

ISOLATION AND CHARACTERIZATION OF THE MEMBRANE ENVELOPE ENCLOSING THE BACTEROIDS IN SOYBEAN ROOT NODULES

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ABSTRACT

The membrane envelope enclosing the bacteroids in soybean root nodules is shown by ultrastructural and biochemical studies to be derived from, and to retain the characteristics of, the host cell plasma membrane. During the early stages of the infection process, which occurs through an invagination, *Rhizobium* becomes surrounded by the host cell wall and plasma membrane, forming the infection thread. The cell wall of the infection thread is degraded by cellulolytic enzyme(s), leaving behind the enclosed plasma membrane, the membrane envelope. Cellulase activity in young nodules increases two- to threefold as compared to uninfected roots, and this activity is localized in the cell wall matrix of the infection threads.

Membrane envelopes were isolated by first preparing bacteroids enclosed in the envelopes on a discontinuous sucrose gradient followed by passage through a hypodermic needle, which released the bacteroids from the membranes. This membrane then sedimented at the interface of 34–45% sucrose (mean density of 1.14 g/cm³). Membranes were characterized by phosphotungstic acid (PTA)-chromic acid staining, ATPase activity, and localization, sensitivity to nonionic detergent Nonidet P-40 (NP-40) and sodium dodecyl sulfate (SDS) gel electrophoresis. These analyses revealed a close similarity between plasma membrane and the membrane envelope. Incorporation of radioactive amino acids into the membrane envelope proteins was sensitive to cycloheximide, suggesting that the biosynthesis of these proteins is primarily under host-cell control. No immunoreactive material to leghemoglobin antibodies was found inside or associated with the isolated bacteroids enclosed in the membrane envelope, and its location is confined to the host cell cytoplasmic matrix.

KEY WORDS *Rhizobium* · leghemoglobin ·
membrane envelope · immunohistochemistry

In symbiotic nitrogen-fixing root nodules of leguminous plants, the bacteroids are enclosed in a

membrane envelope which was first observed by Bergersen and Briggs (5). This membrane envelope compartmentalizes the bacteroids from the host cell cytoplasm, and thus may play an important role in segregating eu- and procaryote metab-

olism and attendant incompatibilities which possibly occur on each side of the membrane. There are three main views regarding the origin of this membrane: (a) that *Rhizobium* is taken into the host by endocytosis and that the membrane is derived from the plasma membrane of the host cell (5, 14, 15, 18, 35, 38, 30); (b) that it is derived from the host endomembrane system, e.g., nuclear envelope (34) or endoplasmic reticulum (ER) (23); and (c) that it is synthesized *de novo* (9-11). Although the view that it originates from the plasma membrane of the host cell is dominant, the membrane probably undergoes both structural and functional changes due to the symbiotic demands of both host and bacteroid. Apparently, it retains the same configuration, i.e., the surface facing the cell wall of the host cell now faces the bacteroid(s) (38, 39); however, it loses the ability to form cell wall material on this surface.

Very little is known about the initial infection process, the mode of "release" of *Rhizobium* into the plant cell, the role of individual partners in various metabolic processes, and the effectiveness of barrier(s) between them, i.e., the role of the membrane enclosing the bacteroids. Development of the membrane envelope appears to be important for "effective symbiosis." If the membrane envelope is not formed, the *Rhizobium* may become parasitic or saprophytic on the host cell (42). It also provides an intracellular compartment where processes such as oxidative phosphorylation in the host cell and nitrogen fixation in bacteroids, which may differ in their pO_2 requirement, can occur in close proximity. Leghemoglobin, which is localized in the host-cell cytoplasm around the membrane envelopes (40), probably helps in meeting these demands.

Towards defining the "functional" characterization of this symbiotic process, we first studied the origin of the membrane envelope in soybean root nodules and developed a method for isolation and purification of this membrane. The results suggest that the membrane envelope is derived from the plasma membrane of the host cell through an invagination process. Dissolution of the enclosed host cell wall takes place with the involvement of cellulolytic enzyme(s) apparently secreted by the host cell. Subsequent proliferation and biosynthesis of this membrane is controlled by the host. Although the membrane undergoes some modifications, it retains several characteristics of the host plasma membrane. To date, leghemoglobin is the

only plant gene product that has been associated with effective nodulation. It is anticipated that analysis of membrane envelope proteins would be a first step towards identification of other specific plant gene product(s), which may be expressed as a result of invasion by *Rhizobium* and are obligatory for symbiosis.

MATERIALS AND METHODS

Growth of Soybean

Soybean (*Glycine max*, var. Kanrich) seeds were obtained from Strayer Seed Farm, Hudson, Iowa and germinated in vermiculite in a growth chamber. Seeds were inoculated with a suspension of *Rhizobium japonicum* (Strain 61A76) grown as described previously (44). Plants were irrigated with nitrogen-free medium and grown under a 12-h photoperiod, days at 28°C and nights at 22°C. Nodules were harvested after 3-4 wk of germination and separated into small (<2 mm in diameter) and large (>2 mm in diameter) nodules.

Ultrastructural Studies

Nodules of different sizes were sliced directly in either a mixture of paraformaldehyde and glutaraldehyde in Sorensen's buffer (24), or in 2.5% glutaraldehyde in the above buffer. They were fixed for 1 h at 0°C or 23°C. After a thorough washing with buffer, the tissue slices were treated with 1% OsO_4 in the same buffer for 1 h at 4°C. This was followed by dehydration in an ethanol series and subsequent embedding in Epon. Ultra-thin sections were cut with a diamond knife and stained with uranyl acetate and lead citrate (3, 4). Some sections were also stained with phosphotungstic acid (PTA) and chromic acid (36). Observations were made on a Zeiss-9S electron microscope.

Localization of Cellulolytic Activity

Slices of fixed tissue were washed in cold buffer for 18 h (3), and then placed in an incubation medium containing 0.1 M phosphate buffer (pH 6.0) and 0.02% carboxymethylcellulose (used as substrate) for 10 min at 25°C. Control tissues were either heat-inactivated by boiling for 10 min, or incubated in buffer only, without substrate. After incubation, the slices were transferred to hot Benedict's solution at 80°C for 10 min and washed in distilled water (see references 3 and 4 for details). They were then osmicated and processed for electron microscopy as described above.

Localization of ATPase Activity

Slices of nodules were fixed in 4% formaldehyde (prepared from paraformaldehyde, reference 24) in 80 mM Tris-maleate buffer (pH 7.0) containing 0.25 M sucrose and 0.05% $CaCl_2$ for 1 h at 0°C. After washing in the above buffer, they were incubated for 1 h at 25°C

in a medium (47) containing 1 mM ATP (Na), 80 mM Tris-maleate buffer (pH 6.0), 7 mM MgSO₄, 3.6 mM Pb(NO₃)₂. Controls were kept in the same medium but with added (a) oligomycin 0.46 mg/ml, (b) ouabain 1 mg/ml, and (c) glutaraldehyde, a strong inhibitor of ATPase activity (21). After incubation the slices were washed in distilled water, osmicated in 1% OsO₄ in 0.2 M cacodylate buffer and processed for electron microscopy.

In Situ Solubilization of Root Nodule Membranes by Nonionic Detergent, NP-40

Tissue slices were fixed as described above and treated with 0.2% Nonidet P-40 (NP-40) (Shell Chemical Co., New York) for 12 h at 0°C. Tissue was washed in buffer (Sorenson's buffer, pH 7.2), osmicated and processed for electron microscopy.

Localization of Leghemoglobin

Antibodies prepared to purified leghemoglobins were conjugated with ferritin and used for localization studies as described previously (40). Ferritin-conjugated albumin and ferritin-conjugated nonimmune serum were used as controls. To test for the association of leghemoglobin with the membrane envelope or its possible occurrence inside the envelope, bacteroids enclosed in the membrane envelope were prepared (Fig. 4, Fraction D) and sonicated, or the membrane was solubilized with 0.2% NP-40 and then reacted with antibodies in Ouchterlony plates (8).

Measurement of Cellulase Activity

Small (<2 mm in diameter) nodules (1 g fresh weight) were homogenized in 2 ml of 0.05 M phosphate buffer (pH 6.5). The debris was removed by centrifugation for 5 min at 300 g and the supernate was further centrifuged for 10 min at 5,000 g. The resulting pellet was resuspended in 2 ml of the above buffer. This fraction contained bacteroids enclosed in membrane envelopes and some "infection threads." One aliquot was treated with 0.2% NP-40 to dissolve the membrane and release the contents, which were layered over a cushion of 35% sucrose. It was centrifuged for 10 min at 23,000 g, and the resulting pellet, which contained free bacteroids, was resuspended in 1 ml of phosphate buffer. Both preparations were sonicated for 2 min, centrifuged to remove cellular debris, and analyzed for cellulase activities. Uninfected root tissue was used as a control. Cellulase was assayed viscometrically at 35° by using carboxymethylcellulose as a substrate (8).

