Plant Physiol. (1977) 60, 771-774

Isolation of Bacteria, Transforming Bacteria, and Bacteroids from Soybean Nodules[1]

Received for publication April 13, 1977 and in revised form June 28, 1977

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ABSTRACT

Postnuclei supernatant of soybean (*Glycine max* cv. Chippewa 64) nodule homogenate was fractionated by stepwise sucrose density gradient centrifugation into supernatant, endoplasmic reticulum and mitochondria, and three distinct bands with 1.22, 1.25, and 1.27 g/cm³ of peak density. Based on their enzymic activities, composition of electron transport components, and ultrastructural characteristics, the lightest band appears to be the mature bacteroids; the intermediate band the transforming bacteria; and the heaviest, the bacteria. The isolation procedure separates nodule symbionts into different functional and developmental fractions, and it may be a valuable tool for studies involving development, regulation, and senescence of bacteroids in the nodule.

The nitrogen-fixing legume nodules contain both bacteria and bacteroids whose ultrastructure, composition, and function are different (4). Under symbiotic conditions the bacteria are able to reproduce, induce nodule development, synthesize nitrogenase, and transform into bacteroids. The major role of bacteroids is fixing atmospheric N_2 to ammonia. The fixed product is exported to the cytosol of plant cells where it is converted into amino acids and amides and transferred to the plant xylem element. In soybean nodules, the bacteria and bacteroids are similar in shape and size (4) and they are commonly isolated in one fraction by differential centrifugation (1, 6, 9). If a procedure could be developed to separate the bacteria from bacteroids in nodule homogenate, then the two biological systems might be studied individually with fewer complications.

A stepwise sucrose density gradient centrifugation was used to separate mature bacteroids, transforming bacteria, and bacteria into different fractions. This paper reports the details of the isolation and described some properties of the fractions.

MATERIALS AND METHODS

Nodulated plants of soybean (*Glycine max* cv. Chippewa 64) were grown in the greenhouse for 28 to 35 days as previously reported (7) except that the growing conditions were a daily cycle of 26 C for 14 hr with 16,000 Lux of light and 10 hr at 18 C in the darkness. Nodules were harvested in ice-cold grinding buffer containing 0.05 M K-phosphate, 0.3 M sucrose, 5 mM Mg-acetate, and 0.2 M Na-ascorbate (pH 7.5). After washing twice in the cold grinding buffer, 10-g nodules were hand-ground in 10 ml grinding buffer and 3 g Polyclar AT (insoluble PVP; General Aniline and Film Corporation N.Y., acid-washed and grinding-buffer-saturated) in a mortar with pestle. The homogenate was filtered through four layers of Miracloth, and the filtrate centrifuged at 200*g* for 10 min to remove starch granules, nuclei, and aggregates (1, 6, 9). The postnuclei supernatant (about 7 ml) was layered on a stepwise sucrose density gradient. The gradient was made in 0.05 M K-phosphate buffer (pH 7.5) and composed of 10 ml 45%, 13 ml 50%, 13 ml 52% and 10 ml 57% (w/w) sucrose. The gradient tubes were then centrifuged in a SW 25-2 rotor at 100,000*g* for 4 hr in a Beckman L2-HV ultracentrifuge.

The centrifuged gradient was fractionated to 56 tubes of 1 ml each with an ISCO fractionator for the protein profile of the separation. Protein content was determined by Lowry's method of insoluble protein (14) and corrected for sucrose interference (10). Density was estimated by a Bausch and Lomb refractometer.

For ultrastructural observations, peak tubes of density about 1.22 g/cm³ (bacteroid), 1.25 g/cm³ (transforming bacteria), and 1.27 g/cm³ (bacteria) were pooled separately, washed, fixed with 2% glutaraldehyde in Sorenson's phosphate buffer, post-fixed in 1% osmium tetraoxide in Sorenson's phosphate buffer, dehydrated in acetone series, embedded in Epon, stained in saturated uranyl acetate in 50% ethanol for 30 min, poststained in 0.02% lead citrate (18), and examined with a Philips EM 200 electron microscope (8).

