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Metabolism of ^{14}C-Labeled Photosynthate and Distribution of Enzymes of Glucose Metabolism in Soybean Nodules[1]

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PAUL H. REIBACH[2] AND JOHN G. STREETER
Department of Agronomy, Ohio State University/Ohio Agricultural Research and Development Center, Wooster, Ohio 44691

ABSTRACT

The metabolism of translocated photosynthate by soybean (*Glycine max* L. Merr.) nodules was investigated by $^{14}CO_2$-labeling studies and analysis of nodule enzymes. Plants were exposed to $^{14}CO_2$ for 30 minutes, followed by $^{12}CO_2$ for up to 5 hours. The largest amount of radioactivity in nodules was recovered in neutral sugars at all sampling times. The organic acid fraction of the cytosol was labeled rapidly. Although cyclitols and malonate were found in high concentrations in the nodules, they accumulated less than 10% of the radioactivity in the neutral and acidic fractions, respectively. Phosphate esters were found to contain very low levels of total label, which prohibited analysis of the radioactivity in individual compounds. The whole nodule-labeling patterns suggested the utilization of photosynthate for the generation of organic acids (principally malate) and amino acids (principally glutamate).

The radioactivity in bacteroids as a percentage of total nodule label increased slightly with time, while the percentage in the cytosol fraction declined. The labeling patterns for the cytosol were essentially the same as whole nodule-labeling patterns, and they suggest a degradation of carbohydrates for the production of organic acids and amino acids. When it was found that most of the radioactivity in bacteroids was in sugars, the enzymes of glucose metabolism were surveyed. Bacteroids from nodules formed by *Rhizobium japonicum* strain 110 or strain 138 lacked activity for phosphofructokinase and NADP-dependent 6-phosphogluconate dehydrogenase, key enzymes of glycolysis and the oxidative pentose-phosphate pathways. Enzymes of the glycolytic and pentose phosphate pathways were found in the cytosol fraction.

In three experiments, bacteroids contained about 10 to 30% of the total radioactivity in nodules 2 to 5 hours after pulse-labeling of plants, and 60 to 65% of the radioactivity in bacteroids was in the neutral sugar fraction at all sampling times. This strongly suggests some absorption and metabolism of sugars by bacteroids in spite of the lack of key enzymes. Bacteroids did possess enzymes for the formation of hexose phosphates from glucose or fructose. Radioactivity in α,α-trehalose in bacteroids increased until, after 5 hours, trehalose was a major labeled compound in bacteroids. Thus, trehalose synthesis may be a major fate of sugars entering bacteroids.

The utilization of translocated photosynthate by soybean root nodules is an important, but little understood, physiological process. Vernon and Aronoff (28) demonstrated that sucrose was the major compound translocated from the leaves of soybean plants. Bach *et al.* (2) reported studies on the translocation and subsequent distribution of ^{14}C-photosynthate in soybean nodules. They found that the labeled compounds included glucose, fructose, malate, succinate, and citrate. Somewhat curiously, no label was found in sucrose in nodules. However, 12 h after supplying $^{14}CO_2$ was the earliest sampling time, precluding a description of the distribution of label shortly after the entry of labeled sucrose into nodules.

More recent studies involving the use of $^{14}CO_2$ and shorter sampling intervals have been undertaken with other leguminous plants. Working with *Phaseolus vulgaris* nodules, Antoniw and Sprent (1) found radioactivity in sucrose, glucose, fructose, an unknown carbohydrate, malate, citrate, malonate, and an unknown organic acid. Nodules were sampled only at 2.5 h after a 30-min exposure to $^{14}CO_2$. By separating the bacteroids and cytosol with differential centrifugation, they were also able to demonstrate small amounts of labeled sucrose, glucose, and fructose in the bacteroids. The only bacteroid organic acid which contained label was tentatively identified as 6-P-gluconate.

Lawrie and Wheeler (13) studied the distribution of labeled metabolites in the nodules of *Vicia faba* after plant photosynthesis with $^{14}CO_2$ for 30 min. The neutral fraction contained 70% of the total radioactivity in nodule extracts and label in glucose plus fructose accounted for 87% of the radioactivity in the neutral fraction.

The identity and quantity of most of the mono- and disaccharides in soybean nodules has recently been established (22, 24). With this knowledge, with methods for the separation of these carbohydrates by TLC (23, 24), and with a method for the rapid purification of viable bacteroids (19), a re-examination of the distribution of $^{14}CO_2$-labeled photosynthate in soybean nodules seemed desirable. Our goal was to determine the distribution of radioactivity in whole nodule extracts and in bacteroids and cytosol fractions at the shortest possible times after supplying $^{14}CO_2$ to shoots. When significant quantities of radioactivity were found in sucrose in bacteroids, a study of the distribution of enzymes of glucose metabolism in nodules was also undertaken.

MATERIALS AND METHODS

Plant Material. Beeson soybeans (*Glycine max* L. Merr.) were planted in pots of washed silica sand. Seeds were inoculated at the time of planting with a commercial inoculant (Agricultural Laboratories, Columbus, OH) of *Rhizobium japonicum* containing strains 110, 8-0, 8-T, and 138. Plants were irrigated with a nutrient solution containing no N (22). For experiments 1 and 3, plants were grown outdoors during July and August and were supplied with $^{14}CO_2$ outdoors on clear days for 30-min periods between 9:00 AM and 12:00 noon. For experiment 2, plants were grown in the greenhouse in October and November and metal halide lamps providing 1000 μE m^{-2} s^{-1} (400–700 nm) were used to provide

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[2] Present address: Department of Plant Sciences, Texas A & M University, College Station, TX 77843.

supplemental light and a photoperiod of 14 h. Plants were supplied with $^{14}CO_2$ in the greenhouse for 30-min periods between 9:00 AM and 12:00 noon on a day in December when there was occasional, partial cloudiness. Plants were 6 to 8 weeks old in all experiments.

