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Labeling of Bacteroids Following Incubation of Intact Soybean Nodules with ^{14}C-sucrose for Short Time Periods

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Abstract

A system for isolation of bacteroids from soybean nodules in $\leq$ 5 min was established and was used to study transfer of radioactivity to bacteroids following incubation of intact, detached nodules with ^{14}C-sucrose for 5 or 10 min. Most of the radioactivity in bacteroids was found in sucrose with smaller amounts in glucose and organic acids. However, the radioactivity in the neutral fraction of bacteroids as a proportion of neutral counts in the cytosol was invariant as a function of time. This suggested rapid binding of labeled sugars to bacteroids during bacteroid isolation, and this was confirmed by showing that bacteroids were labeled even when ^{14}C sucrose was added just at the time of nodule maceration. Also, the proportional labeling of the neutral fraction in bacteroids was similar when ^{14}C-sucrose was added to nodule homogenate following nodule maceration and just prior to the isolation of bacteroids. Previous published evidence for labeling of carbohydrate pools in bacteroids probably represents adsorption of sugars to bacteroids during their isolation, and these earlier results are probably not an accurate indication of actual sugar uptake by bacteroids in the nodule.

Keywords: *Bradyrhizobium japonicum*, *Glycine max*, carbon transport

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1. Introduction

A fundamental question about the operation of symbiotic nitrogen-fixing systems is the nature of the reduced carbon compounds transferred from the host plant to the microsymbiont. Many different types of evidence are available on this question, and some of the evidence suggests that sugars are important in supporting nitrogen fixation in bacteroids. However, the bulk of the evidence supports the view that dicarboxylic acids such as succinate and malate are the major forms of reduced carbon supplied to bacteroids in legume nodules (Streeter, 1991).

For example, bacteroids deficient in mechanisms for dicarboxylate uptake and metabolism form Fix^- nodules (summarized in Streeter, 1991), whereas bacteroids having genetic deficiencies for carbohydrate transport and metabolism form Fix^+ nodules (Cervenansky and Arias, 1984; Glenn et al., 1984; Guezzar et al., 1988; Ronson and Primrose, 1979). Also, uptake and metabolism of sugars by isolated bacteroids are very slow relative to the uptake and metabolism of dicarboxylic acids (deVries et al., 1982; Glenn and Dilworth, 1981; Hudman and Glenn, 1980; Reibach and Streeter, 1984; Salminen and Streeter, 1987a; Saroso et al., 1984). Thus, Km values for sugar uptake are high and Vmax values are low relative to values for the dicarboxylic acids (Reibach and Streeter, 1984; Salminen and Streeter, 1987a, 1991). Analyses of enzyme activities in bacteroids are also consistent with these results because activities for glycolysis are generally low relative to levels of TCA cycle enzymes (Copeland et al., 1989; Reibach and Streeter, 1983; Salminen and Streeter, 1987a; Saroso et al., 1986).

Analysis of the relative rate of conversion of various substrates to CO_2 by isolated bacteroids is especially useful because this approach integrates several steps in transport and metabolism (Streeter, 1991). Results indicate slow respiration of sugars relative to the respiration of dicarboxylic acids (Salminen and Streeter, 1987a, 1991). However, several studies have shown that relative respiration rates for different classes of substrates depends heavily on the O_2 concentration used during the analysis (Gadzhi-Zade et al., 1990; Guerin et al., 1990; Herrada et al., 1989; Shramko et al., 1990; Trinchant et al., 1981). Specifically, sugars may provide high rates of respiration and/or nitrogenase activity when bacteroids are incubated at very low oxygen levels; authors of these reports argue that these conditions are the most relevant to the *in situ* operation of nodules.

These results with low O_2 focus attention on the need to asses the situation in intact nodules. Interestingly, when intact plants are labeled with $^{14}CO_2$ or $^{13}CO_2$ and the distribution of label in nodules determined, most of the

label in bacteroids is found in the neutral fraction, sucrose being the most highly labeled single compound (Kouchi and Yoneyama, 1986; Reibach and Streeter, 1983; Romanov et al., 1985). In these earlier studies, incubation times have generally spanned periods from 30 to 120 min. In an attempt to define the movement of sucrose label in nodules, more precisely, we supplied ^{14}C-sucrose to intact nodules and used a technique for the very rapid isolation of bacteroids. This made possible the analysis of label distribution in bacteroids after incubation periods of only 5 or 10 min. Results reported here with short incubations and rapid bacteroid isolation indicate that the labeling of bacteroids with radioactive sucrose is probably an artifact.

