When the concentration of MgATP was varied in the presence of a fixed excess (2 mM) of $MgCl_2$ typical Michaelis-Menten kinetics were observed and a K_m value of 0.062 ± 0.003 mM was determined.

The activity of the plant cytosolic fructokinase in reaction mixtures which contained 1 mM ATP was maximum when the concentration of $MgCl_2$ was 1.5 mM (Fig. 3). Higher concentrations of $MgCl_2$ inhibited the enzyme. Mn^{2+} could replace Mg^{2+} at low concentrations but concentrations of Mn^{2+} greater than 0.5 mM were inhibitory (Fig. 3). When the concentration of MgATP was increased in the presence of a fixed excess of 0.5 mM $MgCl_2$ a hyperbolic plot was observed and a value of 0.095 ± 0.005 mM was determined for the K_m of fructokinase for MgATP.

Effect of pH. The plant cytosolic hexokinase had a broad pH optimum from 8.2 to 8.9. Activities of 90% or greater of the maximum were observed between pH 7.6 and 9.2. The pH optimum of fructokinase was 8.2 with 90% or greater of the maximum activity between pH 7.4 and 9.3.

Effect of Cations. The effect of monovalent cations was studied after the enzyme preparations and the coupling enzymes had been dialyzed against 10 mM Tris-HCl buffer (pH 8.2). The reaction mixtures prepared in this manner contained 0.5 mM K^+, 2 mM Na^+, and 1 mM NH_4^+.

The addition of 50 mM KCl, RbCl, or NH_4Cl to the reaction mixtures stimulated the activity of the plant cytosolic hexokinase by 15%. Increasing the concentration of the salts to 100 mM did not produce any further increase in activity. NaCl, LiCl, and CsCl (final concentration 100 mM) did not stimulate hexokinase activity.

Monovalent cations were required by fructokinase for maximum activity. K^+ was most effective in stimulating activity, with the addition of 50 mM KCl giving a stimulation of 4.3-fold (Table III). Increasing the concentration of K^+ in the reaction mixtures to 100 mM did not result in any further stimulation of activity. Rb^+ also stimulated activity but to a lesser extent than K^+ (Table III). NH_4^+ was even less effective while Na^+, Li^+, and Cs^+ gave only slight stimulations (Table III).

Table III. *Effect of Monovalent Cations on Fructokinase from the Plant Cytosolic Fraction of Soybean Nodules*

Addition	Fructokinase Activity	
	25 mM	50 mM
	U/mg protein[a]	
K^+	8.04	9.50
Rb^+	6.58	8.10
NH_4^+	4.18	6.46
Na^+	2.41	2.51
Li^+	2.46	2.66
Cs^+	2.91	3.17

[a] With no addition the fructokinase activity was 2.22 U/mg protein.

Al^{3+} (final concentration 0.1 mM, added as $KAl[SO_4]_2$) had no effect on hexokinase under the standard assay conditions (pH 8.2). At pH 6.6, in reaction mixtures which contained 20 mM Mes-KOH buffer, 0.1 mM Al^{3+} inhibited activity by 35%. There was no effect on fructokinase when 0.1 mM Al^{3+} was added to reaction mixtures at pH 8.2 or 6.6.

Effect of Products. Under the standard assay conditions MgADP at concentrations of 1 and 2 mM inhibited activity of the plant cytosolic hexokinase by 45 and 62%, respectively. Fructokinase was inhibited 15 and 37% when MgADP was added to standard reaction mixtures at concentrations of 1 and 2.5 mM, respectively. Fructokinase was inhibited 20% by 10 mM fructose-6-P but 10 mM glucose-6-P was not inhibitory.

Effect of Metabolites. The following metabolites (final con-

centration 5 mM) had no effect on fructokinase under the standard assay conditions: UDP, UDPglucose, glucose-1-P, sucrose, 3-P-glycerate, P-enolpyruvate, pyruvate, ethanol, succinate, α-ketoglutarate, glutamine, allantoin, NO_2^-, and NO_3^-.