$^{14}CO_2$ Fixation. Pots of plants (6 plants/pot) were sealed in polyethylene bags (61 × 26 × 21 cm). $^{14}CO_2$ was liberated from vials of $Ba^{14}CO_3$ by the addition of 2.0 ml of 50% (v/v) lactic acid. Plants in each pot were supplied with approximately 400 μCi $^{14}CO_2$. The bags were shaken in order to distribute the radioactivity evenly to all plants. $^{14}CO_2$ fixation continued for 30 min, at which time the bags were removed and any $^{14}CO_2$ remaining was vented. Photosynthesis was allowed to continue in the presence of ambient $^{12}CO_2$ for the designated times.

Metabolite Extractions. All nodules on six plants were harvested and results are expressed on a total radioactivity per six plants (or per pot) basis. Nodules were harvested, weighed, and ground in 95% ethanol with a mortar and pestle. The samples were centrifuged at 27,000*g* and the supernatant was collected. The pellets were extracted four times with 75% ethanol and the supernatants were combined. The ethanol was removed with a rotary evaporator, and the samples were dissolved in H_2O and stored at 4°C with a few drops of chloroform, or frozen, until further analysis.

Bacteroid-Cytosol Isolation. Bacteroids were obtained from a subsample of nodules which varied in weight but which comprised about 20% of the main sample. Bacteroids were isolated by Percoll gradient centrifugation as previously described (19), with the following modifications. Nodules were crushed in buffer at 4°C and the residue was removed by filtration through glass wool. The entire filtrate was loaded onto the Percoll mixture. After centrifugation, the bacteroids were removed and transferred to tubes containing 95% ethanol (80°C). The cytosol fraction was also collected into tubes containing 95% ethanol (80°C). The resulting precipitates were removed by centrifugation and the supernatants were collected. The pellets were extracted three times with 75% ethanol and centrifuged, and the supernatants were combined. The ethanol was removed by rotary evaporation and the solids were dissolved in water and stored as above.

Metabolite Analysis. Extracts from experiment 1 were fractionated by Dowex column chromatography. Samples were passed through Dowex 50 (H^+, 1 × 5 cm) and Dowex 1 (formate, 1 × 5 cm), and effluent was taken as the neutral fraction. The Dowex 1 column was eluted with 2 N HCl to give the organic acid-phosphate ester fraction. The Dowex 50 column was eluted with 50% (v/v) NH_4OH to give the basic fraction containing amino acids. For experiments 2 and 3, the extracts were fractionated on ion-exchange Sephadex columns, according to the procedure of Redgwell (18). The anion exchanger was QAE-A-25-formate (Sigma Chemical Co). The cation exchanger was SP-C-24-H^+ (Sigma). This procedure allows the extract to be fractionated into the neutral, basic, acidic, and phosphate ester fractions.

The neutral sugars were separated by TLC (23, 24). (Allantoin moves with the solvent front in the second direction of this chromatography system and radioactivity in allantoin was not measured in these experiments.) After solvent irrigations and drying, the spots were detected by spraying the plates with a saturated KIO_4 solution and heating at 45°C until dry. The cooled plates were then sprayed with a solution containing 1.0 ml saturated $AgNO_3$ and 200 ml acetone, to which H_2O was added until the precipitate dissolved. The use of $AgNO_3$ and, especially, KIO_4 significantly lowers recovery of radioactivity in carbohydrates. Typical recoveries in the TLC analyses are 60 to 70% of the radioactivity spotted and we know that a large portion (at least half) of this loss is due to the spray reagents. However, this procedure is the best method we have been able to devise for the detection of cyclitols, which are very inert (24).

Amino acids were separated by TLC as previously described (21). The organic acids were separated by one-dimensional TLC according to the procedure of Ting and Dugger (26). Camag D-O G without binder was substitutetd for Silica Gel H. Malic and malonic acids were quantitatively determined by GC. Samples or standards were dissolved in 50 μl pyridine plus tartaric acid (10 mg/ml) as an internal standard. The dissolved organic acids were reacted with 50 μl Regisil (RC-2 with 1% trimethylchlorosilane, Regis Chemical Co.) by heating at 70°C for 15 min. Samples were analyzed with a Varian 3700 GC with an SE-52 column. Flow rates were He, 30; H_2, 30; air, 300 cc/min and the temperature program involved an initial temperature of 60°C for 5 min followed by a temperature increase of 3°C/min to a maximum temperature of 190°C. Peak areas were determined with a Varian CDS 111 integrator.

Analysis of Enzyme Activities. Plants were grown in a greenhouse as described above except that sand was sterilized by autoclaving and seeds were inoculated with a liquid culture (23) of *R. japonicum* strain USDA 110 or USDA 138.

Bacteroids and cytosol were purified using Percoll gradients as described by Reibach *et al.* (19), except that the grinding medium was 0.2 M Na phosphate buffer, pH 7.5, containing 2 mM dithioerythritol. (Preliminary trials showed increased activity of most enzymes when dithioerythritol was included in all buffers and showed no advantage of including insoluble PVP at the grinding step.) Bacteroids were washed in this same buffer and finally suspended in a known volume of 0.1 M Na phosphate, pH 7.5, containing 2 mM dithioerythritol. These and all subsequent operations were carried out at 2°C.