Isolation of Membranes

3-wk-old fresh nodules (2 g) were washed in deionized water (0–4°C) and gently homogenized in a mortar and pestle at 4°C in 8 ml of buffer (0.5 M sucrose, 2.5%

Ficoll (Pharmacia Fine Chemicals Inc., Piscataway, N. J.), 5% dextran T₄₀, 50 mM Tris-HCl (pH 7.6), 2 mM MgSO₄, 10 mM EDTA, and 5 mM mercaptoethanol), and processed as described in the flow diagram (Fig. 4). The 5,000 g pellet (Fraction A) was resuspended in the above buffer and layered over a discontinuous sucrose gradient containing 3 ml of 60%, 4 ml of 45%, and 3 ml of 34% (wt/vol) sucrose in 10 mM Tris (pH 7.6), 1 mM MgSO₄, and 5 mM mercaptoethanol. The fraction collected from the 45/60% interface (Fraction D) contained mainly bacteroids enclosed in membrane envelopes. This fraction was further processed as described in Fig. 4 and layered over a step gradient containing 52, 45, and 34% sucrose. After the second centrifugation, the fraction at the interface of 34/45% sucrose was collected, diluted with buffer (10 mM Tris-HCl, pH 7.6, 1 mM MgSO₄, 5 mM mercaptoethanol), and centrifuged at 23,000 g for 10 min. The resulting pellet was processed for electron microscopy or used for enzyme assays. To prepare membranes from control roots, segments (10 mm) from root tips of 5-day-old non-inoculated seedlings were harvested and homogenized in the above buffer. The homogenate was centrifuged at 14,000 g to remove mitochondria (27), and the supernate was layered on the first step gradient (Fig. 4). A fraction enriched in plasma membrane sedimented at the interface of the 34/45% sucrose (mean density, 1.14 g/cm³) (22). All fractions were fixed as described above and processed for electron microscopy. Besides uranyl acetate and lead citrate, PTA-chromic acid staining was also carried out. Staining density of membrane fractions was analyzed on a VP-8 image analyzer (Interpretation Systems, Inc., Lawrence, Kansas).

Enzyme Assays on Membrane Fractions

Cytochrome *c* oxidase: Activity was assayed (22) in a reaction mixture (1.5 ml) containing 50 μl of enzyme (5–10 μg protein), 50 μl of 0.3% digitonin, or 0.2% NP-40, 1.35 ml of 50 mM sodium phosphate buffer (pH 7.5). Reaction was started by adding 50 μl of 0.45 mM reduced cytochrome *c* (reduced by sodium dithionite and passed through a Sephadex G-25 column before use to remove excess of dithionite).

NADH/NADPH – CYTOCHROME *c* REDUCTASE: Reduction of cytochrome *c* was monitored (22) in a reaction mixture (1.5 ml) consisting of 50 μl of enzyme (5–10 μg protein), 50 μl of 50 mM potassium cyanide, 100 μl of 0.45 mM cytochrome *c*, and 1.25 ml of buffer. The reaction was started by addition of 50 μl of either 3 mM NADH or NADPH. Rates of cytochrome *c* oxidation and reduction were determined from the initial linear slopes by using an extinction coefficient for cytochrome *c* of 18.5 mM⁻¹ cm⁻¹ at 550 nm (22).

ATPase: Release of radioactive phosphate from [γ -³²P]ATP was measured in a reaction mixture (200 μl) containing 100 μl of 100 mM Tris-HCl, pH 6.0, 10 μl

of enzyme, 20 μ l of 500 mM KCl, 20 μ l of 20 mM MgSO₄, and 20 μ l of 5 mM ATP containing 500 μ Ci/ml of [γ -³²P]ATP-tetra (triethylammonium) salt, (4.93 Ci/mmol). The reaction was allowed to proceed at 30°C for 30 min after which 400 μ l of a suspension of activated charcoal in 5% TCA was added. The sample was centrifuged for 5 min at 12,000 g, and an aliquot (10–20 μ l) of the supernate was used to determine the released phosphate. Radioactivity was determined in 5 ml of Aquasol (New England Nuclear, Boston, Mass.) in a Beckman LS-333 scintillation counter (Beckmann Instruments, Inc., Palo Alto, Calif.). Controls without enzyme and charcoal were used to determine the non-specific release of inorganic phosphate and the total radioactivity used per assay, respectively.

SDS Gel Electrophoresis of Membrane Proteins

A discontinuous sodium dodecyl sulfate (SDS) system was used (32). Washed membrane fractions were solubilized in a buffer containing 100 mM Tris-HCl (pH 6.8), 2% SDS, 5% mercaptoethanol, and 10% glycerol. A drop of saturated bromophenol blue (marker) was added. Proteins of known molecular weight were processed similarly. Samples were heated for 3 min at 100°C, cooled to room temperature, and layered over a slab of gel (1.5 mm thick). The acrylamide concentration was prepared as an exponential gradient from 10 to 15%. Electrophoresis was carried out at 6 mA for 16 h. The gel was removed and stained for 1 h in 1% Coomassie blue in 10% acetic acid and 25% iso-amyl alcohol, destained for 24 h in the above solvent and photographed.

In Vivo Labeling of Membranes

About 3-wk-old nodules (1 g fresh weight) were incubated in 1 ml of deionized H₂O with 4 μ Ci of L-[U-¹⁴C]leucine (sp act 300 mCi/mmol) for 1 h at 25°C or 350 μ Ci of [³⁵S]methionine (sp act 460 Ci/mmol) for 2 h at 25°C. The samples were shaken at 180 rpm on a gyratory shaker. After incubation, nodules were washed, the membrane enclosing the bacteroids was prepared (Fraction E, Fig. 4), and TCA-insoluble radioactivity was measured (40). To see whether isolated bacteroids which are still enclosed in the membrane envelope (Fig. 4, Fraction D) could synthesize any membrane envelope proteins, they were incubated with 2 μ Ci/ml of [¹⁴C]leucine in 0.5 M sucrose, 0.01 M Tris (pH 7.6), and 1 mM Mg-acetate as above. They were processed as in Fig. 4 to isolate membrane envelope (Fraction E), and the hot TCA-precipitable radioactivity was measured.

RESULTS

Development of the Membrane Envelope

Initial infection of a root cell proceeds through

an invagination process forming a tubelike structure known as an infection thread (31). It consists of *Rhizobium* surrounded by host cell wall material (infection thread wall) which in turn is enclosed by the plasma membrane of the host cell. Fig. 1a shows a cross section of such an infection thread, and Fig. 1b shows its continuity with cell wall and plasma membrane of the host cell (see also references 13, 30, and 38). The infection thread wall is eventually removed, leaving the plasma membrane (membrane envelope) between the host cell cytoplasm and the bacteroids. To find out the mode of release of *Rhizobium* from the infection thread, attempts were made to localize cellulolytic activity in these sections by cuprous oxide precipitation reaction (3, 4). This activity was found in the cell wall region of the infection threads (Fig. 1c), suggesting that cellulase may be involved in dissolving the wall of the infection thread.

Analysis of the cellulase activity in various cell fractions showed (Table I) that there is a two- to threefold increase in total cellulase activity after infection, some of which was present in the fraction containing infection threads and bacteroids enclosed in the membrane envelopes. Since free-living *Rhizobium* and the bacteroids contain very little cellulase activity (see also reference 29), this enzyme appears to be produced by the host cell in response to infection by *Rhizobium*.

Cytochemical Studies on Root Nodule Membranes

PTA-CHROMIC ACID STAINING: To determine the similarities between plasma membrane and the membrane envelope enclosing the bacteroids, PTA, a plant plasma membrane specific stain (36), was used. Fig. 2a shows that both the plasma membrane and the membrane envelope enclosing bacteroids stain positively. Although some staining is seen on the bacteroids, no other endomembranes of the host cell stain with PTA in root nodules. During later stages of development, the staining pattern of the plasma membrane and the membrane envelope loses uniformity, resulting in unstained patches (Fig. 2b). The reason for this unevenness is not clear though both membranes behave similarly.