DISCUSSION

The present investigation has shown that the plant cytosolic fraction of soybean nodules contains a hexokinase and a fructokinase. The amount of hexokinase in the host cytosol was small in comparison to fructokinase. Hexokinase had a high affinity for glucose (K_m 0.075 mM) and a relatively low affinity for fructose (K_m 2.5 mM). The concentration of glucose in soybean nodules is much higher than fructose (16, 17) and therefore this enzyme is likely to be involved mainly with the phosphorylation of glucose. Furthermore, comparison of the values of the parameter V/K_m with glucose and fructose indicates that fructose would be a much poorer substrate than glucose at low concentrations of the hexoses. Hexokinase was sensitive to inhibition by MgADP and this could have significance for the regulation of the enzyme *in vivo*. The plant cytosolic hexokinase of soybean nodules was not strongly inhibited by Al^{3+} at low pH. Al^{3+} is a potent inhibitor of hexokinase II of pea seeds and pea stem hexokinase, with 50% inhibition occurring at Al^{3+} concentrations at 2 and 10 μM, respectively (19, 21).

The fructokinase in the plant cytosolic fraction of soybean nodules phosphorylated fructose only. The enzyme was inhibited at pH 8.2 by concentrations of fructose greater than 0.4 mM. In view of the low concentration of fructose in nodules (16, 17) this inhibition is unlikely to be metabolically important. However the inhibition could be significant in the selection of conditions appropriate for the assay of fructose phosphorylating activity in nodules. Substrate inhibition by fructose also occurs with the fructokinases of pea seeds (5, 22). In the case of pea seed fructokinase I, inhibition is the result of the reaction proceeding through a slower pathway in the kinetic mechanism at high concentrations of fructose (6). The plant cytosolic fructokinase of soybean nodules had a specific requirement for K^+ ions for maximum activity. Rb^+ and NH_4^+ stimulated activity to some extent but Na^+, Li^+, and Cs^+ were not effective. In this regard the soybean nodule fructokinase was similar to fructokinase II of pea seeds, which also has a specific requirement for K^+ ions (22). The requirement of pea seed fructokinase I for monovalent cations is nonspecific (5). The native fructokinase of soybean nodules had a mol wt of 84,000 ± 5,000. This compares with a value of 72,000 ± 4,000 reported for pea seed fructokinase I, which has a monomeric structure (6).

The situation with respect to the enzymes that phosphorylate glucose and fructose in the plant cytosol of soybean nodules is thus comparable to other plant tissues, such as pea seeds (5, 20–22) spinach leaves (3), and developing castor oil seeds (10). These tissues all contain a number of hexose kinases which preferentially phosphorylate either glucose or fructose.

Essentially all of the fructokinase activity in the soybean nodules used in this investigation, with *R. japonicum* strain CB1809 as the symbiont, was in the plant cytosolic fraction (11). Similar results have been reported by Reibach and Streeter using soybean nodules infected with *R. japonicum* strain USDA 110 or USDA 138 (14). Very little fructose phosphorylating activity is in the bacteroid fraction of these nodules (11, 14). Thus when sucrose is cleaved in the host cytoplasm by invertase or sucrose synthase the fructose produced is likely to be phosphorylated directly by fructokinase in the same metabolic compartment rather than be taken up by the bacteroids. The low concentration of fructose in soybean nodules suggests that there is adequate fructokinase activity in the host cytoplasm to meet the requirements for fructose phosphorylation. The fructose-6-P formed by fructokinase may be metabolized further in the host cytoplasm. A number of enzymes of the glycolytic and pentose phosphate pathways have been detected in the plant cytosolic fraction of soybean nodules (14).