Five drops of Triton X-100 (Sigma) were added and the bacteroids were sonicated 4 × 3 min. The mixture was centrifuged at 48,000*g* for 15 min. This supernatant and the cytosol collected from the Percoll gradient were filtered through columns of Sephadex G-25 (Pharmacia) using 0.01 M Na phosphate, pH 7.0, containing 1 mM dithioerythritol, for equilibration and elution of the columns. Preparation of the fractions required about 2 (cytosol) to 4 (bacteroid extract) h and enzymes were assasyed as soon as the filtered enzyme preparations were available.

All reagents were of the highest purity available from Sigma Chemical Co. Purified enzymes were also purchasesd from Sigma as follows: glucose-6-P dehydrogenase, type XI; P-glucose isomerase, type III; aldolase, type I; α-glycerol-P dehydrogenase, type I; triose-P isomerase, type III; pyruvate kinase, type II; lactate dehydrogenase, type II.

P-glucomutase (EC 2.7.5.1) was assayed as described in Bergmeyer (3; vol 2, p 798), except that histidine was omitted. Glucose-dependent hexokinase (EC 2.7.1.1) was assayed as described in Bergmeyer (3; vol 1, p 473), except that 1 mM dithioerythritol and 30 mM KCl were included in the reaction mixture. The reaction mixture for fructose-dependent hexokinase was the same as that just described except that fructose replaced glucose and two units (Sigma) of P-glucose isomerase were included. P-glucose isomerase (EC 5.3.1.9) was assayed as described in Bergmeyer (3; vol 1, p 501), except that buffer pH was 7.9 and substrate (fructose-6-P) concentration was 10 mM. The substrate was checked for contamination with glucose-6-P before initiating the reaction with extract. P-fructokinase (EC 2.7.1.11) was assayed using the reaction mixture described by Stephenson *et al.* (20) except that 1 mM dithioerythritol was included. There was no activity with either bacteroid extract or cytosol when PPi was substituted for ATP (8). Fructose-1,6-bisP aldolase (EC 4.1.2.13) was assayed using the mixture described by Stephenson *et al.* (20), except that $CoSO_4$ was omitted. Glucose-6-P dehydrogenase (EC 1.1.1.49) was assayed as described in Bergmeyer (3; vol 2, p 642), using <0.1 mg protein to avoid accumulation of product. 6-P-gluconate dehydrogenase (EC 1.1.1.44) was assayed as described in Bergmeyer (3; vol 2, p 632), except that pH 8.0 was used and dithioerythritol was substituted for cysteine. Enzyme activity with NAD substituted for NADP

(15) was also determined.

All of the above assays are based on measurement of ΔA_{340} due to formation or consumption of NAD(P)H. A_{340} was recorded at 30°C for 5 to 10 min using a Gilford model 250 spectrophotometer and the period of linear ΔA used for calculation of enzyme activity. Sephadex-filtered enzyme preparations were used. The ΔA prior to adding the substrate was nil for most enzyme assays. Protein content of enzyme preparations was estimated with a dye-binding method (7).

Unsuccessful attempts were made to detect the following enzyme activities: fructokinase (EC 2.7.1.3) using the coupled assay of Raushel and Cleland (17); glucose dehydrogenase (EC 1.1.99.a), using intact bacteroids, bacteroid fragments (after sonication), and bacteroid extracts with the assay described by Hauge (10); a combined assay for 6-P-gluconate dehydratase (EC 4.1.2.2) and 2-keto-3-deoxy-6-P-gluconate aldolase (EC 4.1.2.14) described by Stephenson *et al.* (20). This latter assay, which tests for the formation of pyruvate from 6-P-gluconate, was used with bacteroid extracts and bacteroid fragments (after sonication), and no activity was detected in either case.

RESULTS AND DISCUSSION

Results are reported for three $^{14}CO_2$-labeling experiments. Experiment 1 involved two replicates and samples taken at 1-h intervals from 1 to 5 h after the $^{14}CO_2$ pulse. Experiment 2 provided more intensive sampling of the period of increasing radioactivity in nodules. There were three replicates and samples were taken at 0.5-h intervals from 1 to 3.5 h after the $^{14}CO_2$ pulse. Experiment 3 provided additional data during the period of decreasing radioactivity in nodules. There were three replicates and samples were taken at 1-h intervals from 2 to 5 h after the $^{14}CO_2$ pulse. For the determination of radioactivity in bacteroids and cytosol, only two replicates were analyzed regardless of the number of replicates in the experiment.

Whole Nodules. Radioactivity was detected in nodules 1 h after the end of the $^{14}CO_2$ fixation period and reached a peak after 3 h (Fig. 1). These results are consistent with the time courses obtained with *V. faba* (13) and demonstrate that recently synthesized carbohydrates are rapidly translocated to nodules. The largest amount of radioactivity was found in the neutral fraction, with smaller and approximately equal amounts in the organic acid and amino acid fractions (Fig. 1).