LOCALIZATION OF ATPase ACTIVITY: The reaction product of ATPase activity is localized in a similar manner on the plasma membrane and the membrane enclosing the infection thread (Fig. 3a). This is consistent with the continuity of these

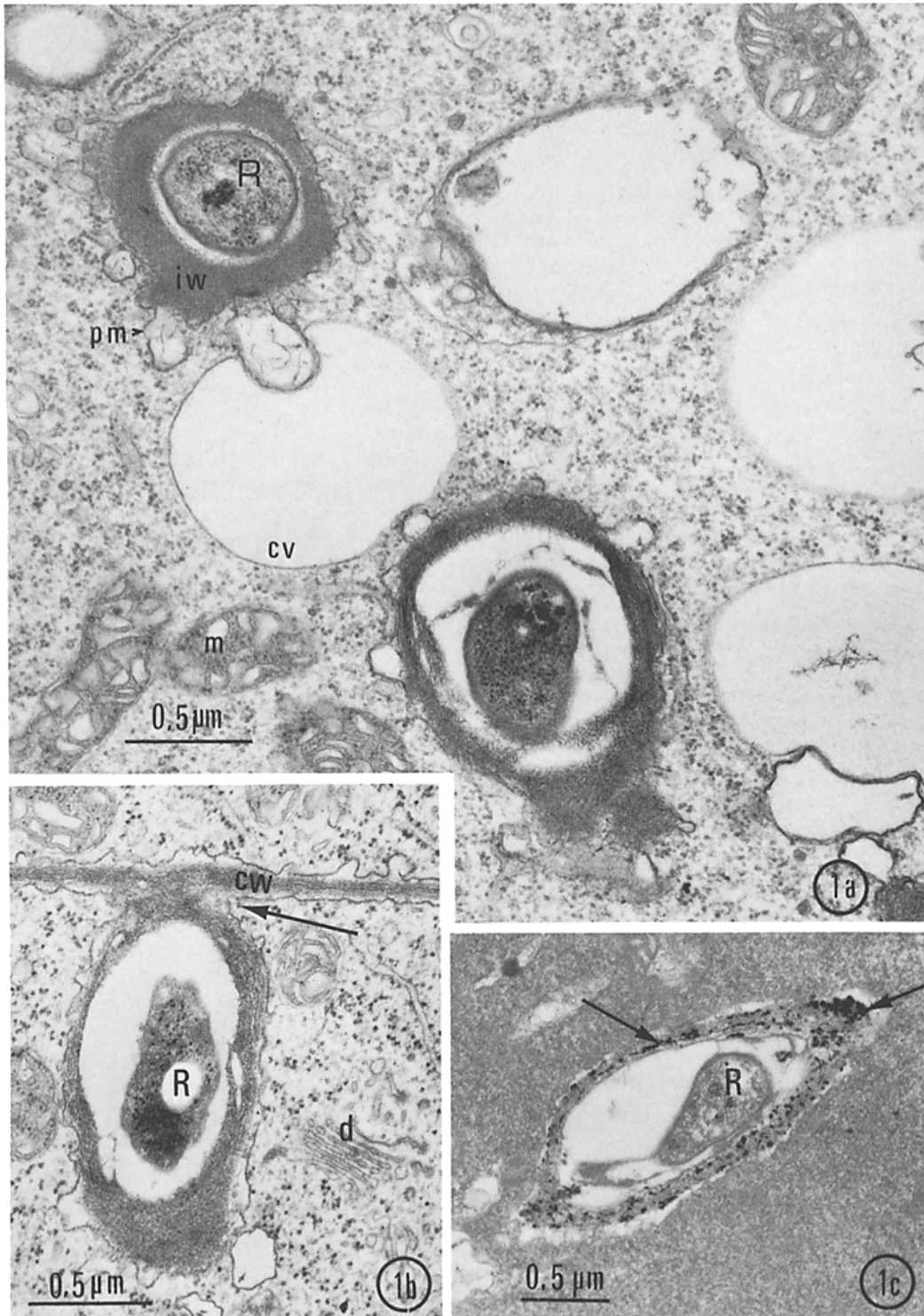


FIGURE 1 Ultrastructure of a young nodule. (a) cross section of the infection thread, bacteroid enclosed in the cell wall material and plasma membrane; (b) tangential section of the infection thread entering the cell (arrow indicates the continuity of the host plasma membrane); (c) localization of cellulolytic activity in the infection thread (arrows indicate reaction product of the cellulase activity in the cell wall material enclosing the bacteroids). *R*, *Rhizobium*; *cw*, cell wall; *iw*, infection thread wall; *pm*, plasma membrane; *d*, dictyosomes; *m*, mitochondria; *cv*, cytoplasmic vacuole.

TABLE I
Cellulase Activity in Young Root Nodules

Tissue fraction	Cellulase activity Enzyme U*/g tissue
Control root (5,000 g supernate)	163.7
Root nodules (5,000 g supernate)	370.9
Free bacteroids	63.6
Bacteroids enclosed in the envelope	134.5‡
Total activity in root nodules	505.4

Free bacteroids and bacteroids enclosed in the envelope were prepared as described in Materials and Methods.

* 1 U of cellulase activity is defined as the amount of enzyme required to cause 1% loss in viscosity in 2 h at 35°C (8).

‡ This value does not represent all the activity which may be present in this fraction, since some of the membrane envelopes break during fractionation procedure.

membranes in Fig. 1 *b*. Similar reaction product(s) were observed on the membrane envelope in mature nodules (Fig. 3 *b*). This pattern of localization appears to be specific to plasma membrane and its derivative-membrane envelope; no other endomembrane shows this similarity. Tu (38) observed this similarity by localization of "adenyl cyclase," the enzyme whose presence is questionable in this tissue (see Discussion). ATPase activity was found to be inhibited in glutaraldehyde-fixed tissue (21). Oligomycin controls also showed some inhibition while ouabain had little effect. These results are subject to the permeability of these inhibitors in root nodules.

SOLUBILIZATION OF MEMBRANES IN SITU BY NONIONIC DETERGENT: Treatment of the fixed tissue with a nonionic detergent (NP-40) selectively solubilized the host membranes. Fig. 3 *c* shows that both plasma membrane and the membrane envelope disappear after NP-40 treatment but that the bacterial membrane is intact. Organization of other cellular organelles is reasonably maintained after this treatment. Since this detergent solubilizes some lipids of these membranes, these data suggest that host membranes may be similar in their composition and are different from bacteroidal membranes. Other endomembranes such as the ER and the Golgi apparatus are also solubilized with this treatment.

Isolation of the Bacteroids Enclosed in the Membrane Envelope

Fig. 4 shows a flow diagram of the procedure

developed to isolate intact membrane envelopes enclosing the bacteroids. Extremely gentle homogenization is necessary to prevent breakage or bursting of the membrane envelopes. Even with great care, at least half of the membrane envelopes are disrupted by this procedure, and they do not pellet at 5,000 g along with the bacteroids. The broken envelopes can be recovered from the supernate; however, they are contaminated with plasma membrane and other endomembranes. The fraction (Fraction D, Fig. 4) obtained at the interface of the 45/60% sucrose gradient in the first step gradient from the 5,000 g pellet contains mostly bacteroids enclosed in the membrane envelopes (Fig. 5 *a*). Most of the other contaminating membranes sediment at the 34/45% sucrose interface in the first gradient. The membrane envelopes broke during resuspension of the 5,000 g pellet are also found at this interface (our unpublished results). Some bacteroids pass through the 60% sucrose cushion, and ultrastructural observations of these fractions show that they are mainly free of membrane envelopes. It appears that there are at least three populations of bacteroids in root nodules that can be identified on the basis of electron-transparent granules (probably β -hydroxybutyrate), and they may represent a gradient in transformation of bacteroids. Due to the high density of Fraction D (Fig. 4), other endomembranes do not sediment with this fraction, and therefore it serves as a clean starting material for membrane envelope preparation.

Isolation of the Membrane Envelope

The fraction (Fraction D, Fig. 4) containing bacteroids enclosed in membrane envelope (Fig. 5 *a*) was processed as in Fig. 4 and passed through a no. 26 gauge hypodermic needle several times to break membrane envelopes. Recentrifugation of this material in a second step-gradient yields a fraction (Fraction E, Fig. 4) that sediments at the 34/45% interface, a characteristic density of plasma membrane (27). The electron micrograph of these preparations (Fig. 5 *b*) shows membrane envelopes that appear to be free of contamination with rough ER, Golgi apparatus, and mitochondria. Control plasma membrane preparations from uninfected soybean roots also sediment at this density (see also references 22 and 27). The major visible difference between the control plasma membrane and the membrane enclosing the bacteroids is the capacity to form vesicles. Control plasma membrane forms vesicles that are smaller than those from membrane envelopes.

