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Poly-β-hydroxybutyrate Utilization by Soybean (*Glycine max* Merr.) Nodules and Assessment of Its Role in Maintenance of Nitrogenase Activity[1]

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ABSTRACT

Soybean (*Glycine max*) nodule bacteroids contain high concentrations of poly-β-hydroxybutyrate and possess a depolymerase system that catalyzes the hydrolysis of the polymer. Changes in poly-β-hydroxybutyrate content and in activities of nitrogenase, β-hydroxybutyrate dehydrogenase, and isocitrate lyase in nodule bacteroids were investigated under conditions in which the supply of carbohydrate from the soybean plants was interrupted. The poly-β-hydroxybutyrate content of bacteroids did not decrease appreciably until the carbohydrate supply from the host plants was limited by incubation of excised nodules, incubation of plants in the dark, or by senescence of the host plant. Isocitrate lyase activity in bacteroids was not detected until poly-β-hydroxybutyrate utilization appeared to begin. The presence of a supply of poly-β-hydroxybutyrate in nodule bacteroids was not sufficient for maintenance of high nitrogenase activity under conditions of limited carbohydrate supply from the host plant. An unusually high activity of β-hydroxybutyrate dehydrogenase was observed in bacteroid extracts but no significant change in the activity of this enzyme was observed as a result of apparent utilization of poly-β-hydroxybutyrate by nodule bacteroids.

Poly-β-hydroxybutyrate, a polyester composed of the D-(−) stereoisomer of β-hydroxybutyrate, was discovered by Lemoigne (19) in 1923 as a major component of an aerobic bacillus and later was found to occur as a storage material in a variety of microorganisms (7) and in root nodules of legumes (9).

PHB[3] samples isolated from many sources have similar properties. The product of PHB hydrolysis in hot concentrated H_2SO_4 exhibits an absorption peak at 235 nm (17). When hydrolyzed in mild acid, the product shows an optical rotation of −14.4° (18, 27). The polymer is insoluble in many organic solvents but is soluble in hot chloroform (20). Samples of PHB from different bacteria showed melting points ranging from 114 to 188 C (20, 29) and molecular weights ranging from 1,000 to 256,000 (22). A molecular weight of 128,000 was reported (2) for PHB isolated from an unidentified *Rhizobium*.

Sierra and Gibbons (30) demonstrated PHB depolymerase activity in *Micrococcus halodenitrificans* by measuring the anaerobic release of CO_2 from a bicarbonate buffer. Activity was strongly dependent upon the presence of either Na^+ or Li^+ (31). PHB granules, according to Merrick and Doudoroff (24), are hydrolyzed to BOHB by a complex enzyme system present in the soluble fraction of PHB-depleted cells of *Rhodospirillum rubrum*. This system consisted of a thermostable activator, a thermolabile depolymerase, and an esterase. Although the principal product of hydrolysis by the depolymerase was BOHB, small quantities of esterfied products also were released.

Evidence for the metabolic pathway of utilization of BOHB was provided by Sierra and Gibbons (30), who demonstrated that BOHB was oxidized to acetoacetate by crude extracts from *M. halodenitrificans*. Acetoacetate was further metabolized when ATP, Mg^{2+}, coenzyme A, and oxaloacetate were added to the extracts, suggesting that acetyl-CoA was an intermediate of the reaction and that utilization occurred through the tricarboxylic acid cycle.

The catalysis of nitrogen reduction by cell-free extracts of nitrogen-fixing microogranisms and nodule bacteroids requires a supply of ATP (12, 23, 25) and an appropriate reductant (4, 8, 12, 14, 25). It has been estimated (3, 10) that 3 to 19 mg of carbohydrate are consumed by legume root nodules for each milligram of N_2 fixed. Moreover, two or more carbon atoms are required for the export of each fixed nitrogen atom, in the forms of amino acids and amides, from the nodules to the host (33).

It has been established that up to 50% of the dry weight of *Rhizobium japonicum* bacteroids consists of PHB (14), and that β-hydroxybutyrate oxidation in *in vitro* experiments is capable of supplying the electrons for the support of bacteroid nitrogenase activity (14). The purpose of this investigation, therefore, was to study the utilization of PHB in soybean root nodules and to assess the possible role of the polymer as a source of energy for maintenance of nitrogenase activity in nodule bacteroids.