Glucose would be formed in the nodules when invertase acts on sucrose. The amount of hexokinase in the plant cytosolic fraction of the soybean nodules was small and this may mean that the phosphorylation of glucose in the host cytoplasm of these nodules is limited. Thus there could be glucose available for uptake by the bacteroids. The distribution of glucose phosphorylating activity in nodules infected with *R. japonicum* strains CB 1809 (11), USDA 110 or USDA 138 (14) indicates that glucose can be metabolized by these bacteroids.

LITERATURE CITED

1. Andrews P 1966 The gel filtration behaviour of proteins related to their molecular weights over a wide range. Biochem J 96: 595–606
2. Bach MK, WE Magee, RH Burris 1958 Translocation of photosynthetic products to soybean nodules and their role in nitrogen fixation. Plant Physiol 33: 118–124
3. Baldus B, GJ Kelly, E Latzko 1981 Hexokinases of spinach leaves. Phytochemistry 20: 1811–1814
4. Brown CM, MJ Dilworth 1975 Ammonia assimilation by *Rhizobium* cultures and bacteroids. J Gen Microbiol 85: 39–48
5. Copeland L, DD Harrison, JF Turner 1978 Fructokinase (fraction III) of pea seeds. Plant Physiol 62: 291–294
6. Copeland L, SR Stone, JF Turner 1984 Kinetic studies of fructokinase I of pea seeds. Arch Biochem Biophys 233: 748–760
7. Duggleby RG 1981 A nonlinear regression program for small computers. Anal Biochem 110: 9–18
8. Kidby DK 1966 Activation of plant invertase by inorganic phosphate. Plant Physiol 41: 1139–1144
9. Meunier J, J Buc, J Ricard 1971 Isolation, purification and characterization of wheat germ hexokinases. FEBS Lett 14: 25–28
10. Miernyk JA, DT Dennis 1983 Mitochondrial, plastid and cytosolic isozymes of hexokinase from developing endosperm of *Ricinus communis*. Arch Biochem Biophys 226: 458–468
11. Morell MK, L Copeland 1984 Enzymes of sucrose metabolism in soybean nodules. Alkaline invertase. Plant Physiol 74: 1030–1034
12. Morell MK, L Copeland 1985 Sucrose synthase of soybean nodules. Plant Physiol 78: 149–154
13. Rawsthorne S, FR Minchin, RJ Summerfield, C Cookson, J Coombs 1980 Carbon and nitrogen metabolism in legume root nodules. Phytochemistry 19: 341–355
14. Reibach PH, JG Streeter 1983 Metabolism of ^{14}C-labeled photosynthate and distribution of enzymes of glucose metabolism in soybean nodules. Plant Physiol 72: 634–640
15. Robertson JG, MP Taylor 1973 Acid and alkaline invertase in roots and nodules of *Lupinus angustifolius* infected with *Rhizobium lupini*. Planta 112: 1–6
16. Streeter JG 1980 Carbohydrates in soybean nodules II. Distribution of compounds in seedlings during the onset of nitrogen fixation. Plant Physiol 66: 471–476
17. Streeter JG 1981 Seasonal distribution of carbohydrates in nodules and stem exudate from field-grown soya bean plants. Ann Bot 48: 441–450
18. Streeter JG 1982 Enzymes of sucrose, maltose and α,α-trehalose catabolism in soybean root nodules. Planta 155: 112–115
19. Tanner GJ, L Copeland, JF Turner 1983 Subcellular localization of hexose kinases in pea stems: Mitochondrial hexokinase. Plant Physiol 72: 659–663
20. Turner JF, QJ Chensee, DD Harrison 1977 Glucokinase of pea seeds. Biochim Biophys Acta 480: 367–375
21. Turner JF, L. Copeland 1981 Hexokinase II of pea seeds. Plant Physiol 68: 1123–1127
22. Turner JF, DD Harrison, L Copeland 1977 Fructokinase (fraction IV) of pea seeds. Plant Physiol 60: 666–669