Rhizobial Root Nodules of Soybean as Revealed by Scanning and Transmission Electron Microscopy

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ABSTRACT

Gross-structural and fine-structural organization of the soybean root nodule (incited by *Rhizobium japonicum*) were correlated by the application of scanning (SEM) and transmission electron microscopy (TEM). The root nodule was comprised of cortical tissue and central tissue. The latter consisted of bacteroidal cells (Rhizobium-infected) and interstitial cells (Rhizobium-free). The bacteroidal cells were arbitrarily categorized into three infection stages: early, intermediate, and advanced. In the early stage, each bacteroid was enclosed in a membrane envelope; in the intermediate stage, several bacteroids were enclosed in a membrane envelope; and in the advanced stage, the bacteroids lacked a membrane envelope and the host cellular membrane system had deteriorated. All three infection stages of bacteroidal cells were present simultaneously in all root nodules observed. It is suggested that, in the advanced stage, the nitrogen-fixing ability might be lacking. Since the number of the bacteroidal cells in the advanced stage increased with the root nodule size, the efficiency of a root nodule in nitrogen fixation should not be judged on the basis of nodule size alone. Rather, efficiency should be evaluated on the basis of the total surface area of envelope membranes.

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Rhizobial infection in the root cortical tissue cells leads to the formation of nodules on roots of leguminous plants. The rhizobia in the nodules, although generally referred to as symbionts, are parasites in the strict sense because they acquire their nutrient from the host cells. Moreover, the host tissue responds to the interaction and proliferates to form a gall-like structure. However, unlike other cases that result in the production of galls and tumor-like structures, the *Rhizobium*-legume interaction produces beneficial effects on the host. Thus, the parasitism occurs in the absence of disease (8). Although disease is not incited by the rhizobial infection, the physiology of the host cells undoubtedly changes due to the host-parasite interaction, and the degree of physiological alteration of the host cells may differ during various stages of infection.

Since physiological alterations are generally associated with changes in fine- and/or gross-structural organization, an understanding of the fundamental processes of nodule development and structural organization in various stages of rhizobial infection should help to further the understanding of host-parasite relationships involving other organisms and their hosts.

Root nodules of soybean have been studied by transmission electron microscopy (TEM) (2, 6, 13), but those studies have dealt only with the bacterial component of the host cells. In addition, such studies are limited to a rather small area of tissue, often consisting of only a few cells. Thus, it is difficult to correlate the organization of the limited number of cells seen with TEM with the overall organization of root nodules. Attempts to correlate the observations made by TEM with those made by light microscopy have met with only limited success, because the light microscope has limited resolution. Therefore, the relationship between fine-structural and gross-structural organization of soybean root nodule with respect to its development is not yet fully understood.

Scanning electron microscopy (SEM) can be used to shed light on this problem. It can provide a three dimensional surface view of a structure. In contrast to TEM, however, structure within the organelles is difficult to visualize. Thus, the combination of TEM and SEM offers an excellent means of studying structural organization.

The present investigation is intended to further the understanding of the host-parasite relationship between soybean and *Rhizobium japonicum* by using SEM and TEM.

MATERIALS AND METHODS.—Soybean [*Glycine max* (L.) Merr. 'Bansei'] and *Rhizobium japonicum* (Kirchner) Buchanan were used throughout this investigation. *R. japonicum* was obtained in fresh root nodules from H. Tachibana, USDA, Department of Botany and Plant Pathology, Iowa State University, Ames, Iowa.

Seeds of soybean were sown in a steamed soil mixture consisting of sand, peat and loam (1:1:2). Two seeds were sown in each 15 cm diameter pot. In order to induce nodulation, fresh root nodules were crushed and minced with water and then added to the seeded pots. These pots were kept at 21 ± 1 C in a growth chamber programmed to 14 hours of light and 10 hours of darkness with a fluorescent light intensity of 26,900 lx (2,500 ft-c) at bench level.

Root nodules were harvested 2.5 months after sowing. The size of nodules varied from approximately 0.5 to 4.5 mm in diameter. Three different sizes of nodules were selected, i.e., approximately 0.5 mm, 1.0 mm, and 2.0 mm. Each size group contained at least 15 root nodules. Each group was then divided into three subgroups, with five nodules in each subgroup. These three subgroups were subjected to different schemes of fixation for SEM and TEM as described below.