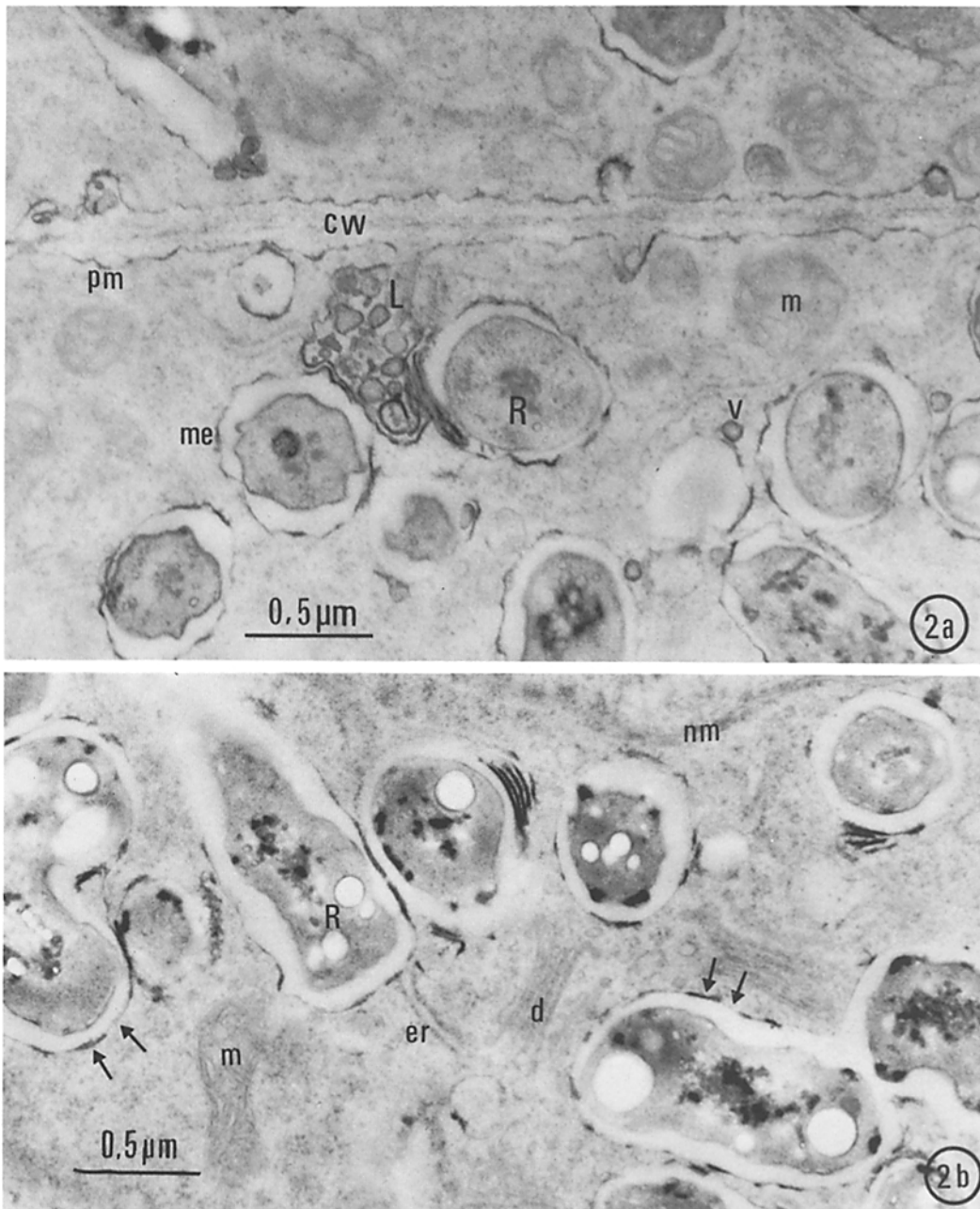


FIGURE 2 PTA-chromic acid stain of root nodule membranes. (a) young nodule (2 wk after infection), note the similarity of stain between the plasma membrane and the membrane envelope enclosing the bacteroid. Lomasomes, and other PTA-positive vesicles apparently fused with the membrane envelope. (b) Mature nodule, note the unevenness of staining in the membrane envelope (arrows) and the lack of staining of other endomembranes, ER, Golgi apparatus, etc. *cw*, cell wall; *R*, *Rhizobium*; *v*, vacuole; *L*, lomasomes; *m*, mitochondria; *d*, dictyosomes; *me*, membrane envelope; *pm*, plasma membrane; *nm*, nuclear membrane; and *er*, endoplasmic reticulum.

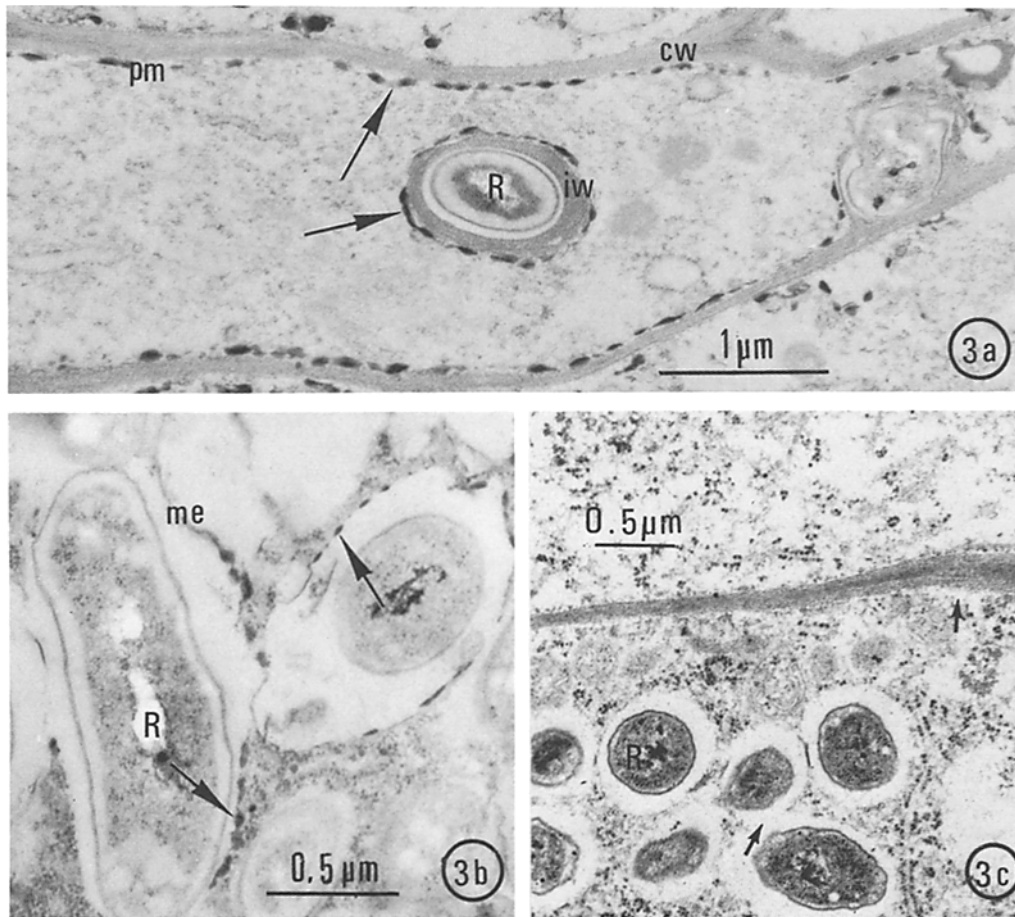


FIGURE 3 Cytochemical staining of root nodule membranes. (a) ATPase localization in young nodules. Note the similarity of staining between plasma membrane and the membrane envelope enclosing the infection thread (arrows indicate reaction product); (b) ATPase localization on the membrane envelope enclosing the bacteroids in mature nodules (arrows indicate reaction product); (c) *in situ* solubilization of the plasma membrane and membrane envelope enclosing the bacteroids by a nonionic detergent NP-40. Note the disappearance of both membranes after treatment (arrows). *R*, *Rhizobium*; *pm*, plasma membrane; *iw*, infection thread wall; *me*, membrane envelope; and *cw*, cell wall.

Characterization of Membrane Fractions

PTA-chromic acid staining of the membrane envelope fraction (Fraction E, Fig. 4) shows (Fig. 6) that this fraction is PTA-positive. However, similar to the *in vivo* staining (Fig. 2*b*), isolated membrane envelope does not stain uniformly. Analysis of the PTA-positive area in these preparations with the help of an image analyzer gives a value of 34% which obviously represents the lowest limit, since about half of the membrane does not stain with PTA either *in vivo* or *in vitro* (cf. Fig. 2*b* and Fig. 6). Treatment of this fraction with nonionic detergent (NP-40) solubilizes most

of the membrane proteins, suggesting that it is not contaminated by bacterial membranes that appear to be resistant to this treatment (our unpublished data and see also Fig. 3*c*). Activities of cytochrome *c* oxidase (a mitochondrial marker enzyme) and NADPH- and NADH-cytochrome *c* reductase (ER [33] and tonoplast [28] marker enzymes, respectively) in the final membrane envelope preparation (Fraction E, Fig. 4) are very low (Table II) as compared to the starting material (Fraction A, Fig. 4) for membrane envelope preparations, showing the lack of contamination of other plant endomembranes in the purified membrane envelope preparation.