MATERIALS AND METHODS

CHEMICALS

Reagent grade chemicals or those of the highest grade available were obtained from commercial sources. NAD, sodium DL-β-hydroxybutyrate, DL-isocitrate lactone (hydrolyzed as recommended by the manufacturer), 2-mercaptoethanol, EDTA,

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[3] Abbreviations: PHB: poly-β-hydroxybutyrate; BOHB: D(−)-β-hydroxybutyrate.

and tris were purchased from Sigma Chemical Corporation; anhydrous hydrazine from Matheson, Coleman and Bell; chloroform and acetone from J. T. Baker Chemical Company. Calcium carbide, used to generate acetylene, and 2,4-dinitrophenylhydrazine were obtained from Allied Chemical Company, and levigated alumina from Beckman Spinco Division.

SOURCE OF PLANT MATERIALS

Soybean plants (*Glycine max* Merr. var. Chippewa) inoculated with a commercial strain of *Rhizobium japonicum* (kindly supplied by Dr. Joe Burton of Nitragin Company) were cultured in a greenhouse in pots of perlite supplied with a nitrogen-free nutrient solution (1). The perlite was sterilized by autoclaving for 4 hr at 15 p.s.i. before use. The plants, about 10 per pot, were provided with supplemental light for 16 hr each day and were flushed daily with nitrogen-free nutrient solution except every 4th day when they were flushed with water to remove any accumulated salts.

ISOLATION, ASSAY, AND CHARACTERIZATION OF PHB

Lipid granules suspected to be PHB were isolated from soybean root nodules by the method of Williamson and Wilkinson (35). Nodules were macerated in 0.05 M tris-Cl buffer, pH 8.4, using a mortar and pestle. The macerated material was squeezed through four layers of cheesecloth and centrifuged at 300*g* for 10 min. The residue, containing unbroken plant cells and cell debris, was discarded, and the supernatant fraction was centrifuged at 8000*g* for 15 min. The residue, containing mainly bacteroids, was washed twice with cold glass-distilled water. The washed bacteroids were dried to a constant weight at 85 C and were hydrolyzed overnight at room temperature with 0.2 ml of commercial Clorox (5.25% sodium hypochlorite) per mg of dried bacteroids. PHB granules released from the bacteroids were collected by centrifuging at about 8000*g* for 20 min and washed successively with glass-distilled water and redistilled acetone.

The supernatant fraction from the Clorox hydrolysis step was recentrifuged, and the small residue was washed successively with the supernatants from the glass-distilled water and the acetone washings. All the residues were combined and dissolved in boiling chloroform. After cooling to room temperature, the chloroform solution was filtered through Whatman No. 1 filter paper. PHB in the filtrate was determined by the method of Law and Slepecky (17) and expressed as percentage of dry weight of bacteroids.

A more highly purified preparation of PHB was used for examination of properties. PHB for this purpose was crystallized from the filtered chloroform solution by adding five volumes of redistilled acetone at −20 C. The crystals were collected by filtration and washed with cold redistilled acetone. For the second crystallization, the crystals were redissolved in boiling chloroform, and the acetone precipitation procedure was repeated.

The melting point of PHB crystals was determined by slowly heating them in a capillary tube immersed in a mineral oil bath. PHB was hydrolyzed to BOHB with anhydrous hydrazine and HCl according to Ottaway (27). Optical rotation of the hydrolytic product at 22 C was measured with a sodium lamp as a light source. The hydrolytic product was tested as a substrate for BOHB dehydrogenase isolated from soybean nodule bacteroids, under assay conditions to be discussed subsequently. The isolated PHB was converted to crotonic acid with hot concentrated sulfuric acid (17). The absorption spectrum of the crotonic acid in concentrated sulfuric acid was recorded with a Cary model 11 spectrophotometer.

PREPARATION AND ASSAY OF ENZYMES

PHB Depolymerase. The bacteroid residues used for assaying PHB depolymerase activity were prepared anaerobically according to Klucas *et al.* (15). After the bacteroids were broken in the French pressure cell, the ruptured cells were centrifuged at 48,000*g* for 50 min, the supernatant fraction was discarded, and the residue containing PHB granules and other debris was suspended, aerobically, in 0.05 M tris-Cl buffer, pH 8.5 (1 ml of buffer for 1 g of residue). This suspension exhibited PHB depolymerase activity. The pH of the suspension was maintained at 8.5 (by addition of 0.1 M KOH) during incubation of reaction mixtures at 30 C. Samples (5 ml) were removed at appropriate intervals and placed in a boiling water bath for 3 min, the precipitated protein and PHB granules were removed by centrifugation, and BOHB in the supernatant fraction was determined by an enzymatic method in the presence of hydrazine and excess NAD (34).