For assay of enzyme activities and composition of electron transport components, the three fractions were collected in bulk and stored in a freezer (−18 C) for 1 to 14 days. The stored fractions were washed twice in the phosphate buffer containing 0.15 M sucrose. The washed fractions were suspended in 6 ml tris buffer (0.05 M, pH 7.5) and passed three times through a French press at 15,000 p.s.i. of pressure. The suspension was centrifuged at 10,000*g* for 30 min to remove whole cells, particles of poly-β-hydroxybutyrate, and cell walls (1). The supernatant was used as enzyme preparation for β-hydroxybutyrate dehydrogenase (20) and Cyt *c* oxidase (16). The concentration of electron transport components was determined in the preparation by the procedure of Appleby (1, 2). The protein content of the preparation was estimated by the method of Lowry for insoluble protein in cold alkali (14).

For the assay of nitrogenase, the three fractions were isolated and washed similarly but they were kept under anaerobic conditions at all times. The isolated fractions were used right after preparation. Two days' storage at −18 C reduced the activity in the bacterial fraction to less than 10%, in the transforming bacteria fraction to 20 to 30%, and in the bacteroid fraction to 20 to 40%. The conventional shaking incubation with gas phase (3, 6, 19) was used for the assay because of the

[1] This study was supported in part by a research operating grant from the National Research Council of Canada to R. C. P.
Oregon Agricultural Experiment Station Technical Paper No. 4498.

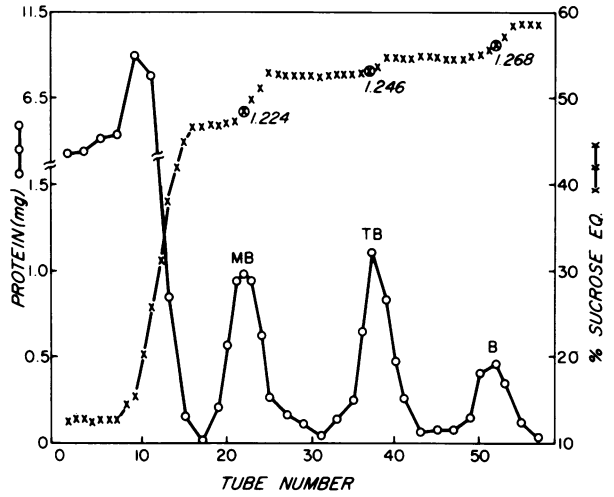


FIG. 1. Protein content peak density (g/cm³), and sucrose equivalent concentration of fractions separated by the stepwise sucrose density gradient centrifugation of the postnuclei homogenate from 2-g nodules of 28-day-old soybean plants. MB: mature bacteroids; TB: transforming bacteria; B: Bacteria.

difficulty in sampling the reaction product in the no-gas phase incubation (5). Each fraction was incubated in 2 ml HEPES-K-phosphate buffer (25 mM each, pH 7.5) containing 5 mM Mg-acetate, 50 mM Na-succinate, and 0.2 mM purified oxyleghemoglobin with 10% C_2H_2, 3% O_2 and 87% argon as the gas phase (3, 19). After 30 min incubation at 30 C with shaking at 100 rpm, the gas phase was analyzed by gas chromatography. Purified oxyleghemoglobin was prepared from the cytosol of soybean nodules according to the procedure of Appleby and Wittenberg (3, 19) and stored at −90 C. The concentration of leghemoglobin used in each assay was determined in the soluble fraction by the pyridine hemochromogen method (6). The dry wt and protein wt of the material used in each assay were analyzed according to Bergersen *et al.* (6).