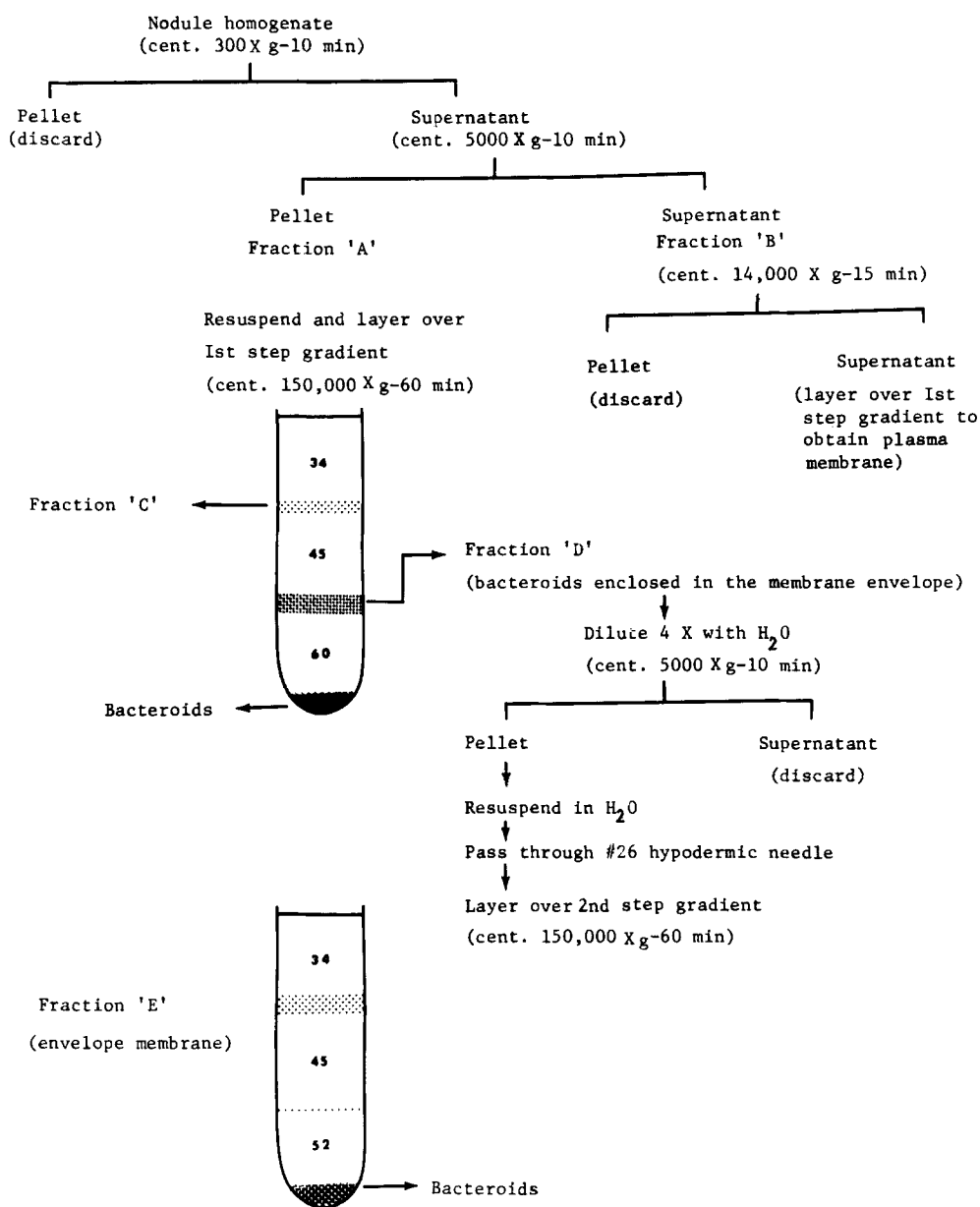


FIGURE 4 Flow diagram for the isolation of membrane envelope enclosing the bacteroids. (See Table III for identification of various fractions.)

Although there is no well-characterized marker for plant plasma membranes, we followed the activity of Mg^{++} -dependent, K^+ -stimulated ATPase activity at pH 6.0, which appears to be specific for plasma membrane (27). Table II shows that the specific activity of this enzyme increased in the final membrane envelope fraction. Based upon the recovery of this enzyme, the total yield of

membrane envelope is about 20%; however, recovery may actually be higher since Fraction A may contain other ATPases similar to the crude fraction, resulting in a higher activity. Due to the high density of Fraction D, the contamination by other endomembranes is drastically reduced in the purified membrane envelope preparation (Table II).

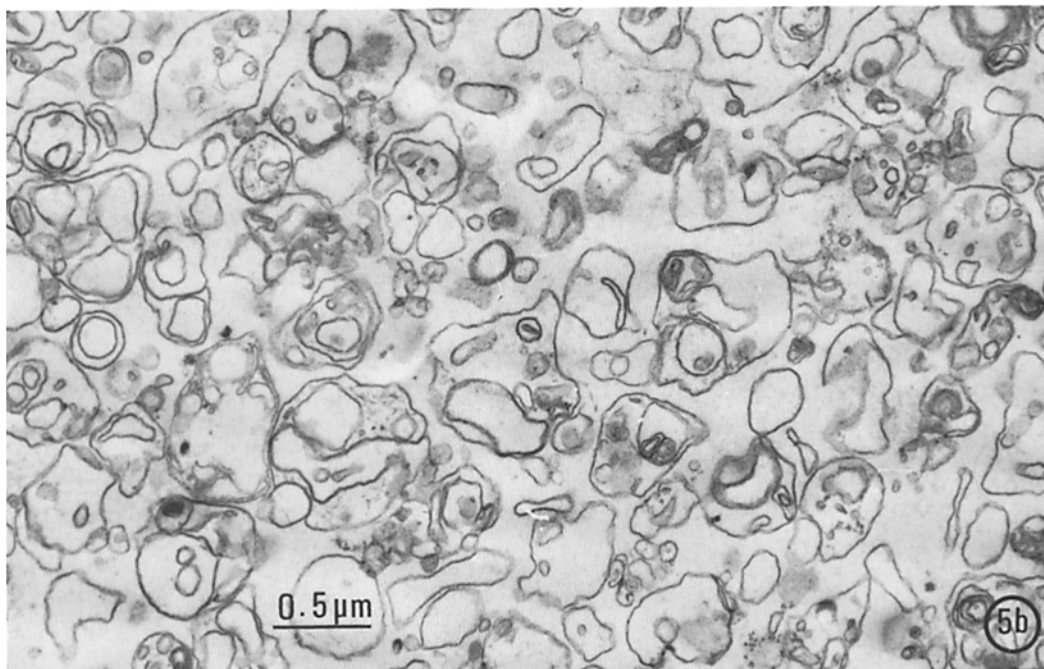
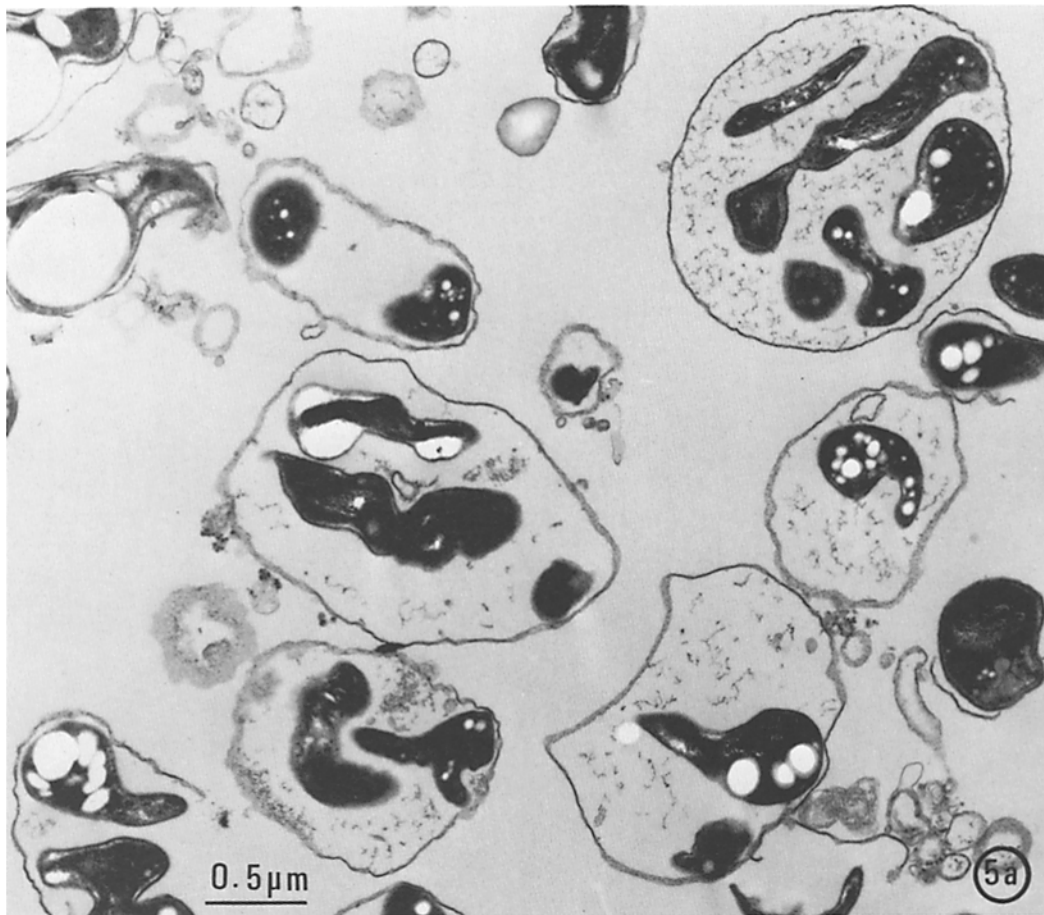


FIGURE 5 (a) Electron micrograph of Fraction D (Fig. 4) (i.e., bacteroids enclosed in the membrane envelope). (b) Electron micrographs of membranes prepared from Fraction D (i.e., membrane envelope, Fraction E [Fig. 4]).