Nitrogenase. Activity of nitrogenase in nodules was assayed by reduction of acetylene to ethylene. Soybean nodules (0.5 g) were placed in 21-ml serum bottles. Two layers of cheesecloth wetted with 0.2 ml of water were placed in each bottle to supply moisture. Bottles were capped with rubber serum stoppers, and reactions were incubated at 25 C for 30 min under 0.25 atm O_2, 0.65 atm argon, and 0.1 atm acetylene. The reactions were initiated by the injection of acetylene and were terminated by adding 0.5 ml of 50% trichloroacetic acid with a syringe. Ethylene was assayed by the procedure utilized by Koch and Evans (16). Appropriate control reactions were included.

BOHB Dehydrogenase. Crude extract for BOHB dehydrogenase assay was prepared by macerating soybean nodules in 0.05 M tris-Cl buffer, pH 8.0, with a mortar and pestle (4 ml of buffer for 1 g of nodules). The macerated material was squeezed through four layers of cheesecloth and centrifuged at 300*g* for 10 min to remove intact host cells and cell debris. The bacteroids were collected by centrifugation at 8,000*g* for 15 min; washed twice with 0.05 M tris-Cl buffer, pH 8.0; and resuspended in 0.025 M tris-Cl buffer, pH 8.0, containing 5 mM $MgCl_2$ (5 ml of buffer for 1 g of bacteroids). The suspended bacteroids were mixed with levigated alumina (2 g/g of bacteroids) and macerated with a mortar and pestle. A crude cell-free extract was obtained by centrifuging at 35,000*g* for 30 min. All steps were carried out at 0 to 4 C.

BOHB dehydrogenase activity was assayed in a mixture that contained, in μmoles per 3 ml: tris-Cl, pH 8.0, 100; $MgCl_2$, 3; NAD, 1.2; sodium DL-β-hydroxybutyrate, 20; and crude bacteroid extract containing about 0.2 mg of protein.

The reactions were started by adding enzyme, and the activity at 25 C was followed spectrophotometrically by measuring the increase in the absorbancy of NADH at 340 nm with a Cary model 11 spectrophotometer.

Isocitric Lyase. The first steps for preparation of bacteroid crude extract for assay of isocitrate lyase activity were the same as those for the preparation of BOHB dehydrogenase. The method was based on that of Johnson *et al.* (13). After being washed twice with 0.05 M tris-Cl buffer, pH 8.0, the bacteroids were suspended in 0.025 M tris-Cl buffer, pH 8.0, containing 0.01 M 2-mercaptoethanol and 0.1 mM Na_2EDTA (5 ml of buffer for 1 g of bacteroids). After addition of 0.2 g of cold levigated alumina per g of cells, the cell suspension, in an ice bath, was sonicated for 2 min in a MSE sonicator. To minimize heating, the suspension was cooled to 1 C before it was sonicated for an additional 2 min. Cell-free extracts were obtained by centrifugation at 35,000*g* for 30 min. Endogenous pyridine nucleotides were removed by addition, with stirring, of 5 mg of activated charcoal per ml of crude extract (5).

Charcoal and adsorbed pyridine nucleotides were removed by centrifugation.

Isocitrate lyase activity was determined by the procedure of Daron and Gunsalus (6). The complete reaction contained, in μmoles per 3 ml: tris-Cl, pH 7.9, 200; $MgCl_2$, 6; cysteine-HCl, 4; sodium isocitrate, 15; and charcoal-treated crude extract containing about 4 mg of protein. Reactions were conducted under N_2 at 25 C in 21-ml serum bottles capped with rubber stoppers. Reactions were started by adding isocitrate and were terminated after 10 min by injecting 0.2 ml of 80% (w/v) trichloracetic acid with a hypodermic syringe. The precipitated protein was removed by centrifugation, and glyoxylate in the supernatant fraction was determined colorimetrically as the 2,4-dinitrophenylhydrazone (6). The absorbancy of the complete reaction mixture was corrected for the absorbancy of the reaction mixture lacking substrate.

Other Assays. Protein was determined by the biuret method (11) and nitrogen by the micro-Kjeldahl procedure (32).

RESULTS

SOME PROPERTIES OF PHB

The melting point of the purified granules from nodule bacteroids was between 169 and 170 C, and an absorption peak at 235 nm was observed after heating the granules in concentrated sulfuric acid. These values agree with those reported for authentic PHB (22, 17). The lipid granules from soybean nodules were resistant to mild acid and base hydrolysis and, consequently, were degraded by hydrazinolysis (27). Optical rotation (−14.8°) of the hydrolytic product agreed well with the values reported by Ottaway (27) and Lehninger and Grevill (18) for authentic BOHB.The product of hydrolysis also was observed to function as a substrate for highly purified BOHB dehydrogenase isolated from nodule bacteroids. These results provide conclusive evidence that the lipid granules isolated from the bacteroids contained PHB.