RESULTS AND DISCUSSION

A typical protein profile of the fractionated postnuclei nodule homogenate is shown in Figure 1. Tubes 1 through 8 contained red leghemoglobin and nodule soluble proteins. Tubes 9 through 13 probably were the mixture of plant ER and mitochondria as evidenced by their characteristic densities (1.13 and 1.18, respectively) (12). Tubes 19 through 29 consisted mainly of mature bacteroids (Fig. 2 and Table I). Tubes 31 through 41 contained mostly transforming bacteria (TB) (Fig. 4 and Table I). Tubes 49 through 55 were composed chiefly of bacteria (B) (Fig. 6 and Table I). Their respective peak densities were 1.224, 1.248, and 1.266 g/cm³. All three fractions exhibited a gram-negative stain characteristic typical of *Rhizobium*. When cultured cells of *Rhizobium japonicum* were centrifuged alone on the sucrose gradient, a major band with a peak of 1.266 g/cm³ and a minor band of 1.246 g/cm³ were observed. When co-centrifuging 0.2 ml packed cultured cells with the postnuclei supernatant, no additional bands were observed. These observations substantiate the identity of the two heavier bands as bacterial in nature.

Figures 2 and 3 show the composition and ultrastructure of the isolated mature bacteroid fraction. The single membrane and the large granules of poly-β-hydroxybutyrate were evident. Occasionally, polyphosphate particles were present in this fraction (4). DNA fibers were sparse and ribosomes rare. All of these observations agree with the *in situ* ultrastructural characteristics of bacteroids in soybean nodules (4, 11). The MB fraction contained mostly mature bacteroids, with parabacteroid membranes and mitochondria the occasional contaminants.

The ultrastructure of the fraction with density peak of 1.248 g/cm³ consisted mainly of transforming bacteria (Figs. 4 and 5). The smaller granules of poly-β-hydroxybutyrate, the distinct DNA fibers and ribosomes, and the occasional-walled double membrane indicated an intermediate developmental stage between mature bacteroids (Figs. 2 and 3) and bacteria (Figs. 6 and 7). Some membrane and microbodies have also been observed as rare contaminants of the fraction.

The compact appearance of the B fraction (Figs. 6 and 7) may be attributed to the concentrated sucrose solution which was used in the gradient for separation. Even though the washing process that served as deplasmolysis procedure had been applied twice after gradient separation, the cytoplasmic region was dense compared to the cultural bacteria. Ribosomes and DNA fibers were, however, discernible. The thick cell wall of bacteria can occasionally be observed.

Some characteristics of enzyme activity and electron transport components of the three isolated fractions are summarized in Table I. β-Hydroxybutyrate dehydrogenase is a marker enzyme of "bacteroids" in nodule tissue (H. J. Evans, personal communication); the bacteroid fraction indeed exhibited the highest specific activity. Transforming bacteria fraction acquired two-thirds of the activity of that in bacteroids, whereas the bacterial fraction contained only 40% of the enzyme activity of the bacteroid. Bacteria cells of *R. japonicum* are known to contain β-hydroxybutyrate dehydrogenase and to accumulate poly-β-hydroxybutyrate in culture (J. M. Vincent, personal communication). Therefore, it is not surprising that the bacterial fraction of the nodule contains the enzyme.

Cyt *c* oxidase occurred only in cultured cells of *R. japonicum* (2). The highest specific activity was observed in the bacterial fraction (Table I). The small activity found in bacteroid fraction might be the contaminant. The transforming bacteria fraction had 70% of the enzyme activity of the bacteria fraction indicating an intermediate developmental state between the bacteria and mature bacteroids.

The nitrogenase activity in the bacteroid fraction as measured by the acetylene reduction assay approached the rate of soybean bacteroids isolated by the single-step centrifugation (3, 6, 19). In the reaction mixtures 0.2 mM oxyleghemoglobin was used. If more oxyleghemoglobin was added to the reaction mixture, the rate might be higher because the nitrogenase activity of soybean bacteroids was linear in response to added oxyleghemoglobin from 0.1 mM to 1 mM (6). The transforming bacterial and the bacterial fractions had 50 to 14%, respectively, of the nitrogenase observed in the bacteroid fraction. Since cultured *Rhizobium* cells under reduced O_2 tension and with special inducing sub-

FIG. 2. Electron micrograph of bacteroid fraction. Note prominent poly-β-hydroxybutyrate granules (B) and nucloid regions (arrows). (× 8,180).