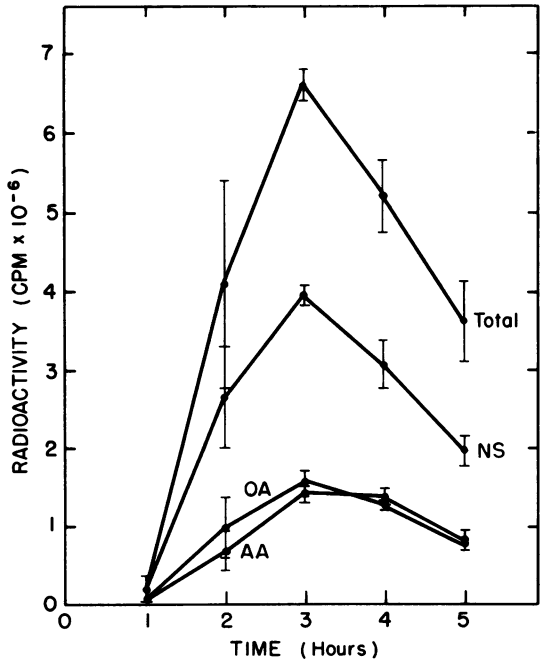

FIG. 1. Radioactivity in the neutral (NS), basic (AA), and acidic (OA) fractions and the sum of the total radioactivity (Total) in these fractions. Extracts of whole nodules, from intact soybean plants exposed to $^{14}CO_2$ for 30 min, were fractionated on Dowex ion-exchange columns. Sampling times were measured from the end of the 30-min $^{14}CO_2$ pulse. The mean and range of two replicates are given (Experiment 1).

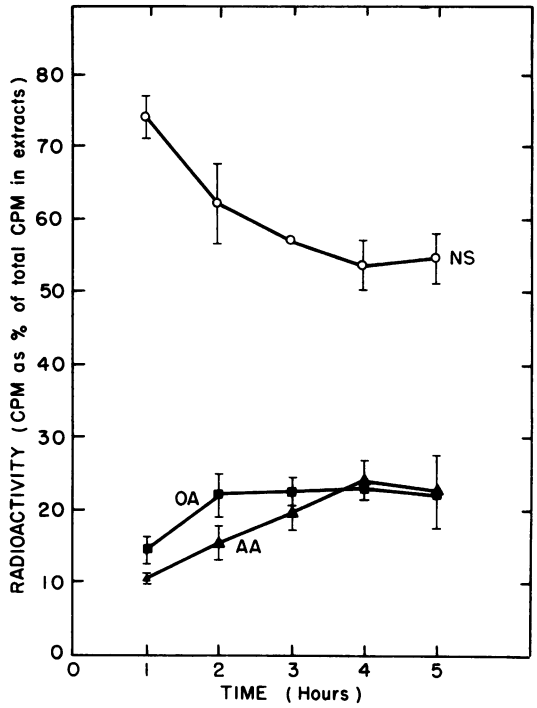

FIG. 2. Percentage of total radioactivity in the neutral (NS), basic (AA), and acidic (OA) fractions. Percentages were calculated from the data in Figure 1. The mean and range of two replicates are given.

From Figure 1, it is difficult to discern the change in the proportions of radioactivity in the three fractions with time. However, when the results for each fraction are expressed as per cent total radioactivity, a decline from about 75% to about 55% can be seen for the neutral fraction (Fig. 2). There was a concomitant increase in the proportion of total radioactivity in organic acids and amino acids with a slightly greater proportion in organic acids between 1 and 3 h (Fig. 2). These proportions are in agreement with those found with a single 2.5-h sample of *P. vulgaris* nodules (1). However, Lawrie and Wheeler (13) found much smaller proportions of radioactivity in acidic and basic fractions of *V. faba* nodules at all sampling times from 0.5 to 6 h.

Sucrose contained most of the radioactivity in the neutral fraction of all samples analyzed (Table I). A small proportion of the radioactivity was found in glucose and a very small proportion was found in fructose. The proportion of the label in sucrose declined slightly with time while the proportion of the label in trehalose increased.

Only a small amount of radioactivity was found in the cyclitols at all times (Table I). These compounds, namely D-pinitol, D-*chiro*-inositol, and *myo*-inositol, comprise a major portion (40 to 50%) of the soluble carbohydrate in soybean nodules and their concentrations increase dramatically during the onset of N_2 fixation (22, 24). The weak labeling of the cyclitols at periods of up to 5 h indicates that they are not rapidly synthesized from recently acquired photosynthate. This conclusion, which was supported by subsequent experiments, agrees with the recent report of Kouchi (12) who found little accumulation of ^{13}C in cyclitols in any part of soybean plants up to 23 h after supplying plants with $^{13}CO_2$.

A second experiment was conducted to provide more frequent

Table I. *Labeling of Carbohydrate Compounds in Soybean Nodules 2 to 5 Hours after Pulse Labeling of Shoots with $^{14}CO_2$*

Data are for whole nodule extracts. A sample was also obtained 1 h after pulse labeling but there was insufficient radioactivity for analysis of individual compounds. The average of two replicates is shown; range values were generally <5% of the average. The mean recovery of radioactivity from thin layer plates was 65% in these sample (see "Materials and Methods"). Experiment 1 is shown.

Time after Pulse	Radioactivity					Total Radioactivity in Neutral Fraction
	Fructose	Glucose	Sucrose	Trehalose	Cyclitols	
h	*cpm as % total in neutral fraction*					*cpm × 10^{-6}*
2.0	0.87	3.5	59	1.5	2.8	2.67
3.0	0.77	3.3	55	4.2	4.3	3.97
4.0	0.54	3.2	51	4.2	4.4	3.11
5.0	0.23	3.3	50	5.2	3.1	1.98

Table II. *Labeling of Carbohydrate Compounds in Soybean Nodules 1.0 to 2.0 Hours after Pulse Labeling of Shoots with $^{14}CO_2$*

Data are for whole nodule extracts. The mean ± SE (in parentheses) of three replicates are shown. The mean recovery of radioactivity from thin layer plates was 77% in these samples (see "Materials and Methods"). Experiment 2 is shown.