2. Materials and Methods

Plant growth

Soybean seeds (*Glycine max* (L.) Merr.) cv Ripley were planted in silica sand and were inoculated with a water culture of *Bradyrhizobium japonicum* USDA 110 (Crist et al., 1984). Plants were grown in a glassshouse and were irrigated three times daily with nutrient solution lacking combined nitrogen (Streeter, 1981). Nodules used for experiments were from plants 35 to 45 days after planting.

Feeding of ^{14}C-sucrose

Uniformly labeled sucrose having 99% radiochemical purity and 632 mCi/mmole was purchased from NEN/DuPont immediately prior to use in order to avoid any sucrose hydrolysis in storage. Each sample labeled consisted of 300 mg of nodules which were detached from freshly harvested plants immediately prior to feeding. Nodules were placed in a liquid scintillation vial cap (1 cm depth, 2.5 cm diameter). A 300 μl pool of ^{14}C-sucrose containing about 12 μCi of radioactivity was pipetted around the nodules; specific radioactivity was that listed above. Nodules were approximately 1/4 immersed in liquid; thus, most of the nodule surface was exposed to the air but the nodule surface was wetted with radioactive sucrose solution.

After incubations of 5 or 10 min, excess liquid was drawn off and nodules were immediately transferred to a small conical piece of screen and washed with 4×5 ml of water to remove surface radioactivity. Nodules were blotted dry and transferred to a chilled mortar and ground in 600 μl of 0.2 M sorbitol. The homogenate was transferred to a 5 cc plastic syringe and filtered through two disks of Miracloth.

Isolation of bacteroids

A silicone oil centrifugation technique based on the method of Klingenberg and Pfaff (1967) was developed. Microfuge tubes containing 40 μl of 10% (v/v) perchloric acid with an upper 70 μl layer of silicone oil (Sigma Chemical Co., M-9389) were prepared in advance of the experiment (Fig. 1). For the rapid

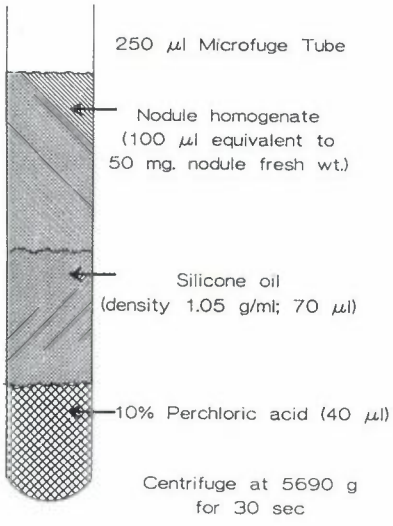

Figure 1. System for rapid separation of bacteroids and cytosol by centrifugation of crude nodule homogenates over silicone oil in a microfuge. See text for details.

separation of bacteroids and cytosol, 100 μl of crude nodule brei was pipetted on top of the silicone oil and the tubes were spun in a microfuge at 5690 g for 30 s. The top layer following centrifugation was pipetted into a clean 1.5 ml microfuge tube. Next, 100 μl of water was pipetted into the 250 μl tube to rinse the surface of the silicone oil. This was combined with the initial top layer and centrifuged at 12,400 g for 1 min to remove mitochondria and other particulate matter. A measured aliquot of this supernatant ("cytosol") was combined with 95% ethanol.

The optimum combination of initial sorbitol concentration and centrifugal force $\times$ time were established by trial and error. The conditions described were found to provide the maximum recovery of bacteroids (about 94%) plus optimum removal of mitochondria (about 96% removal). These estimates are based on the analysis of the marker enzymes β-hydroxybutyrate dehydrogenase, a bacteroid marker, and cytochrome C oxidase, a mitochondrial

marker (Reibach and Streeter, 1984). The time between the grinding of nodules and centrifugation of bacteroids into the perchloric acid layer was 4.6±0.2 min.

Extraction and analysis of metabolites

Silicone oil was drawn off the top of the original microfuge tube and discarded. The bacteroid sample in perchloric acid was transferred to another tube, neutralized with K_2CO_2, and mixed with 10 volumes of 95% ethanol. Bacteroids and precipitate from the cytosol were repeatedly extracted with 80% ethanol and extracts were combined, dried by vacuum evaporation, and taken up in 2 ml of water.