PHB DEPOLYMERASE

Figure 1 provides evidence that BOHB was liberated from PHB by a depolymerase system. Analysis of samples removed from the reaction mixture after 30 min of incubation revealed a concentration of 0.03 M BOHB. Further activity of the depolymerase appeared to cease after 60 min of incubation (Fig. 1). Although no systematic effort was made to characterize the PHB depolymerase, the results indicated that the depolymerase from bacteroids was associated with a particulate fraction of the ruptured cells.

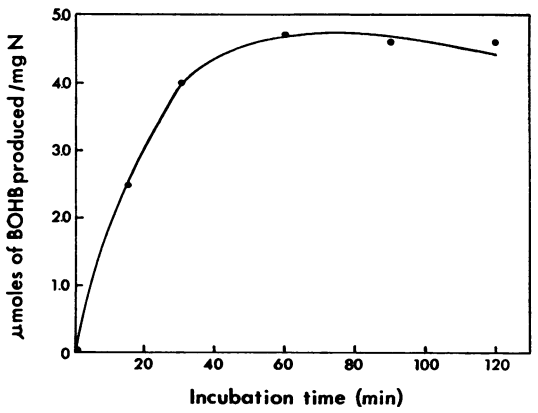

FIG. 1. Time course of poly-β-hydroxybutyrate depolymerase activity in nodule bacteroids. The experimental conditions were described under "Materials and Methods."

PHB UTILIZATION AND NITROGENASE ACTIVITY

Enzyme Activities and PHB Content during Incubation in Dark. Since the PHB content of nodule bacteroids was observed (14) to range up to 50% of the bacteroid dry weight, it was of interest to investigate the possibility that PHB might support nitrogenase activity, particularly under conditions where the supply of carbohydrate from the host plants had been interrupted.

When soybean plants were placed in a growth chamber, in the dark, the nitrogenase activity of nodules decreased rapidly (Fig. 2). After 3 days in the dark, the activity was about 37% of that observed when the experiment was initiated. In contrast, the PHB content of bacteroids changed very little during a 3 day incubation period (Fig. 2). After incubation in the dark for 5 days, the PHB content decreased at a more rapid rate and the nitrogenase activity declined to 20% of that originally observed. Isocitrate lyase activity appeared after 5 days of incubation but not before, suggesting that PHB was converted to C-4 dicarboxylic acids and metabolized through the glyoxylate cycle. After 12 days of incubation in the dark, isocitrate lyase activity in bacteroids increased further. BOHB dehydrogenase activity in extracts of bacteroids increased slowly up to the 6th day of incubation but remained fairly constant thereafter.

Enzyme Activities and PHB Content of Detached Nodules. Changes in PHB content, nitrogenase, BOHB dehydrogenase, and isocitrate lyase activities of nodules after they had been severed from the host plants are shown in Figure 3. The PHB content of detached nodules remained fairly constant until 35 hr after detachment, then declined. The specific activity of BOHB steadily increased in bacteroids throughout the period of incubation. The sudden appearance of isocitrate lyase activity after 34 hr of incubation coincided with the initiation of a noticeable decline in total PHB content. These results again suggest that products of PHB degradation were utilized through the glyoxylate cycle.

Nitrogenase activity of nodules decreased to about 35% of the original activity during the first 10 hr after nodule excision, whereas PHB content decreased approximately 1% during this period (Fig. 3).

Enzyme Activities and PHB Content through Growth Period. Investigation of the utilization of PHB and measurements of nitrogenase activity of soybean nodules throughout the growth period were conducted in hopes of providing information regarding any possible interaction between the two processes. Nitrogenase activity increased rapidly, reaching a maximum at 33 days after seeds were planted but fell equally rapidly after the maximal rate was attained (Fig. 4). In contrast, the PHB content of nodules increased strikingly up to 25 days, and then increased more gradually until plants reached an age of 80 days and finally declined. Again isocitrate lyase activity was detected at about the same time that the PHB content of bacteroids began to decline.