Time after Pulse	Radioactivity					Total Radioactivity in Neutral Fraction
	Fructose	Glucose	Sucrose	Trehalose	Cyclitols	
h	*cpm as % total in neutral fraction*					*cpm × 10^{-6}*
1.0	12.5 (8.4)	11.9 (3.8)	51 (6)	2.6 (1.1)	9.1 (3.4)	0.097 (0.039)
1.5	2.0 (0.4)	8.9 (1.5)	55 (2)	1.4 (0.5)	3.6 (0.9)	1.39 (0.20)
2.0	1.8 (0.1)	9.9 (0.5)	59 (4)	3.2 (0.8)	4.7 (0.8)	1.66 (0.35)

sampling of nodules during the period of rapid accumulation of radioactivity. Another important feature of experiment 2 was the separation of the acidic fraction into phosphate ester and organic acid fractions by using Sephadex ion-exchange columns (18). The distribution of radioactivity in whole nodule extracts was similar to that shown in Figure 1, with about 2×10^5 cpm in nodules after 1 h followed by a rapid increase in radioactivity which peaked at 3 h. One difference in experiment 2 was that the labeling of the organic acid fraction was clearly faster than the labeling of the amino acid fraction (data not shown). Radioactivity in amino acids was $<2 \times 10^4$ cpm (relative to total cpm of about 2×10^6) until after 2 h.

Most of the radioactivity in the amino acid fraction was in glutamate in all experiments (data not shown). Serine and alanine also contained substantial amounts of radioactivity in 1- to 2-h samples; this result is consistent with the formation of these amino acids from intermediates between carbohydrates and Krebs cycle acids. Aspartate contained substantial radioactivity at sampling times >2 h. In one experiment, asparagine accumulated substantial radioactivity after 4 h but in another experiment this did not occur.

The distribution of radioactivity in the neutral fraction of whole nodule extracts from experiment 2 is shown in Table II. Only the results for the first three sampling times are shown because there was little change in the distribution of radioactivity after 2 h. There seemed to be a high proportion of label in fructose and cyclitols in the 1-h sample, relative to the 2-h sample from experiment 1 (Table II *versus* Table I). However, the amount of radioactivity in the 1-h samples was small and the standard errors were large (Table II). After 1.5 h, when radioactivity in the neutral fraction had increased 14-fold, the distribution of radioactivity was similar to that seen in experiment 1. It is doubtful that the data in Table II indicate rapid but transient labeling of cyclitols. One clear difference between the two experiments was the higher proportion of label in glucose and fructose in experiment 2, perhaps indicating more rapid utilization of hexoses in the first

Table III. *Labeling of Organic Acids in Soybean Nodules 1.0 to 3.5 Hours after Pulse Labeling of Shoots with $^{14}CO_2$*

Data are for whole nodule extracts. The mean of three replicates is shown, except for 2 h (two samples) and 2.5 h (single observation). The mean recovery of radioactivity from thin layer plates was 95% in these samples. Experiment 2 is shown.

Time after Pulse	Radioactivity								Total Radioactivity in Organic Acid Fraction
	Fumarate	Succinate	Malonate	α-Ketoglutarate	Malate	Citrate	Others[a]	Origin	
h	*cpm as % total in acidic fraction*								*cpm × 10^{-6}*
1.0	7.2	8.9[b]		12.8	63	2.7	20	7.4	0.047
1.5	2.0	3.7[b]		2.3	38	7.6	24	2.4	0.263
2.0	3.3	5.0	3.5	1.5	40	5.8	34	4.8	0.205
2.5	3.5	2.6	1.8	1.5	39	6.8	45	5.0	0.629
3.0	3.9	2.9	2.5	1.0	45	11.1	18	4.4	0.576
3.5	3.5	3.3	2.8	1.3	38	12.2	15	4.2	0.520
Average SE	0.7	1.0	0.8	1.2	5	1.6	3	1.6	

[a] Other unknown compounds from TLC plates.

[b] For 1- and 1.5-h samples, succinate and malonate were not resolved; SE (1.0 h) = 2.6; SE (1.5 h) = 0.2.

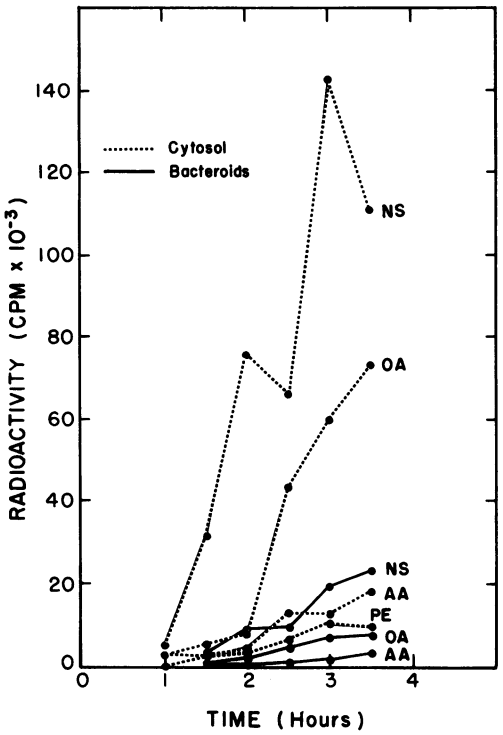

FIG. 3. Radioactivity in the neutral (NS), basic (AA), acidic (OA), and phosphate ester (PE) fractions in bacteroids and cytosol of soybean nodules. Bacteroids and cytosol were obtained from Percoll density gradients and extracts were fractionated using Sephadex ion-exchange columns. Sampling times were measured from the end of the 30-min $^{14}CO_2$ pulse. The mean of two replicates is shown (Experiment 2).

experiment.