Amino acid, organic acid and neutral fractions were obtained by passage of aqueous samples extracts through tandem columns of Dowex 50 H^+ (top) and Dowex 1 – formate (bottom) (Salminen and Streeter, 1987b). Amino acids and organic acids were separated by thin-layer chromatography as described earlier (Salminen and Streeter, 1987b). Spots representing individual compounds were scraped from plates, and radioactivity was determined by liquid scintillation.

Sucrose, glucose, and fructose were separated by thin layer chromatography using 250 μm layers prepared from a mixture of 25 g silica gel (with 5% $CaSO_4$ binder) plus 50 ml of 0.1 M $NaHSO_3$ (Adachi, 1965). After spreading and drying, plates were heated for 1 hr at 110–120°C and cooled prior to use. Plates were spotted along the bottom with an appropriate aliquot of the radioactive neutral fraction plus 5 μg each of sucrose, glucose, and fructose standards. Plates were irrigated (ascending) for 3 1/2 hr at 22°C with a solvent mixture containing ethyl acetate:dimethyl sulfoxide:pyridine (4:1:1) (Kindl and Hoffmann-Ostenhof, 1966). Bulk solvent was evaporated in a hood and plates were dried overnight at 40–50°C. Sugars were located by spraying plates with a solution containing 2.5 ml anisaldehyde, 45 ml of 95% ethanol, 2.5 ml of concentrated sulfuric acid, plus 0.5 ml of glacial acetic acid and heating the plates at 100°C for 15 min. Spots were scraped from the plates and radioactivity was determined by liquid scintillation.

3. Results

Following incubations of intact nodules with ^{14}C-sucrose, about 100,000 dpm to 150,000 dpm were recovered in the total nodule sample (300 mg fresh weight) after 5 or 10 min, respectively. Ninety to 95% of this radioactivity was in the cytosol fraction, as expected; but there was sufficient label in bacteroids to permit analysis of label distribution in fractions and in individual compounds.

About 85% of the label in bacteroids was in the neutral fraction; 9% (5 min) to 13% (10 min) of the radioactivity in bacteroids was in the organic acid fraction and an even smaller proportion of radioactivity was in the amino acid fraction (Table 1). Virtually all of the radioactivity in the organic acid fraction in bacteroids was in malate; radioactivity in the amino acid fraction was so small that data for individual compounds were not considered reliable.

Roughly 60 to 75% of the neutral fraction label in bacteroids was in sucrose. Generally, radioactivity could not be detected in fructose in bacteroids after 5 min incubations and was still a minor component of radioactivity in the neutral fraction after 10 min. Thus, aside from sucrose, glucose was the main component in the neutral fraction which was labeled. Expressed as a proportion of the dpm in sucrose in bacteroids, values for glucose in bacteroids were 50% after 5 min and 63% after 10 min (Table 2). Proportional labeling of glucose in the cytosol was somewhat less than labeling in the bacteroids, but the ratios increased with time in both fractions (Table 2).

The tendency of the labeling of the neutral fraction in the bacteroids to follow labeling in the cytosol is emphasized by the organization of the data

Table 1. Labeling of metabolite fractions in bacteroids following incubation of whole soybean nodules with ^{14}C-sucrose for 5 or 10 min. There were two experiments, each with three replicate feedings and the results of the two experiments were very similar. Data shown are for the mean ± S.E. of six observations.

Time (minutes)	Fraction	Radioactivity (dpm)	% of total dpm in 3 fractions
5	neutral	3270±360	88.7±1.8
	organic acid	337±50	9.1±1.2
	amino acid	74±27	2.1±0.8
10	neutral	5270±580	80.7±4.0
	organic acid	871±169	12.8±1.5
	amino acid	450±213	6.5±3.0

Table 2. Relative labeling of sucrose and glucose in bacteroids and cytosol after feeding intact soybean nodules with ^{14}C-sucrose for 5 or 10 min. There were two experiments, each with three replicates; data shown are for the combined experiments, i.e., mean ± S.E. of six replicates.