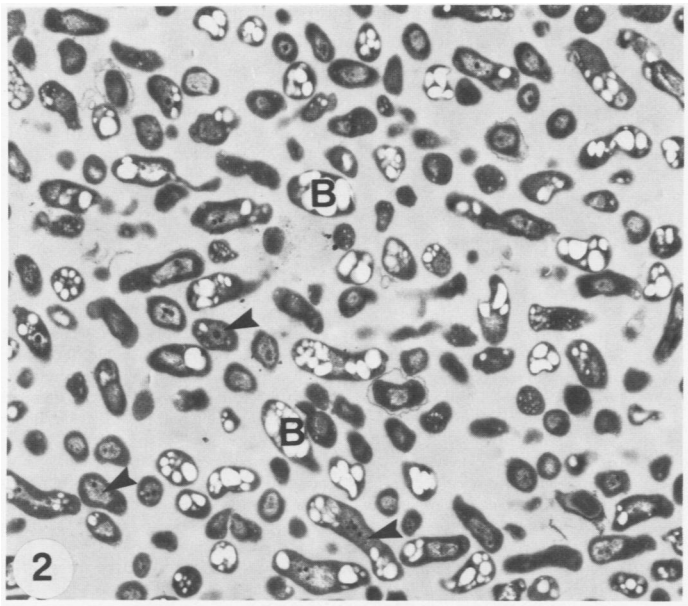
FIG. 3. Electron micrograph of bacteroid fraction. Note polyphosphate granules (P), nucloid region (N), and numerous poly-β-hydroxybutyrate granules (B) (× 28,800).

FIG. 4. Electron micrograph of transforming bacterial fraction. Some small poly-β-hydroxybutyrate granules (arrows) are present (× 8,400).

FIG. 5. Electron micrograph of transforming bacterial fraction showing the nucloid region (N) with numerous fibrils, outer membrane (large arrows) and polyribosome (small arrows) (× 53,240).

FIG. 6. Electron micrograph of bacterial fraction (× 13,590).