The distribution of radioactivity in the organic acid fraction of whole nodules from experiment 2 is shown in Table III. The highest proportion of the label was found in malate, which is in agreement with earlier studies (1, 2, 13). There appeared to be high proportions of label in fumarate and α-ketoglutarate after 1 h relative to later times, although total counts in these compounds after 1 h were very small. The rapid labeling of α-ketoglutarate would be consistent with the rapid labeling of glutamate which was observed in all experiments. A significant feature of the distribution of radioactivity in organic acids was the relatively small amount of label in malonate (Table III). Malonate has been reported to be a major organic acid in soybean nodules (25) and, in agreement with this point, analysis of some of our nodule samples indicated an average of 1.46 mg malonate and only 0.88 mg malate/g fresh weight. (Smaller quantities of succinate, fumarate, α-ketoglutarate, and citrate were also detected.) The small proportion of radioactivity in malonate at all times (Table III) indicates that this compound was not synthesized rapidly from recently acquired photosynthate.

Very small amounts of radioactivity were detected in the phosphate ester fraction in experiment 2 (Fig. 3). Whereas we had established analytical methods for separating these compounds, there was insufficient radioactivity in samples to warrant analysis of individual compounds. The small amount of radioactivity in the phosphate ester fraction suggests that, in contrast to *P. vulgaris* nodules (1), 6-P-gluconate is not highly labeled in soybean nodules after supplying shoots with $^{14}CO_2$.

Bacteroids *versus* Cytosol. The distribution of radioactivity in various fractions from bacteroids and cytosol from experiment 2 is shown in Figure 3. The most rapid accumulation of radioactivity occurred in the neutral fraction of the cytosol followed by the organic acid fraction of the cytosol. The labeling patterns shown in Figure 3 indicate that there was very little direct fixation of $^{14}CO_2$ by nodules with the procedures used to supply $^{14}CO_2$ in these experiments. Analysis of individual compounds in the neutral and organic acid fractions from the cytosol showed a pattern of labeling essentially the same as shown by analyses of whole nodules (Tables I, II, and III).

The distribution of radioactivity among fractions in bacteroids showed a pattern which was similar to that in the cytosol, namely neutral fraction > organic acids > amino acids (Fig. 3). We were surprised to find that 60 to 65% of the total radioactivity in bacteroids was in the neutral fraction at all sampling times. Analysis of the distribution of radioactivity in individual compounds in the neutral fraction was undertaken with samples from experiment 1 because they contained two to five times as much radioactivity as the samples from experiment 2.

Sucrose contained the largest proportion of the radioactivity in the neutral fraction of bacteroids after 2 to 4 h (Table IV). Small amounts of radioactivity were found in glucose, and even smaller amounts in fructose and the cyclitols. Trehalose initially contained a small proportion of the radioactivity, but this proportion increased with time as the proportion of label in sucrose declined (Table IV). In the other experiment where there was sufficient radioactivity in bacterioid neutral sugars to permit analysis of individual compounds (experiment 3), a time-dependent increase in the labeling of trehalose was not observed (data not shown). However, these samples were obtained during the period of decline in total radioactivity in nodules and trehalose was the most highly labeled compound after sucrose. The combined results suggested that sucrose is a major compound entering bacteroids or that it is rapidly synthesized in bacteroids from labeled precursors.

Assuming that most of the energy consumed in nodules is consumed in bacteroids, one would predict a time-dependent transfer of ^{14}C from cytosol to bacteroids. The relative amounts of

Table IV. *Labeling of Carbohydrates in Bacteroids Isolated from Soybean Nodules 2 to 5 Hours after Pulse Labeling of Shoots with $^{14}CO_2$*

A sample was obtained 1 h after pulse labeling, but there was insufficient radioactivity for analysis of individual compounds. Bacteroids were obtained from a subsample of nodules which represented, on the average, 22% of the main sample. The mean of two replicates is shown. The mean recovery of radioactivity from thin layer plates was 60% in these samples (see "Materials and Methods"). Experiment 1 is shown.

Time after Pulse	Radioactivity					Total Radioactivity in Neutral Fraction
	Fructose	Glucose	Sucrose	Trehalose	Cyclitols	
h	*cpm as % total in neutral fraction*					*cpm × 10^{-4}*
2	2.2	4.5	50	7.5	2.6	11.0
3	1.3	5.0	42	13.5	3.3	12.0
4	1.0	5.4	34	23.5	2.2	8.9
5	0	1.4	14	27.5	1.0	9.7