Time (minutes)	Radioactivity in glucose as per cent of the radioactivity in sucrose	
	Bacteroids	Cytosol
5	49.6±5.1	35.6±6.6
10	63.3±12.2	56.1±6.2

Table 3. Labeling of the neutral, organic acid and amino acid fractions of bacteroids relative to radioactivity in these fractions in the cytosol of soybean nodules following incubations with ^{14}C-sucrose. There were two experiments, each with three replicates, and results for the two experiments were very similar. Data shown are for the mean ± S.E. of six observations.

	Radioactivity in bacteroids as % of the radioactivity in the cytosol		
Time (minutes)	Neutral fraction	Organic acid fraction	Amino acid fraction
5	4.50±0.43	8.95±1.29	7.64±2.08
10	4.61±0.30	13.70±1.30	12.73±3.30

shown in Table 3. Only about 4.5% of the label in the neutral fraction dpm were found in bacteroids and this did not change significantly with time. In contrast, larger proportional amounts of label were found in bacteroids for the organic acid and amino acid fractions, and these proportions did increase as a function of incubation time (Table 3).

The data in Table 3 led us to suspect that the relatively high level of neutral fraction counts in bacteroids (Table 1) might merely represent radioactive sugars bound to bacteroids during the isolation of bacteroids from ^{14}C-labeled nodules. To test this possibility, 121,000 dpm of ^{14}C-sucrose was added to 300 mg nodules during the grinding step in 0.2 M sorbitol. (This amount of labeled sucrose was approximately equivalent to the radioactivity recovered *in the nodules* after the 5 and 10 min incubations of intact nodules with high levels of radioactive sucrose.) Bacteroids were rapidly isolated as before and total dpm were separated into the three standard fractions. Neutral fraction dpm in bacteroids were again about 4.5% of neutral fraction dpm in the cytosol. Thus, even though time was not allowed for uptake or metabolism of ^{14}C-sucrose by intact nodules, proportional radioactivity in bacteroids was the same as when nodules were incubated for 5 or 10 min (Fig. 2). The relationship between labeling of the neutral fractions in bacteroids and cytosol is also illustrated by the inset in Fig. 2 which shows that the two parameters are closely correlated.

A second type of zero-time control was employed wherein 121,000 dpm of ^{14}C-sucrose were added to the nodule homogenate sample just prior to placing the sample in microfuge tubes for centrifugation (see Fig. 1). In these samples, labeling of the neutral fraction in the purified bacteroids constituted 4.20±0.003% of the dpm in the neutral fraction of the cytosol. This proportion is very similar to that shown in Fig. 2.

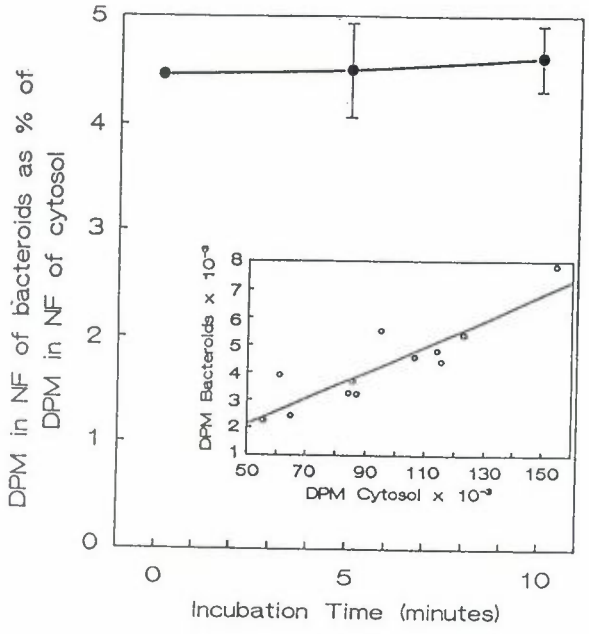

Figure 2. Time dependence of the labeling of the neutral fraction (sugars) in bacteroids following feeding of soybean nodules with ^{14}C-sucrose. DPM = disintegrations per minute; NF = neutral fraction. Results for the point near time zero represent radioactivity in the neutral fraction of bacteroids following addition of ^{14}C-sucrose during the grinding of nodules. For these data, standard error bars were within the plotted point. *Inset* Relationship between neutral fraction labeling in bacteroids and cytosol showing individual observations. The best-fitting linear repression was plotted; the correlation coefficient for the data, 0.887, is significant at the 1% level of confidence.