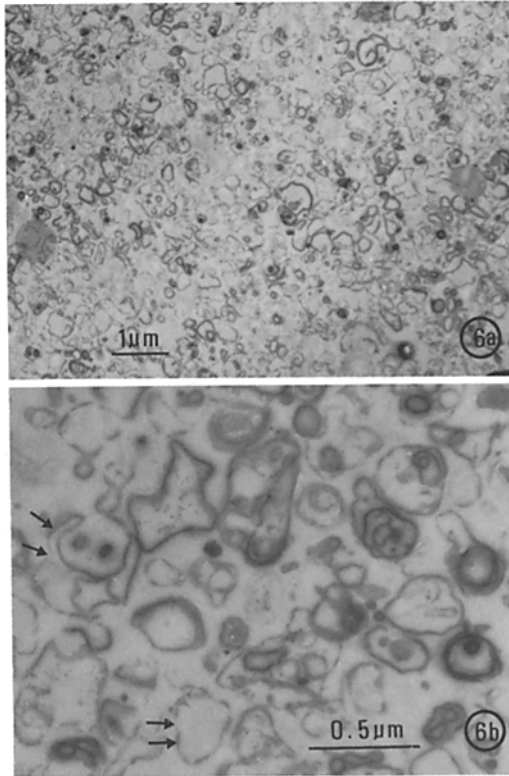


FIGURE 6 (a) PTA-chromic acid staining of Fraction E (Fig. 4), i.e., membrane envelope enclosing bacteroids (see also Fig. 5b); (b) higher magnification of a. Note the unevenness of staining within one membrane vesicle (arrows) (cf. Fig. 2b).

We also investigated the possibility of contamination of membrane envelope (Fraction E, Fig. 4) by bacteroid membrane. Fraction A (Fig. 4) was isolated and incubated in the presence of ^{14}C -amino acids. This fraction was then mixed with fresh nodules, and membrane envelopes were prepared as outlined in Fig. 4. Data in Table III shows that there is no significant contamination by bacteroid membrane of the membrane envelope fraction (Fraction E, Fig. 4). Since only 30% of the total radioactivity was in bacteroid membranes, the maximum contamination of Fraction E would be 0.03% (Table III).

Electrophoretic analysis of the membrane proteins in SDS-gels is shown in Fig. 7. Several proteins are common to those of control plasma membrane; however, some distinct peptides can be observed in the membrane envelope fraction. Common protein bands indicate that the integrity of the original membrane is maintained. These

data also show the purity of the membrane, i.e., they are free of cytoplasmic contamination because leghemoglobin, which represents 30% of the total cytoplasmic protein (40), is completely absent in the membrane fraction prepared from Fraction D or Fraction B (Fig. 4). Similar analyses of the membrane preparation from isolated bacteroids indicate (data not shown) that there is no common band similar to membrane envelope or plasma membrane proteins.

Biosynthesis of the Membrane Envelope

Incorporation of radioactive amino acids *in vivo* into the membrane proteins in the presence of eucaryotic and procaryotic protein synthesis inhibitors (Table IV) indicates that the majority of the membrane proteins are synthesized in the host cell cytoplasm. Preliminary SDS-gel electrophoretic analysis of the membrane proteins synthesized in the presence of cycloheximide shows that all of the bands in the membrane envelope fraction are reduced by this treatment (D. P. S. Verma and V. Zogbi, unpublished data). *In vitro* labeling of Fraction D followed by isolation of membrane envelope (Fraction E) does not label the membrane envelope proteins. This is consistent with the idea that the host cell controls the proliferation of the membrane envelope (14, 25). Most of this membrane appears to be synthesized during early stages of root nodule development. In mature nodules, bacterial proliferation ceases (20).

Immunological Studies on the Site of Leghemoglobin

We have shown (44) that leghemoglobin is synthesized by the plant and is localized in the host cell cytoplasm (40). Since ferritin-conjugated antibody may not have penetrated the intact membrane envelopes (40), we observed its distribution in the sections where membrane envelopes were broken. Fig. 8 shows that the ferritin-antibody conjugates are localized on and around the outer surface (facing cell cytoplasm) of the envelope, and that when this envelope is broken (see arrow) the ferritin appears to be attached on the outer surface of the newly formed membrane envelope vesicles. No ferritin was found inside the membrane envelopes or on the surface facing the bacteroids (cf. references 6, 12, and 19). Controls with ferritin-conjugated albumin or ferritin-conjugated nonimmune serum gave no reaction throughout the host cell. To see whether leg-

TABLE II
Cytochrome *c* Oxidase/Reductase and ATPase Activities of Various Membrane Fractions

Fractions (Fig. 4)	Cytochrome <i>c</i> reductase		Cytochrome <i>c</i> oxidase		ATPase*	
	NADH	NADPH	Total activity	Sp act	Total activity	Sp act
	% activity		$\mu\text{mol/min/g}$ tissue	$\mu\text{mol/min/mg}$ protein	$\text{cpm} \times 10^6/\text{g}$ tissue	$\text{cpm}/\mu\text{g}$ pro- tein
Crude	100	100	4.28	0.26	—‡	—‡
Fraction A (5,000 g pellet)	6.4	3.3	1.04	0.15	16.6	1,101
Fraction D First-45/60% interface	0.6	0.2	0.49	0.14	6.3	3,010
Fraction E Second-34/45% interface	0.02	0.01	0.002	0.09	3.4	3,440

Membrane fractions were prepared as in Fig. 4 and assayed for various enzymes as described in Materials and Methods.

* Fractions were treated with 0.2% NP-40 to solubilize membranes and centrifuged at 10,000 *g* to remove bacteria. Protein was determined with the Bio-Rad reagent, using ovalbumin as standard in 0.2% NP-40.

‡ Crude extract contains several types of ATPase activities that interfere with the determination of plasma membrane-specific ATPase (pH 6.0), and thus these values are misleading in calculating fold purification or recovery of the final membrane preparation, Fraction E. Based upon the values in fraction A, which served as a crude starting material for purification of membrane envelope, a 20% yield is obtained; however, this value is low since Fraction A is also contaminated with other ATPase activities that sediment at 34–45% sucrose interface on the first gradient (Fig. 4).

TABLE III
Contamination of the Membrane Envelope Preparation by Bacteroid Membrane

Purification steps (Fig. 4)	Fraction no.	Total radioactivity recovered	Characterization
		%	
5,000 g Pellet	Fraction A	99.6	Bacteroids with membranes
14,000 g Pellet	—	—	Mitochondria, plastids, some membranes
14,000 g Supernate	Fraction B	0.13	
First step gradient			
34/45% Interface	Fraction C	0.01	Broken membrane envelope, ER and plasma membrane vesicles
45/60% Interface	Fraction D	80.5*	Bacteroids enclosed in the membrane envelope
60% Pellet	—	16.7	Bacteroids without membrane envelope
Second step gradient			
34/45% Interface	Fraction E	0.01	Membrane envelope
45/52% Interface	—	0.02	—
52% Pellet	—	99.9	Bacteroids

Fraction A was prepared (Fig. 4) from 1 g (fresh weight) of nodules, resuspended in 2 ml of buffer, and was incubated with ^{14}C -amino acid mixture for 1 h at 25°C. After incubation, it was layered over a cushion (2 ml) of 45% sucrose (wt/vol) and centrifuged at 23,000 *g* for 10 min. This sucrose-washed Fraction A containing 5×10^8 cpm (out of which 30% was in bacteroid membranes) was mixed with fresh nodules, and membranes were prepared as in Fig. 4. Radioactivity in each fraction was determined after precipitation with hot-TCA.