DISCUSSION

It is clear from these studies that nodule bacteroids accumulate PHB at concentrations ranging up to 50% of their dry weight. Bacteroid extracts exhibit a depolymerase system that catalyzes the hydrolysis of PHB to BOHB and an unusually active BOHB dehydrogenase that catalyzes the oxidation of BOHB to acetoacetate with the concomitant reduction of NAD to NADH. *In vitro* experiments of Klucas and Evans (14) have established that the oxidation-reduction potential of

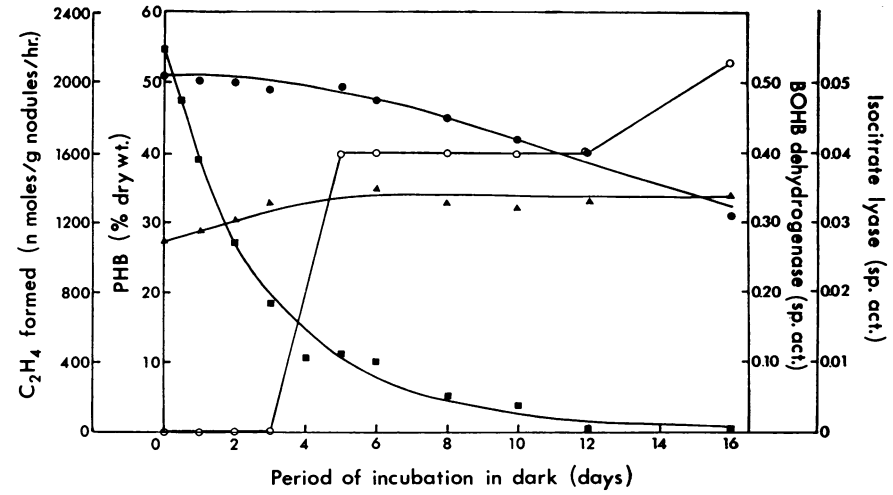

FIG. 2. Changes in the activities of nitrogenase (■), BOHB dehydrogenase (▲), isocitrate lyase (○), and in the PHB content (●) of root nodules during maintenance of the intact soybean plants in the dark. The specific activities (sp. act.) of BOHB dehydrogenase and isocitrate lyase are expressed as μmoles of NAD reduced per min per mg of protein and μmoles of glyoxylate formed per 10 min per mg of protein, respectively. The experiments were conducted with 28-day-old plants grown under the conditions described under "Source of Plant Materials." The plants were placed in a darkened growth chamber maintained at 27 C. Cultures were flushed daily with nitrogen-free nutrient solution (1) except on every 4th day, when they were flushed with water.

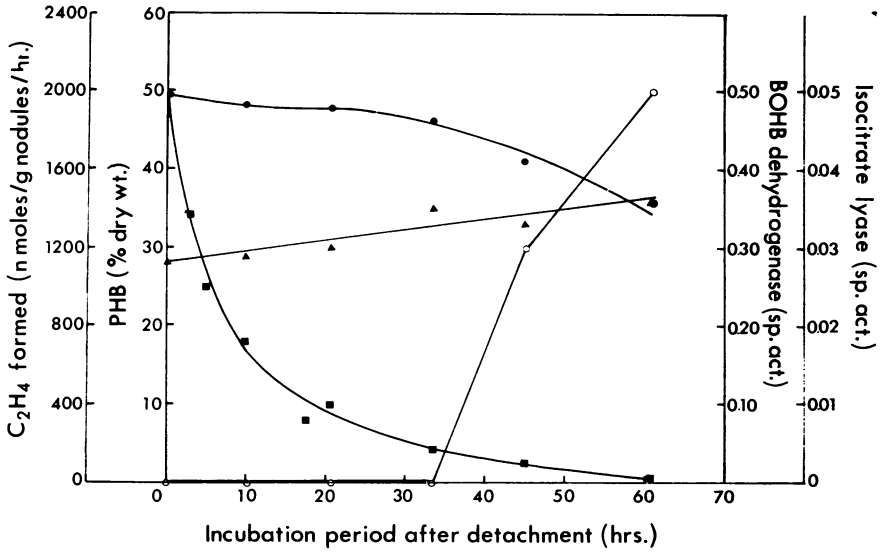

FIG. 3. Changes in the activities of nitrogenase (■), BOHB dehydrogenase (▲), isocitrate lyase (○), and in the PHB content (●) of the detached nodules during incubation. The definitions of specific activities (sp. act.) of BOHB dehydrogenase and isocitrate lyase are included in the legend of Figure 2. The experiments were conducted with 28-day-old plants. The nodules were detached from the plants and washed twice in cold glass-distilled water to remove perlite and other debris. The excised nodules (100 g) were surface-sterilized by gently shaking for 5 min in 1 liter of 2% sodium hypochlorite solution. Subsequent manipulations were carried out under aseptic conditions. The surface-treated nodules were washed with 5 liters of cold glass-distilled water, blotted with cheesecloth, and placed in two 2-liter flasks. Moist cheesecloth was placed in the flasks to supply moisture. Flasks were maintained at 23 C and were flushed continuously with filtered air.