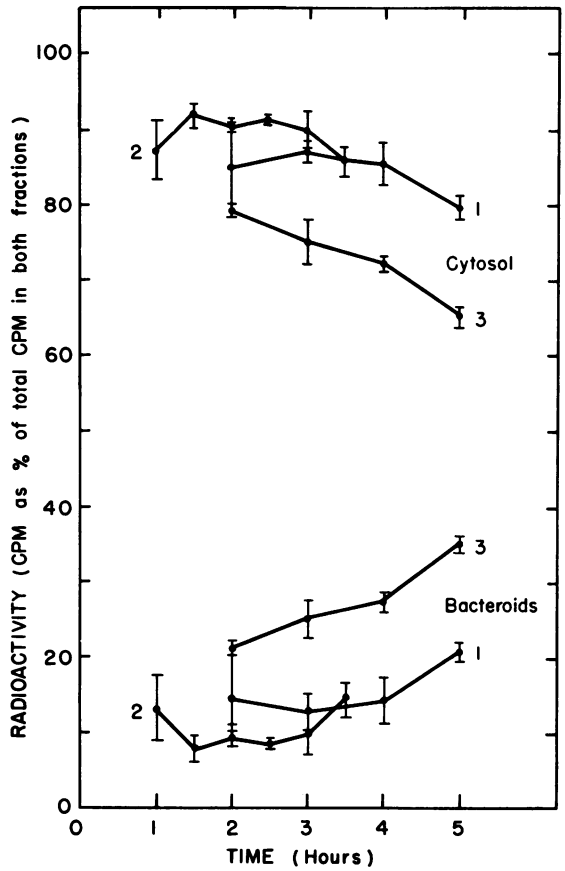

FIG. 4. Percentage of total nodule radioactivity in the bacteroid and cytosol fractions. Whole nodules were separated into bacteroid and cytosol by Percoll gradient centrifugations. The results from three experiments are given and the experiment number is indicated by the numerals. The mean and range of two replicates are given.

radioactivity in the two nodule fractions in three different experiments are shown in Figure 4. The trends shown in Figure 4 suggest a declining proportion of radioactivity in the cytosol and an increasing proportion in bacteroids. This was especially evident for experiment 3, where samples were obtained during a period of declining total radioactivity in nodules. While there were substantial differences among experiments and sampling times, we generally found 10 to 30% of total nodule radioactivity in bacteroids (Fig. 4). In contrast, bacteroids isolated from labeled *P. vulgaris* nodules at a single sampling time contained only 5 to 8% of total radioactivity in the nodules (1).

Distribution of Enzymes. Studies on the distribution of enzymes of carbohydrate metabolism were undertaken mainly because 60 to 65% of the radioactivity in bacteroids was in the neutral fraction of all samples in all experiments. In addition to demonstrating some mechanism for catabolism of glucose in bacteroids, we also wanted to confirm the presumed activity of the glycolytic and pentose-P pathways in the cytosol. Clearly, there was some mechanism for the rapid conversion of sugars to organic acids in the cytosol (Fig. 3).

Results with the two strains of *R. japonicum* were qualitatively identical and quantitatively similar. Thus, enzyme activity was averaged across strains even though this resulted in large standard errors in a few cases where there were apparent differences between strains (Table V). The cytosol of soybean nodules contained 3.5 to 11 times the activity of fructose-dependent hexokinase, P-glucose isomerase, and glucose-6-P dehydrogenase as was found in the bacteroids. In contrast to fructose-dependent hexokinase, the activity of glucose-dependent hexokinase was greater in bacteroids than in the cytosol. Of the enzymes measured, fructose-1,6-bisP aldolase had the lowest activity, with approximately equal amounts of activity in the bacteroids and cytosol.

Two key enzymes of glucose metabolism were not detected in bacteroids, namely, P-fructokinase and (NADP)-6-P-gluconate dehydrogenase. Results for the latter enzyme are in agreement with the literature on cultured *R. japonicum* (11, 14); however, Mulongoy and Elkan (15) reported P-fructokinase in two derivatives of *R. japonicum* 3I1b110. We cannot explain this apparent discrepancy between our results and those of Mulongoy and Elkan. In preliminary experiments with nodules formed using commercial inoculant, we were also unable to detect P-fructokinase in bacteroids. The absence of these two key enzymes suggests that the bacteroids are incapable of metabolizing glucose via the glycolytic pathway or the NADP-dependent oxidative pentose-P pathway.

While bacteroids lacked NADP-dependent 6-P-gluconate dehydrogenase, NAD-dependent enzyme activity was found (Table V). This result is in agreement with the previous observation of this enzyme in cultured cells (16). Mulongoy and Elkan were unable to identify the product of the NAD-linked reaction and the details of this potential route for catabolism of glucose remain unknown.

The results in Table V indicate that glucose catabolism in bacteroids is not via the glycolytic or pentose-P pathways. Also, we did not detect *in vitro* conversion of 6-P-gluconate to pyruvate using bacteroids or bacteroid extracts. However, we cannot completely rule out the presence of the Entner-Doudoroff pathway in bacteroids because the activity of this pathway has been reported to be very low in cultured *R. japonicum* (14), even when cells are grown with gluconate as a carbon source (15). While a different assay (11) might detect some conversion of 6-P-gluconate to pyruvate by bacteroids, our results, when viewed against results with cultured *R. japonicum*, do suggest that the activity for the Entner-Doudoroff pathway in bacteroids is at least very small.