* This value was considered 100% for second step gradient.

hemoglobin is tightly bound to the surface of the membrane envelope or located inside between the bacteroid and membrane envelope, the isolated fractions (Fraction D and Fraction E, Fig. 4) were reacted with Lb-antibody after solubilizing with NP-40. Fig. 9 shows that the leghemoglobin is not present inside the membrane envelopes, nor is it an integral part of the membrane envelope (see

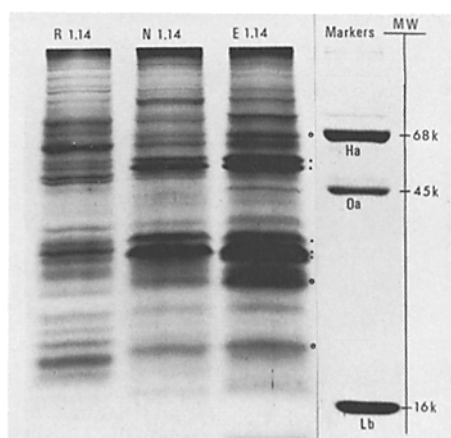


FIGURE 7 SDS-gel electrophoretic analysis of various membrane fractions. R 1.14, plasma membrane from control roots at a density of 1.14 g/cm³; N 1.14, nodule membranes (from supernate, Fig. 4); E 1.14, membrane envelope Fraction E of Fig. 4. Marker proteins; *Ha*, human serum albumin, mol wt (MW) = 68,600; *Oa*, ovalbumin, mol wt = 45,000; and *Lb*, leghemoglobin, mol wt = 16,000.

TABLE IV
Effect of the Inhibitors of Protein Synthesis on the Biosynthesis of Membrane Protein

Treatment	Incorporation of radioactive amino acids into membrane envelope	
	cpm/g nodule	Inhibition %
Exp 1		
Control	1,989	—
Cycloheximide (100 µg/ml)	605	70
Exp 2		
Control	36,650	—
Cycloheximide (200 µg/ml)	4,405	88
Streptomycin* (400 µg/ml)	38,500	—

About 3-wk-old nodules (1 g fresh weight) were labeled with (Exp 1) L-[U-¹⁴C]leucine for 1 h at 25°C or (Exp 2) [³⁵S]methionine for 2 h at 25°C (see Materials and Methods). Membrane envelopes enclosing the bacteroids were isolated as described in Fig. 4, Fraction E. Hot TCA-insoluble radioactivity was measured.

* Streptomycin at this concentration inhibits 70% of the protein synthesis in isolated bacteroids.

also Fig. 7). The only positive reaction was obtained with the cytoplasmic fraction using two different antibody preparations. These results confirm earlier findings (40) that leghemoglobin is localized in the host cell cytoplasm and does not

penetrate the membrane envelope enclosing the bacteroids.

DISCUSSION

Origin and Biosynthesis of the Membrane Envelope

The infection thread, which develops from a tubelike invagination of the host cell wall (31), retains the cell wall material and the plasma membrane of the host cell (Fig. 1). It is not known whether this cell wall is synthesized *de novo* as the infection thread grows or whether it represents the extended, old cell wall. With the removal of this cell wall material, the bacteroids may be considered "released" (18, 30) in the host cell although they are still enclosed in the host plasma membrane, the membrane envelope. The cell wall material appears to be dissolved enzymatically, e.g., by cellulolytic activity which increases two- to threefold and is localized specifically in the cell wall region of the infection thread (Fig. 1c). During development of infection, another cell wall hydrolysing enzyme, e.g., pectinase, is produced by *Rhizobium*, and thus development of infection may be a cooperative action of both host and *Rhizobium* (45).

It is known that bacteroids secrete certain hormones such as indole-acetic acid during the infection process (16, 17) and, although there is no direct evidence, it is likely that cellulase may be induced as a result of the action of these hormone(s) (43). This enzyme is synthesized in the host cell cytoplasm and may be secreted through the plasma membrane enclosing the infection thread (cf. reference 4). Free-living *Rhizobium* and isolated bacteroids have very little cellulase activity (Table I and reference 29).

Although continuity of the plasma membrane around the initial infection thread had been observed (13, 30, 37, 38), it is not certain whether the same membrane continues to proliferate during subsequent development of the membrane envelope or if other endomembranes (9-11, 23, 34) are involved in this process. The thickness of both membranes is identical (mean thickness 84 Å). It retains PTA stainability (a characteristic marker for plasma membrane, reference 36) and stains like the host plasma membrane. Both membranes have ATPase activity with a similar localization (Fig. 3a and b). Tu (38) has observed a similar pattern of localization of adenyl cyclase, and since this activity is not detectable in soybean

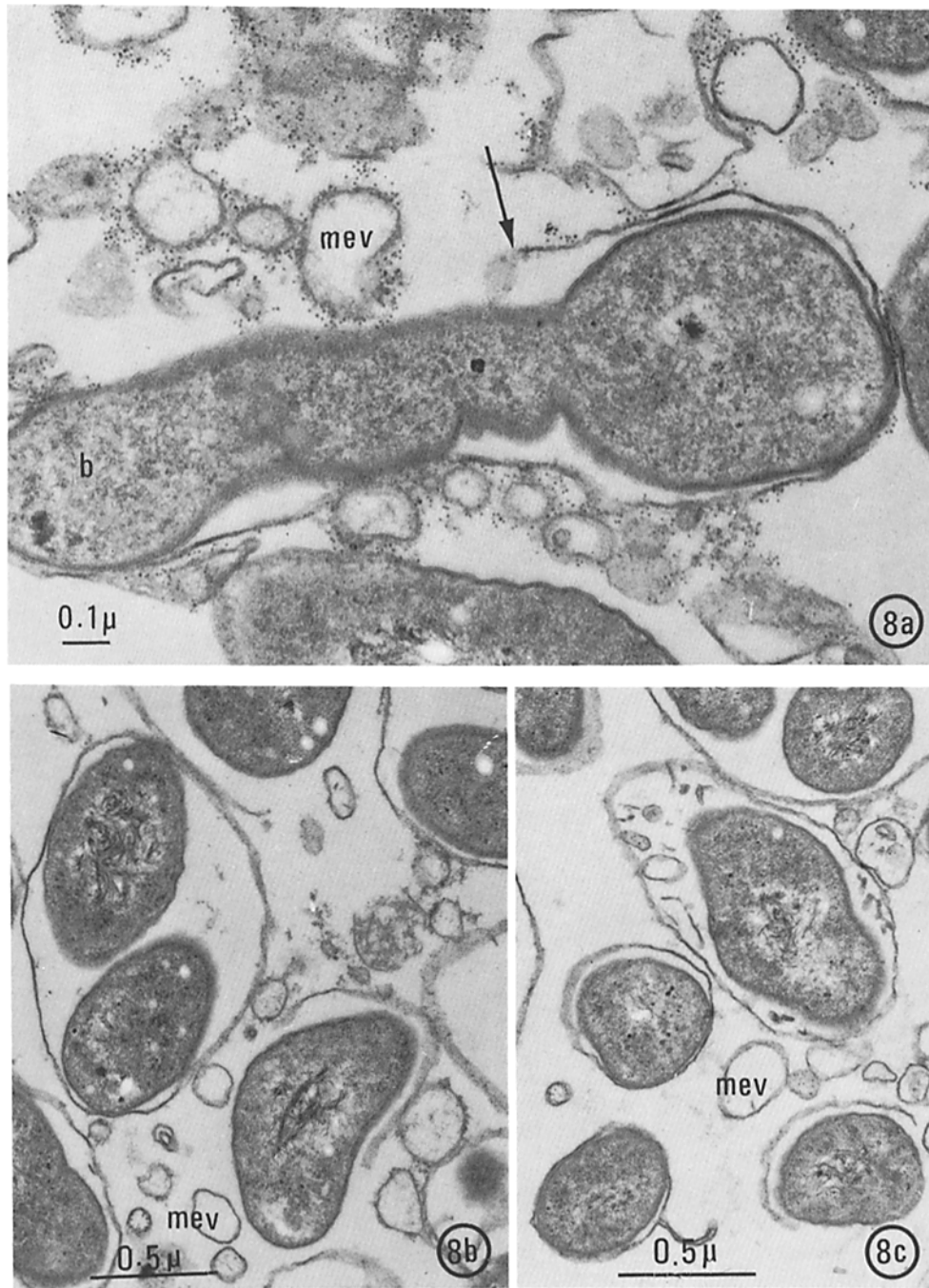


FIGURE 8 (a) Localization of leghemoglobin by ferritin antibody-conjugates. Note the presence of ferritin on the outer side (facing the cell cytoplasm) of the membrane envelope enclosing the bacteroids and its close association with membrane envelope vesicles (*mev*) which are formed due to breaking of the envelope (arrow). (b and c) controls treated with ferritin-conjugated albumin and ferritin-conjugated nonimmune serum, respectively. *b*, Bacteroid; *mev*, membrane envelope vesicles formed during preparation of samples.