FIG. 7. Electron micrograph of bacterial fraction illustrating the nucloid region (N), polyphosphate granules (P), and outer membrane (arrows) (× 41,000).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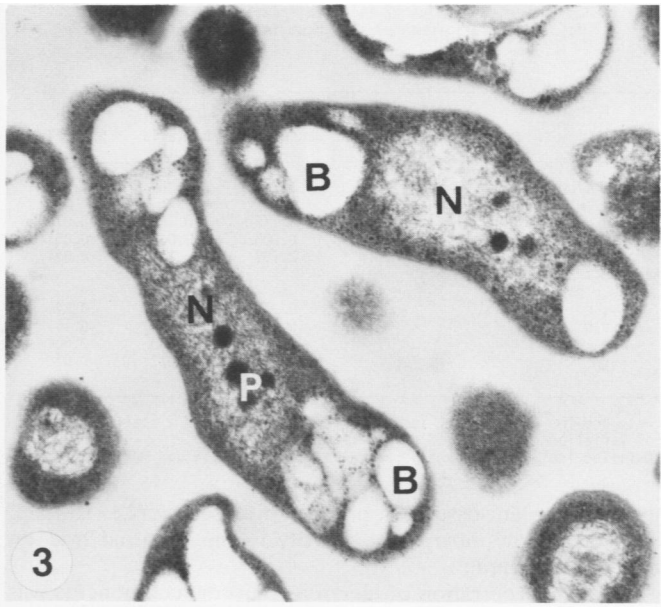
B
2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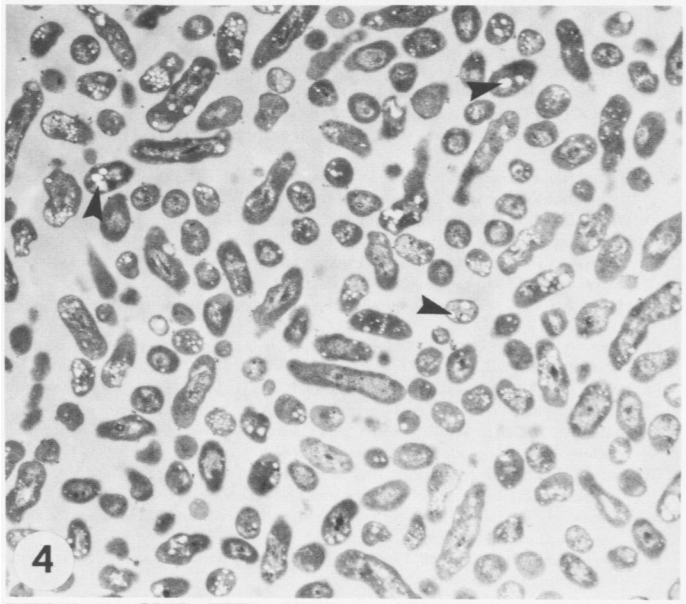
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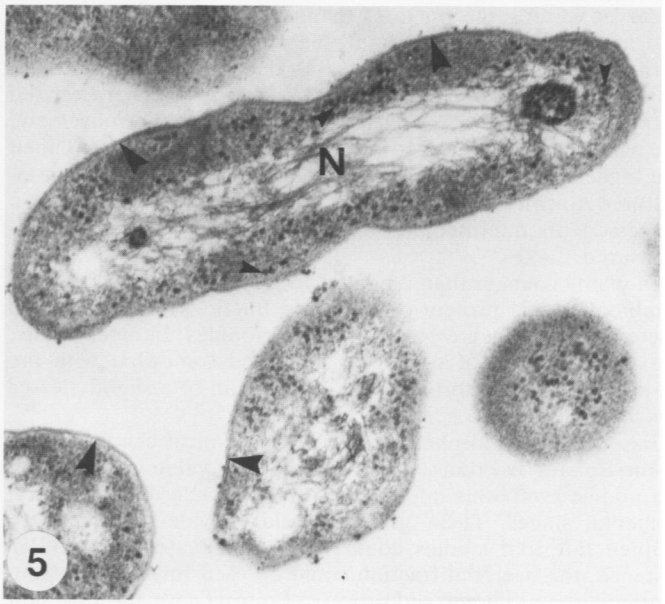
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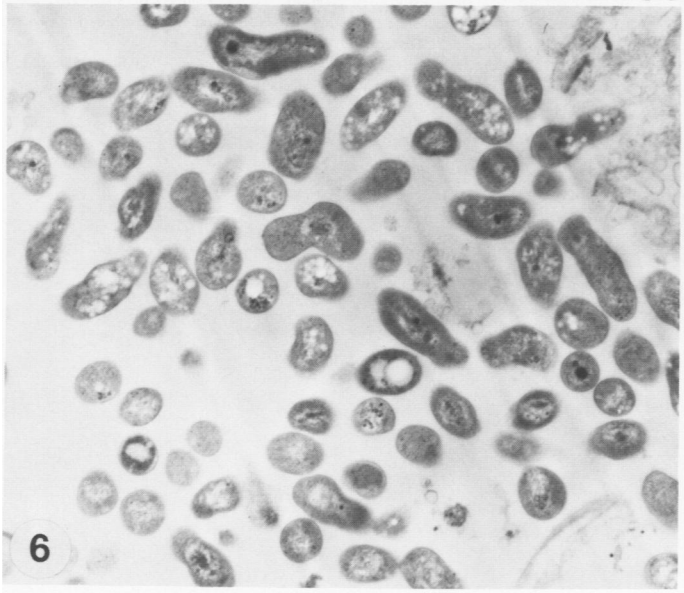
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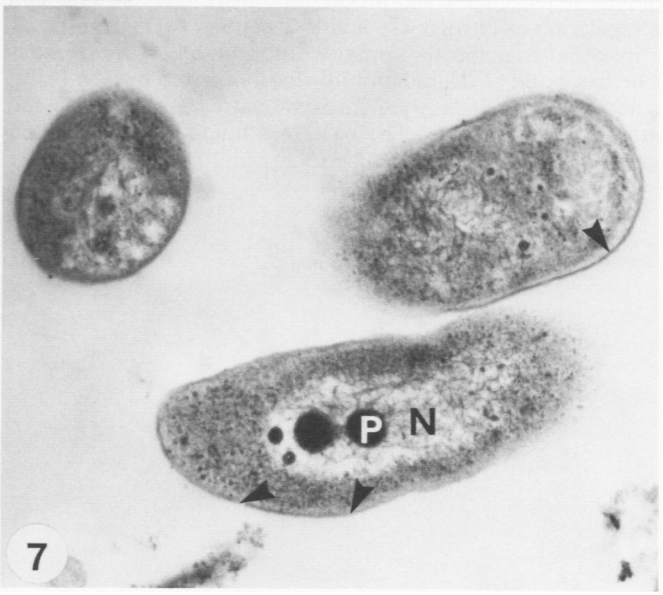
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N
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Table I. Some characteristics of isolated fractions from nodules of 28- to 35-day-old soybean plants grown in greenhouse *[o]