General Discussion. Ten to 30% of the radioactivity in bacteroids + cytosol was recovered in bacteroids (Fig. 4) and 60 to 65% of the radioactivity in bacteroids was in the neutral fraction across all samples in three experiments. It might be argued that this substantial labeling of sugars in bacteroids merely represents accumulation of label in a pool of reduced carbon which is turning over relatively slowly. However, at the shortest times when radioactivity in nodules was great enough to be analyzed, the proportions stated above prevailed. Furthermore, the proportion of the radioactivity in the neutral sugar fraction did not change, as would be expected if accumulation were occurring. Also, it seems unlikely that labeling of bacteroid neutral sugars was an artifact due to absorption of ^{14}C by bacteroids during bacteroid purification. In experiment 2, there as a sharp decline in radioactivity of cytosol neutral sugars between 3 and 3.5 h while radioactivity in bacteroid neutral sugars continued to increase (Fig. 3). Secondly, the distribution of radioactivity within the neutral fraction was different in bacteroids (Table IV) and whole nodule extracts (Table I). We tentatively conclude that the labeling of sugars in bacteroids was due to the direct absorption of radioactive sugars from the cytosol in nodules.

Sucrose was the most highly labeled compound in the bacteroids, except at later sampling times (Table IV). While invertase is completely lacking in *R. japonicum* bacteroids (19, 23), some mechanism for sucrose hydrolysis probably exists in bacteroids because of the faster decline in sucrose radioactivity in bacteroids (Table IV) than in whole nodules (Table I). We were unable to detect P-fructokinase, (NADP)-6-P-gluconate dehydrogenase, or the conversion of 6-P-gluconate to pyruvate in bacteroids, thus also leaving the catabolism of glucose by bacteroids in doubt. However, it should be noted that bacteroids have substantial activity for the conversion of glucose and fructose to their hexose-

Table V. *Activity of Enzymes of Carbohydrate Metabolism in Bacteroids and Cytosol from Soybean Nodules Formed by Rhizobium japonicum*

Data are means (±SE) calculated across two experiments for each of two strains, USDA 110 and USDA 138. All activities are expressed on a μmol substrate basis. Cyt, cytosol; Bact, bacteroids.

Enzyme	Specific Activity				Total Activity in Nodules				
	Cytosol		Bacteroid		Cytosol		Bacteroid		Cyt:Bact
	μmol/mg protein·h				*μmol/g fresh wt·h*				
P-glucomutase	3.6	(0.5)	2.9	(1.0)	22.6	(4.5)	20.2	(10.5)	1.1:1
Hexokinase (glucose)	1.1	(0.2)	2.5	(0.3)	6.5	(0.9)	16.4	(5.2)	1:2.5
Hexokinase (fructose)	2.6	(0.2)	0.42	(0.05)	16.5	(2.5)	2.4	(0.3)	6.9:1
P-glucose isomerase	54.8	(4.4)	16.4	(1.5)	332	(43)	94.4	(16.1)	3.5:1
P-fructokinase	2.7	(0.7)	0		18.1	(6.7)	0		
Fructose-1,6-bisP aldolase	0.39	(0.11)	0.35	(0.06)	2.8	(1.0)	2.4	(0.9)	1.2:1
Glucose-6-P dehydrogenase	4.1	(0.5)	0.50	(0.17)	27.5	(7.1)	2.4	(0.4)	11:1
6-P-gluconate dehydrogenase (NADP)	6.8	(0.4)	0		43.5	(6.5)	0		
6-P-gluconate dehydrogenase (NAD)	0		2.4	(0.2)	0		15.3	(4.5)	

1 and hexose-6 phosphates (Table V). Trehalose in nodules is synthesized in bacteroids (23; Table IV), and glucose-P would be required for the synthesis of trehalose. Also, the conversion of hexoses to hexose phosphates and their subsequent deposition as glycogen is a possible metabolic fate of glucose in bacteroids.

The organic acid fraction of the cytosol was rapidly and heavily labeled from [^{14}C]sucrose entering nodules (Fig. 3). We also found activity for the key enzymes of glycolysis and the pentose-P pathway in the cytosol (Table V). Conversion of phosphoenolpyruvate to four-carbon organic acids probably occurs via phosphoenolpyruvate carboxylase, an enzyme known to be present with high activity in soybean nodules and one which is strictly localized in the cytosol (19). Also, rapid labeling of malate from $^{14}CO_2$ supplied to nodules has been demonstrated (9). Our results are not in conflict with studies showing that organic acids stimulate O_2-dependent respiration and acetylene reduction of isolated *R. japonicum* bacteroids (4, 6). Glucose, fructose, and sucrose are generally not good substrates for stimulating acetylene reduction or O_2 consumption by isolated bacteroids (5). However, work with *R. japonicum* bacteroids did show an enhancement of acetylene reduction by glucose when the O_2 partial pressures were low (27).

A significant feature of the distribution of radioactivity in the neutral fraction was the failure of any of the cyclitols to become significantly labeled. Like sucrose and malate, the cyclitols D-pinitol, D-*chiro*-inositol, and *myo*-inositol represent major pools of reduced carbon in soybean nodules. In experiment 2 (Table II), there was more radioactivity in the cyclitols than in other experiments. Quantitative analysis of carbohydrates in six whole nodule samples (1.5 to 2.5 h) from experiment 2 were used to calculate average specific radioactivities in sucrose, glucose, trehalose, and *myo*-inositol of 40, 22, 14, and 0.45 cpm/μg, respectively. These values emphasize the very small labeling of cyclitols relative to the labeling of glucose and trehalose. In unpublished studies, we have never observed significant labeling of cyclitols after supplying nodule slices with ^{14}C-labeled sucrose, glucose, fructose, maltose, or trehalose for periods of up to 3 h. We conclude that cyclitol metabolism in nodules is very slow relative to the metabolism of sugars and that cyclitols probably do not play a role in supplying reducing power to bacteroids.

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