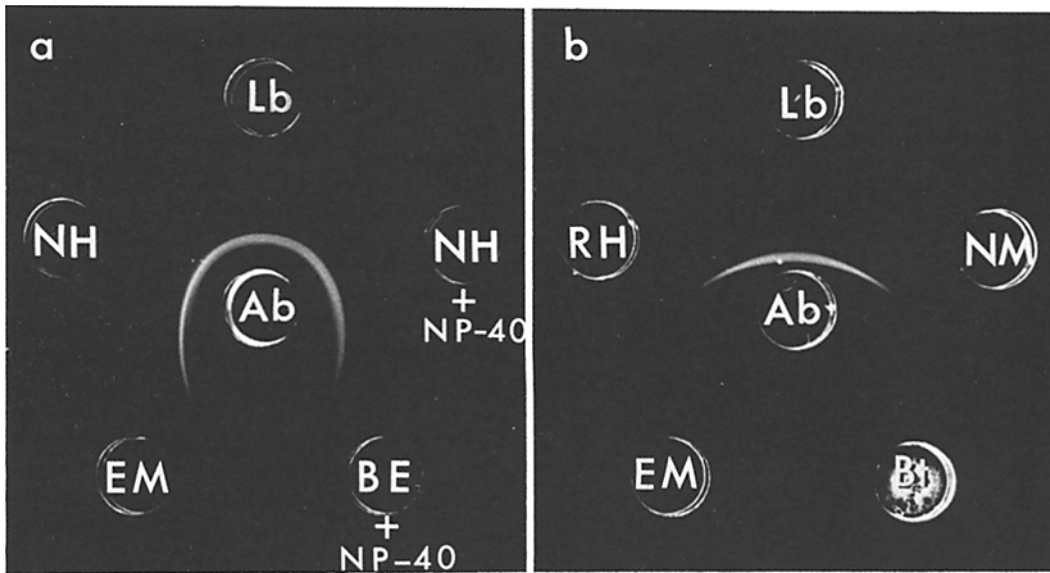


FIGURE 9 Ouchterlony diffusion plate. (a) *Ab*, antibody against leghemoglobin (*Lb*) in rabbit; *NH*, nodule homogenate (postmitochondrial supernate); *Lb*, pure leghemoglobin; *NH + NP-40*, *NH* treated with 0.2% Nonidet P-40; *EM*, membrane envelope (Fraction E of Fig. 4); *BE + NP-40*, Fraction D (i.e., bacteroid enclosed in the membrane envelope and treated with Nonidet P-40). (b) *Ab*, antibody against *Lb* in horse; *RH*, control root homogenate; *NM*, other nodule membranes (Fraction C, Fig. 4); and *Bt*, sonicated bacteroids.

root tissue (49), and since the presence of cAMP in higher plants is questionable (2), it is likely that the activity observed by Tu may be due to ATPase (cf. Fig. 3*a* and *b*). These membranes are also sensitive to nonionic detergent (NP-40) both in vitro and in vivo, while bacterial membranes are not. Similarity of these membranes was further borne out by the recent study of Tu (39) showing similar freeze-fracture face particle decoration on these membranes. Analysis of membrane envelope proteins showed a close similarity with the plasma membrane of uninfected cells. Biochemical data (Table IV) suggest that the membrane envelope proteins are synthesized in the host cell cytoplasm. These results, along with earlier observations (5, 14, 15, 35, 38, 39), confirm that the membrane envelope enclosing the bacteroids originates from plasma membrane of the host and show that most of the known characteristics of the plasma membrane are retained. An extracellular compartment is formed for *Rhizobium* to develop an "intracellular symbiosis."

With proliferation of bacteroids, there is a strong demand for membrane envelope synthesis. The final number of bacteroids enclosed per membrane envelope appears to be controlled by the

host, since the same strain, e.g. *Rhizobium lupini* (strain D25), produces an infection on *Lupinus luteus* L., yielding only one bacteroid per envelope, while infection on *Ornithopus sativus* Brot. yields several bacteroids (25). On the basis of the surface area of the membrane envelopes (6), we have calculated that in soybean there is 20- to 40-fold more membrane contributed by this envelope as compared to the normal cell plasma membrane. In *Lupinus*, there is 20 times more membrane in the infected cells as compared to the cortical cells (35). Although derived from plasma membrane, this membrane may undergo some changes, first, due to the structural demand to enclose a foreign organism, and second, due to its specific function(s), such as transport to and from bacteroids. The osmolarity of the infected cells is very high (about $\approx 2,000$ mosM).

The procedure developed for isolation of this membrane envelope (Fig. 4) provided a fraction (Fraction D) containing bacteroids enclosed in the membrane envelope which, due to its high density, is free from other endomembranes (Fig. 5). Isolation of this "functional compartment" would allow the studies on transport of metabolites to and from bacteroids. This fraction served as a

starting material for a membrane envelope preparation which is substantially free from other endomembranes. The possibility of contamination by bacteroidal membrane is ruled out by the data in Table III.

Leghemoglobin and the Membrane Envelope

We have shown (40) that leghemoglobin is synthesized in the host cell cytoplasm and is present outside the membrane envelope. In sections where the membrane envelope is broken during specimen preparation before antibody treatment, ferritin-conjugated leghemoglobin-antibody appears to be closely associated with these membrane envelope vesicles. Data in Fig. 9 show that leghemoglobin is not bound to the membrane and that no immunoreactive material is present inside these membrane envelopes. Controls in which ferritin-conjugated nonimmune serum or albumin is used do not show any nonspecific adsorption of ferritin. If leghemoglobin were present inside the membrane envelope as suggested by previous workers (6, 12, 19), it would have been retained within the envelopes of paraformaldehyde- and glutaraldehyde-fixed tissue. In isolated envelopes, traces of immunoreactive material would have been present even if the bulk of the leghemoglobin had leaked out during sample preparation. In fixed tissue, 60–65% of the leghemoglobin is retained (our unpublished data).

Recent data (41) on leghemoglobin biosynthesis show that this molecule is not synthesized as a large precursor, suggesting that this protein does not cross the membrane since most secretory proteins appear to be synthesized as precursors. This, along with previous results (40, 44), reinforces the conclusion that leghemoglobin is a plant cytoplasmic protein. The proposed function of leghemoglobin (48) does not require that it be in contact with bacteroids (C. A. Appleby, personal communication).

Possible Role of the Membrane Envelope in Symbiosis

In various infection processes (7), the host tends to develop a membrane structure that forms a "barrier" between the invading pathogen and the host cell cytoplasm. However, in these cases the cell wall tends to dissolve before the pathogen can have any influence on the host (1). In the case of *Rhizobium*, infection occurs through a process

whereby both the host cell wall and the plasma membrane are invaginated together. Development of the infection may be a cooperative action by both host and *Rhizobium*, which produce cell wall hydrolysing enzymes (45). Dissolution of the cell wall takes place at a later stage through the action of cellulolytic enzymes that appear to be synthesized by the plant (Table I). Removal of the cell wall material brings *Rhizobium* in close proximity to the host plasma membrane, and symbiosis is established. It is not known when the nitrogen fixation activity begins in relation to these events, or what determines the dissolution of the wall of infection threads. Although up to 80% of the cell cytoplasm may be occupied by bacteroids enclosed in membrane envelopes, the thin portions of host cytoplasm retained between these vesicles appear very dense and highly organized (42). No destruction of the cytoplasmic matrix is evident in cells packed with bacteria. The membrane envelope may help in maintaining this intracellular organization.

In an effective symbiosis, the bacteroids are never free in the cell cytoplasm (11, 13), and thus from a physiological point of view the bacteroids are still extracellular (13, 14). With the dissolution or nondevelopment of this membrane structure, as in the case of senescing cells (26), noneffective nodules or asymbiotic associations, the bacteria may become parasitic/saprophytic on the host cell cytoplasm (29, 42).

Development of this membrane system also provides a compartment in the cell where two processes, oxidative phosphorylation and N_2 -fixation, which differ in their pO_2 requirement can occur simultaneously with high efficiency. Leghemoglobin, which is localized on the outside of the membrane envelope (Fig. 8, and reference 40), while facilitating the diffusion of oxygen in the host cell, may prevent its diffusion to bacteroids at a higher rate where oxygen-sensitive nitrogenase is present. Our evidence shows that no leghemoglobin is present between the bacteroids and membrane envelope, in contrast to earlier observations (cf. references 6, 12, and 19). An understanding of the number and function of specific plant genes (46), which are required in the development of effective symbiosis, is essential for progress to be made in the successful transfer of nitrogen-fixation genes to other plants that do not possess this capacity.

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Note Added in Proof: A similar procedure has recently been developed for isolation of the membrane envelope (peribacteroid membrane) enclosing the bacteroids from Lupin root nodules (Robertson, J. G. et al., 1978, *J. Cell Sci.* **30**:151-174).

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