Preparation for SEM.—Nodules were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0 for 4 hours, cut into quarters through the long axis, washed for 30 minutes with three changes of the same buffer, postfixed with buffered 2% osmium tetroxide for 4 hours, and dehydrated in a graded ethanol series, then in a graded ethanol-amyl acetate series, and finally in pure amyl acetate. The specimens were then dried in a critical-point dryer according to the procedures described by Boyde and Wood (3).

After drying, the specimens were mounted on aluminum studs with conductive glue, placed on the rotary plate in an Edwards Vacuum Evaporator, and coated with carbon and gold. During the coating, the rotary plate was rotated to achieve a uniform coating. The coated specimens were examined in a Cambridge stereoscan S4 scanning electron microscope.

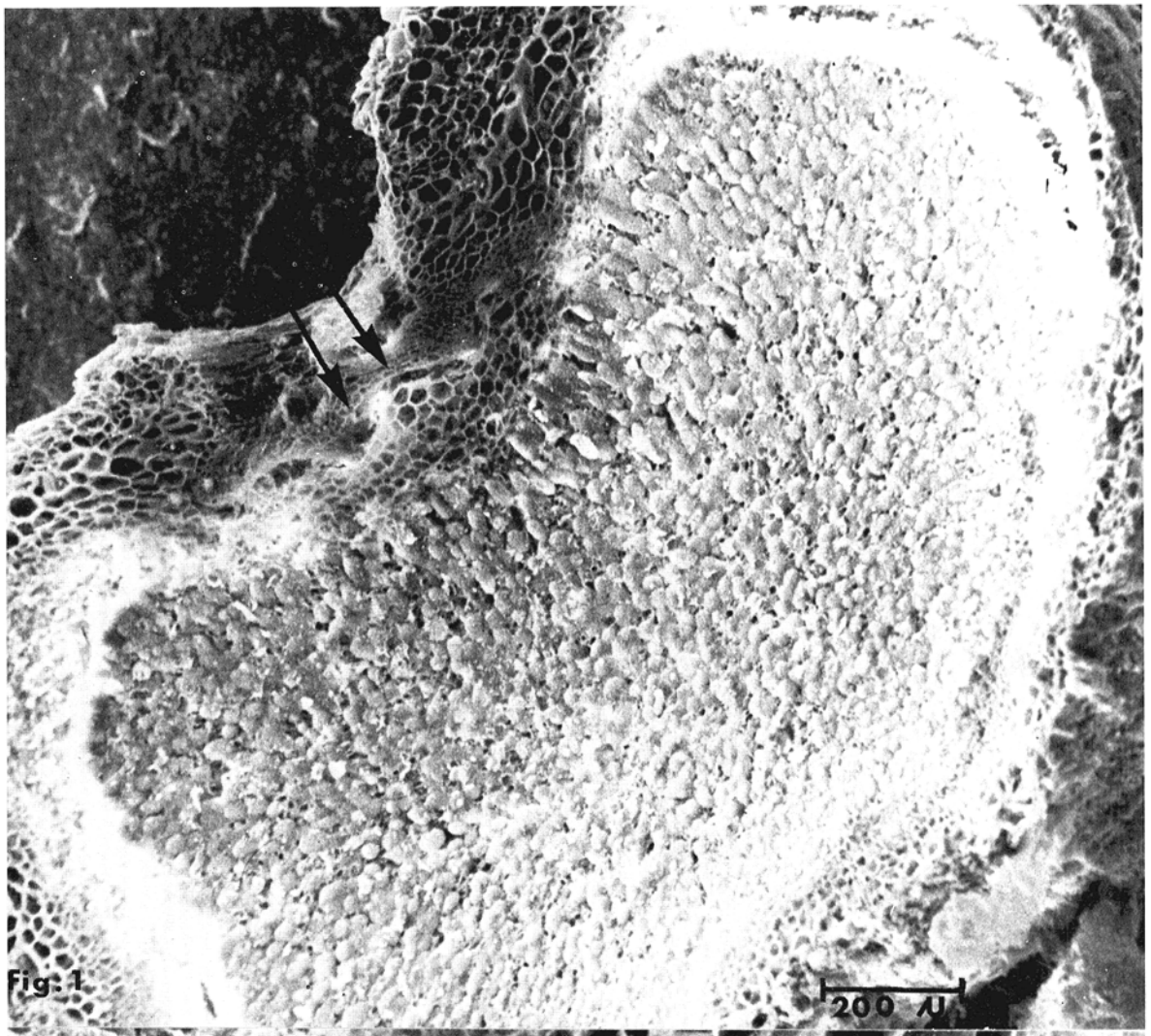
Preparation for TEM.—Nodules were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0. The fixed materials were processed and embedded using the method described by Tu (13). Thin sections were double stained with uranyl acetate and lead citrate and examined in a Philips EM300 electron microscope.