NADH is sufficient to serve as a reductant for bacteroid nitrogenase-dependent acetylene reduction. More recent experiments (36), however, favor NADPH rather than NADH as a reductant for bacteroid nitrogenase under physiological conditions. On the basis of published results (30), one would expect that acetoacetate, the product of BOHB oxidation, would be metabolized through the citric acid cycle in bacteroids, yielding additional reducing power, and ATP that could be utilized in the nitrogen fixation process.

Three different types of experiments were conducted in which the carbohydrate supply to nodule bacteroids was interrupted (Figs. 2, 3, and 4). The rate of nitrogenase-dependent acetylene reduction by nodules decreased sharply as a result of (*a*) incubation of nodulated soybean plants in the dark (Fig. 2), (*b*) incubation of excised nodules in the dark (Fig. 3), or (*c*) the onset of senescence of symbiotically cultured soybean plants. The addition of sucrose, fructose, glucose, pyruvate, succinate or malate to nodules exhibiting low nitrogenase activity failed to restore the activity. These results suggest that some factor other than a source of energy *per se* was involved in the deterioration of nitrogenase activity.

In the different types of experiments the striking decline in nitrogenase-dependent acetylene reduction of nodules clearly preceded any obvious decrease in the gross content of poly-β-

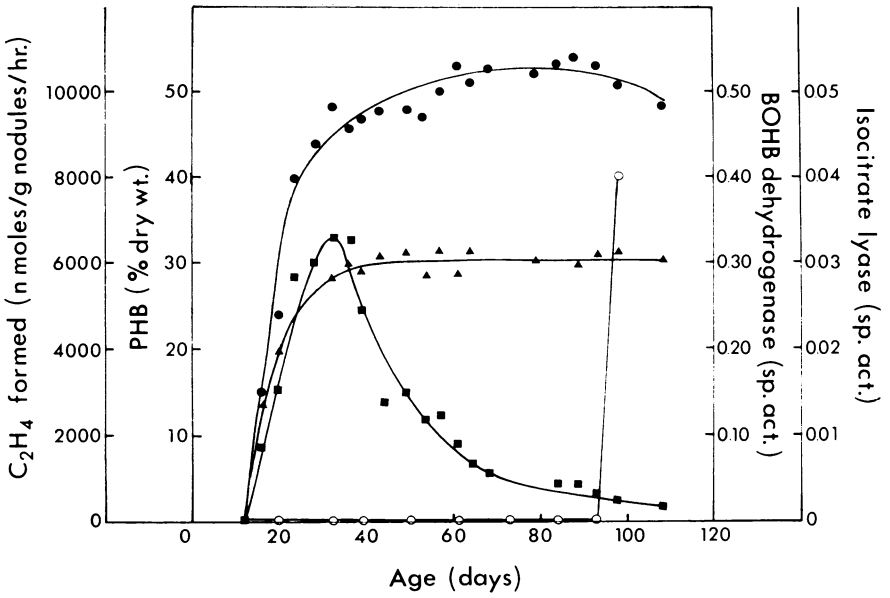

FIG. 4. Changes in the activities of nitrogenase (■), BOHB dehydrogenase (▲), isocitrate lyase (○), and in the PHB content (●) of the root nodules during the growth period of soybean plants. The specific activities (sp. act.) of BOHB dehydrogenase and isocitrate lyase are defined in the legend of Figure 2. The experiments were conducted with nodules from plants of different ages grown under the conditions described under "Source of Plant Materials."

hydroxybutyrate in bacteroids (Figs. 2, 3, 4). This fact lends no support for a direct role of PHB as an energy source for maintenance of nitrogenase activity.

In all three of the experiments, isocitric lyase activity in bacteroid extracts was not detected until a definite decrease in content of PHB in bacteroids became apparent (Figs. 2, 3, and 4). The sudden appearance of isocitric lyase activity suggests the initiation of operation of the glyoxylate cycle, which may provide a mechanism for synthesis of C-4 intermediates from acetyl CoA derived from the degradation of acetoacetate. In previous experiments, Johnson *et al.* (13) failed to detect appreciable isocitric lyase activity in soybean nodule bacteroids, but the enzyme was induced when *Rhizobium* species were cultured in media containing olive oil or certain fatty acids. Under conditions where bacteroids presumably were sustained by PHB alone (Figs. 2, 3, and 4), no obvious mechanism for the maintenance of C-4 intermediates would be available unless isocitric lyase and the glyoxylate cycle were functional. An insufficient supply of C-4 intermediates necessary for synthesis of amino acids and amides in nodules would be expected to lead to the accumulation of fixed NH_4^+, a product known to repress the synthesis of nitrogenase (26).