4. Discussion

The apparent rapid labeling of sucrose and glucose in bacteroids following incubations of intact nodules with ^{14}C-sucrose for short time periods (Tables 1 and 2) was consistent with previously published results for the high radioactivity in sugars in bacteroids following the labeling of intact plants with $^{14}CO_2$ or $^{13}CO_2$ (Kouchi and Yoneyama, 1986; Reibach and Streeter, 1983; Romanov et al., 1985). Our initial interpretation of the results in Tables 1 and 2 was that radioactive sucrose was able to penetrate into the cells of the infected tissues and was being absorbed and metabolized by bacteroids.

Recent analysis of the distribution of enzymes in soybean nodules indicates that nearly all of the sucrose metabolism occurs in the infected zone (Gordon, 1991). Earlier reports indicated that enzymes of sucrose hydrolysis occur

largely (almost entirely?) in the host cytoplasm and not in the bacteroids (Copeland et al., 1989; Reibach and Streeter, 1983; Salminen and Streeter, 1987a; Saroso et al., 1986). Because of the localization of enzymes of sucrose hydrolysis and the large amount of hydrolytic capacity in soybean nodules (Copeland et al., 1989; Reibach and Streeter, 1983), one would not predict that the major labeled sugar in bacteroids would be sucrose (Table 2). Instead, it seems logical that most of the sucrose would be hydrolyzed prior to its reaching the bacteroid surface.

This line of reasoning made us suspect that the concentration of radioactivity in the neutral fraction of bacteroids might be an artifact. Organization of the data as shown in Table 3 made us even more suspicious, because the labeling of the neutral fraction seemed to be a constant proportion of the dpm in the neutral fraction of the cytosol, and the error level in these data was fairly small. Our suspicions were confirmed by the finding that the labeling of the neutral fraction in bacteroids occurred even without allowing for incubation time (Fig. 2). Similarly, addition of labelled sucrose to nodule brei just before centrifugal separation of bacteroids from cytosol gave a result similar to that shown in Fig. 2. Results reported here are consistent with the idea that the major compounds entering the bacteroid cytoplasm are organic acids because the proportional labeling of this fraction was large relative to the neutral fraction, and the proportion in bacteroids increased with time (Table 3).

We conclude that there was rapid binding of labeled sucrose to bacteroids during the isolation of the bacteroids from nodules containing ^{14}C-sucrose. Binding of glucose may also occur but this was not established. In our experiments, the radioactive sugar bound to bacteroids was only a small proportion of the total neutral fraction radioactivity in the nodules; but this small proportion was still large enough to make it appear that sugars were the principle compounds entering the bacteroids (Table 1).

It should be noted that the quantity of sucrose associated with bacteroids was extremely small. We have previously estimated that one gram fresh weight of soybean nodules yields about 65 mg dry weight of bacteroids (Streeter, 1987); thus, a 300 mg sample of fresh nodules should yield about 19.5 mg dry weight of bacteroids. If there are 5000 dpm of sucrose associated with the bacteroids (e.g., Table 1), this is equivalent to about 3.6 pmole of labeled sucrose. Thus, the calculated concentration of labeled sucrose bound to bacteroids in these studies is in the range of 0.2 pmole per mg dry weight.

Use of the word "bound" implies a surface location for the labeled sucrose. An equally likely location for the labeled sucrose would be the periplasmic space, but this was not pursued. That the radioactive sucrose in bacteroids

is somewhere outside of the plasma membrane, is consistent with the previous evidence that isolated *B.japonicum* bacteroids supplied with ^{14}C-sucrose *in vitro* bind some substrate but do not absorb the label in a time-dependent fashion (Reibach and Streeter, 1984).

This sucrose binding phenomenon may explain the previous reports of relatively high labeling of sucrose in bacteroids (Kouchi and Yoneyama, 1986; Reibach and Streeter, 1983; Romanov et al., 1985), and we suggest that these previous results should not be considered as support for the idea that sugars are major compounds transported from the host cytoplasm to the bacteroids. In these earlier studies, techniques of filtration and density gradient centrifugation were used to isolate bacteroids and it is possible that sucrose binding does not occur under these conditions. However, it seems most likely that the interaction between bacteroids and labeled sucrose should not depend on the method of bacteroid isolation. Certainly, if other techniques are used for bacteroid isolation following feedings of radioactive sugars, various types of zero-time controls must be included.

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