RESULTS.—*Gross structure of root nodule.*—In order to investigate the overall structure with respect to the size of the root nodule, nodules of various sizes, 0.5-2.0 mm in diameter, were studied in SEM at low power. The observations outlined below indicate that each size group showed different developmental features which were consistent with their stage of infection.

All root nodules of soybean scanned consisted of two major types of tissues; i.e., cortex and central tissue (Fig. 1, 2-A). The thickness of the cortical tissue was rather constant, irrespective of the nodule size, whereas the proportion of central tissue increased with nodule size.

At higher magnification in SEM, the cortical tissue was seen to consist of an outer and an inner layer of cells. The outer layer was several cells in thickness, and its constituent cells generally lacked cellular contents (Fig. 2-C, 4). The outermost cell zone, which came in contact with the soil, was collapsed (Fig. 2-D, 3). The inner layer was also several cell layers in thickness, and consisted of typical parenchymatous cells containing proplastids of various sizes (Fig. 2-B, 5). The innermost layer of cortical cells was capable of cell division. It extended almost completely around the nodule central tissue, but was not present at the neck, where nodule and root were connected (Fig. 1, arrows). Rhizobia were not found in the cortical tissue cells (Fig. 1, 2).

The area inside the cortical tissue was a large mass of central tissue cells (Fig. 1, 6) comprised of bacteroidal cells and interstitial cells in about a 3:1 ratio. The former,



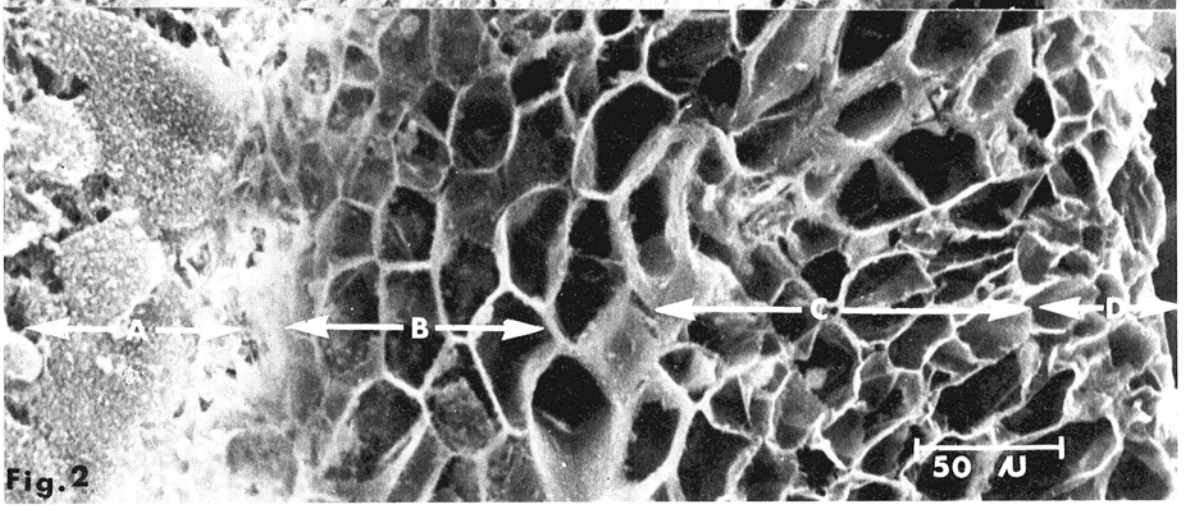


Fig. 1-2. 1) A low magnification scanning electron micrograph of a soybean root nodule cut longitudinally through its long axis showing the overall structure of the root nodule. 2) A portion of soybean root nodule scanned from inside to the surface of the nodule, showing (A) central tissue cells, (B) cortex tissue cells, (C) empty cortex tissue cells, and (D) collapsed empty cortex tissue cells which make up the outermost surface layer of the nodule.