Fraction	Bacteroid	Transforming Bacteria	Bacteria
Density, g/cm^3	1.22	1.25	1.27
B-hydroxybutyrate dehydrogenase, μmole · mg protein^{-1} min^{-1}	0.175	0.115	0.075
Cyt c oxidase, μmole · mg protein^{-1} mjn^{-1}	0.005	0.037	0.051
Nitrogenase, nmole · mg dry wt^{-1} min^{-1}	2.192	1.103	0.321
Electron transport components, μmole · g protein^{-1}			
Cyt c	0.379	0.296	0.241
Cyt b	0.208	0.176	0.133
Cyt a-a_3	0	0.063	0.068
P_{450}	0.094	0.072	0.010

* Data are the average of 3 isolated fractions which had a coefficient of variation less than 15% of the mean.
[o] Detailed isolation and assay procedure, see materials and methods.

stances from plant tissue can produce nitrogenase (13, 15, 17), the observed low nitrogenase activity in the bacterial fraction was not unexpected.

A lower concentration of electron transport components was observed in all three fractions than was observed in bacteroids and cultured bacteria (1, 2) (Table I). The proportion of the various components, however, was comparable with the reported data (1, 2). The bacteroid fraction was characterized with high Cyt *c*, Cyt *b*, and P_{450}, but no Cyt *a*-a_3. Conversely, the bacterial fraction was low in Cyt *c*, *b* and P_{450}, but contained the highest concentration of Cyt *a*-a_3 which is characteristic of cultured *Rhizobium* cells (2). The transforming bacterial fraction possessed an intermediate concentration of the components measured.

In plants younger than 28 days, not only less quantity of total symbionts (total protein of the three fractions) but also more bacterial fraction were observed/2 g of nodules. In older plants, the total quantity of symbionts increased somewhat with the major band shifted to the bacteroid fraction of a slightly lesser density.

Based on the morphological and biochemical characteristics of the isolated fractions, the procedure is of value in separating the nodule symbionts into different functional groups or developmental stages. These groups would provide material with defined fate and studies could be designed accordingly. For instance, the bacterial fraction could be used for studies of the synthetic requirements and process of nitrogenase and P_{450}, and the regulation of nitrogenase and P_{450} synthesis. The transforming bacterial fraction may provide information on the reasons for or the mode of degradation of ribosomes and nucleic acids, the accumulation of poly-β-hydroxybutyrate, and the sustained synthesis of nitrogenase. The bacteroid fraction could be used for discerning the regulatory factors of nitrogenase activity, the function of poly-β-hydroxybutyrate granules, and the senescence process and factors of bacteroids in nodules.

Acknowledgments—We thank H. J. Evans for the sound advice, constant discussion and encouragement, the use of equipment in his laboratory, and the review of this paper. The suggestion by J. C. Vincent of co-centrifuging cultured bacteria in the gradient that led to the discovery of the small bacteria fraction is deeply appreciated. We also thank K. Carter, K. Schubert, and K. Smith for technical assistance, J. G. Guevara for cultured *R. japonicum* cells, and B. C. Lux for the use of his electron microscope. Soybean seeds were kindly provided by P. K. Joo of the Northrup King & Co., Minneapolis, Minn.

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