The work of Pate *et al.* (28) has revealed that the bleeding sap of excised nodules from white clover contain an unusually high concentration of asparagine, aspartic acid, glutamine, and glutamic acid. These high concentrations of amides and amino acids were restricted to conditions where fixed nitrogen was being exported from portions of nodules where N_2 fixation occurred. Similar experiments in our laboratory have revealed that the bleeding sap of excised soybean nodules contained asparagine, aspartic acid, glutamine, and glutamic acid at concentrations of 125, 29, 20, and 5 μmoles/ml of sap, respectively. These data are in agreement with those of Pate *et al.* (28), indicating amides and amino acids as the major forms of export of fixed N_2 from legume nodules. If conditions such as nodule excision, dark exposure, or plant senescence resulted in an improper supply of carbon skeletons for the synthesis of amides and amino acids, then one might expect accumulation of fixed NH_4^+ and repression of nitrogenase synthesis. Further experimentation is necessary to establish whether or not NH_4^+ repression is responsible for the consistently observed decline in nitrogenase activity after nodule excision or other conditions that apparently interfered with the supply of photosynthate from host plants.

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LITERATURE CITED

1. AHMED, S. AND H. J. EVANS. 1960. Cobalt: A micronutrient element for the growth of soybean plants under symbiotic conditions. Soil Sci. 90: 205-210.
2. ALPER, R., D. G. LUNDGREN, R. H. MARCHESSAULT, AND W. A. COTE. 1963. Properties of poly-β-hydroxybutyrate. I. General considerations concerning the naturally occurring polymer. Biopolymers 1: 545-556.
3. BOND, G. 1968. Some biological aspects of nitrogen fixation. *In:* E. J. Hewitt and C. V. Cutting, eds., Recent Aspects of Nitrogen Metabolism in Plants. Academic Press, New York. pp. 15-25.
4. BULEN, W. A., R. C. BURNS, AND J. R. LECOMTE. 1965. Nitrogen fixation: Hydrosulfite as electron donor with cell-free preparation of *Azotobacter vinelandii* and *Rhodospirillum rubrum*. Proc. Nat. Acad. Sci. U.S.A. 53: 532-539.
5. CARPENTER, W. D. AND H. BEEVERS. 1959. Distribution and properties of isocitratase in plants. Plant Physiol. 34: 403-409.
6. DARON, H. H. AND I. C. GUNSALUS. 1962. Citratase and isocitratase. *In:* S. P. Colowick and N. O. Kaplan, eds. Methods in Enzymology, Vol. 5. Academic Press, New York. pp. 622-633.
7. DAWES, E. A. AND D. W. RIBBONS. 1964. Some aspects of the endogenous metabolism of bacteria. Bacteriol. Rev. 28: 126-149.
8. D'EUSTACHIO, A. J. AND R. W. F. HARDY. 1964. Reductants and electron transport in nitrogen fixation. Biochem. Biophys. Res. Commun. 15: 319-323.
9. FORSYTH, W. G. C., A. C. HAYWARD, AND I. B. ROBERTS. 1958. The occurrence of poly-β-hydroxybutyrate in aerobic Gram-negative bacteria. Nature 182: 800-801.
10. GIBSON, A. H. 1966. The carbohydrate requirements for symbiotic nitrogen fixation. A "whole plant" growth analysis approach. Aust. J. Biol. Sci. 19: 499-515.
11. GORNALL, A. G., C. J. BARDAWILL, AND M. M. DAVID. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177: 751-766.
12. HARDY, R. W. F. AND A. J. D'EUSTACHIO. 1964. The dual role of pyruvate and the energy requirement in nitrogen fixation. Biochem. Biophys. Res. Commun. 15: 314-318.

13. JOHNSON, G. V., H. J. EVANS, AND T. M. CHING. 1966. Enzymes of the glyoxylate cycle in *Rhizobia* and nodules of legumes. Plant Physiol. 41: 1330–1336.
14. KLUCAS, R. V. AND H. J. EVANS. 1968. An electron donor system for nitrogenase-dependent acetylene reduction by extracts of soybean nodules. Plant Physiol. 43: 1458–1460.
15. KLUCAS, R. V., B. KOCH, S. A. RUSSELL, AND H. J. EVANS. 1968. Purification and some properties of the nitrogenase from soybean (*Glycine max* Merr.) nodules. Plant Physiol. 43: 1906–1912.
16. KOCH, B. AND H. J. EVANS. 1966. Reduction of acetylene to ethylene by soybean root nodules. Plant Physiol. 41: 1748–1750.
17. LAW, J. H. AND R. A. SLEPECKY. 1961. Assay of poly-β-hydroxybutyric acid. J. Bacteriol. 82: 33–36.
18. LEHNINGER, A. L. AND G. D. GREVILL. 1953. The enzymic oxidation of D and L-β-hydroxybutyrate. Biochim. Biophys. Acta 12: 188–202.
19. LEMOIGNE, M. 1923. Production d'acid β-oxybutyrique par certaines bacteries du groupe du *B. subtilis*. C. R. Acad. Sci. Paris 176: 1761.
20. LEMOIGNE, M. 1927. Etudes sur l'autolyse microbienne. Origine de l'acide β-oxybutyrique forme par autolyse. Ann. Inst. Pasteur 41: 148–152.
21. LEMOIGNE, M. 1946. Fermentation β-hydroxybutyrique. Helv. Chim. Acta 29: 1300–1306.
22. LUNDGREN, D. G., R. ALPER, C. SCHNAITMAN, AND R. H. MARCHESSAULT. 1965. Characterization of poly-β-hydroxybutyrate extracted from different bacteria. J. Bacteriol. 89: 245–251.
23. MCNARY, J. E. AND R. H. BURRIS. 1962. Energy requirements for nitrogen fixation by cell-free preparations from *Clostridium pasteurianum*. J. Bacteriol. 84: 598–599.
24. MERRICK, J. M. AND M. DOUDOROFF. 1964. Depolymerization of poly-β-hydroxybutyrate by an intracellular enzyme system. J. Bacteriol. 88: 60–71.
25. MORTENSON, L. E. 1964. Ferredoxin and ATP requirements for nitrogen fixation in cell-free extracts of *Clostridium pasteurianum*. Proc. Nat. Acad. Sci. U. S. A. 52: 272–279.
26. OPPENHEIM, J. AND L. MARCUS. 1970. Induction and repression of nitrogenase and internal membranes in *Azotobacter vinelandii*. Bacteriol. Proc. 148–149.
27. OTTAWAY, J. H. 1962. A preparation of D(−)-β-hydroxybutyric acid. Biochem. J. 84: 11–12.
28. PATE, J. S., B. E. S. GUNNING, AND L. G. BRIARTY. 1969. Ultrastructure and functioning of the transport system of the leguminous root nodules. Planta 85: 11–34.
29. SCHLEGEL, H. G. AND G. GOTTSCHALK. 1962. Poly-β-hydroxybuttersaure, ihre verbreitung, funktion und biosynthese. Agnew. Chem. 74: 343–347.
30. SIERRA, G. AND N. E. GIBBONS. 1962. Role and oxidation pathway of poly-β-hydroxybutyrate in *M. halodenitrificans*. Can. J. Microbiol. 8: 255–269.
31. SIERRA, G. AND N. E. GIBBONS. 1963. Sodium requirements of poly-β-hydroxybutyrate depolymerase of *M. halodenitrificans*. Can. J. Microbiol. 9: 491–497.
32. UMBREIT, W. W., R. H. BURRIS, AND J. F. STAUFFER. 1964. Manometric Techniques, Ed. 4. Burgess, Minneapolis.
33. WIERINGA, K. T. AND J. A. BAKKHUIS. 1957. Chromatography as a means of selecting effective strains of *Rhizobia*. Plant Soil 8: 254–260.
34. WILLIAMSON, D. H. AND J. MELLANBY. 1963. D(−)-β-Hydroxybutyrate. *In:* H. U. Bergmeyer, ed., *Methods of Enzymatic Analysis*. Academic Press, New York, pp. 459–461.
35. WILLIAMSON, D. H. AND J. F. WILKINSON. 1958. The isolation and estimation of the poly-β-hydroxybutyrate inclusion of *Bacillus* species. J. Gen. Microbiol. 19: 198–209.
36. WONG, P. P., H. J. EVANS, R. V. KLUCAS, AND S. A. RUSSELL. 1970. Investigations into the pathway of electron transport to the nitrogenase from nodule bacterioids. Plant Soil. In press.