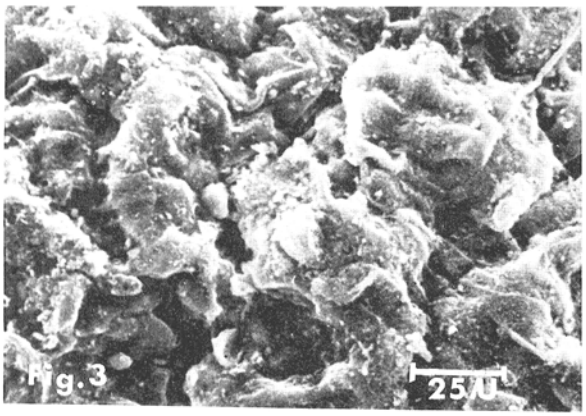
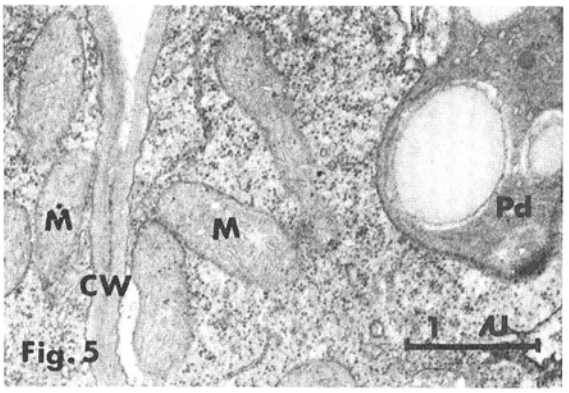


Fig. 3



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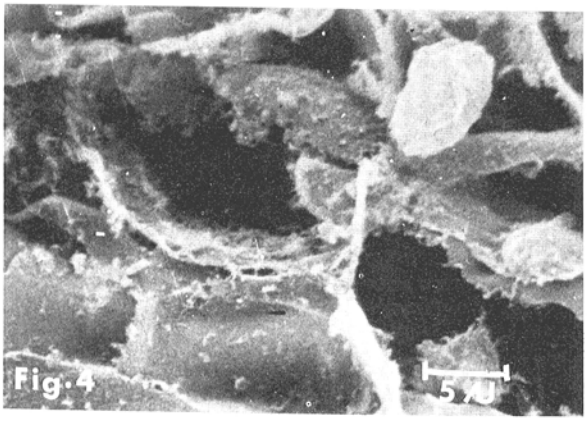

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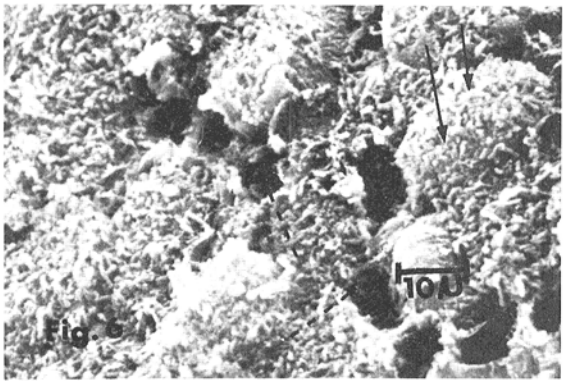

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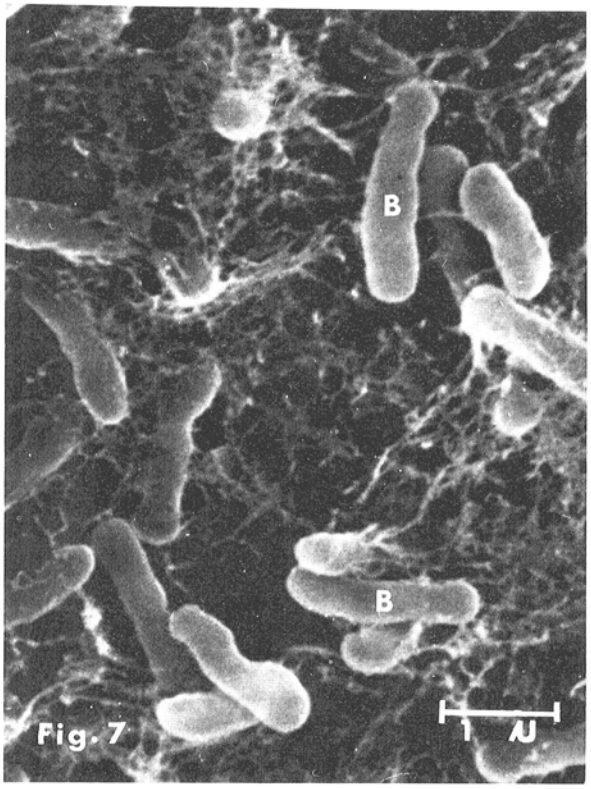

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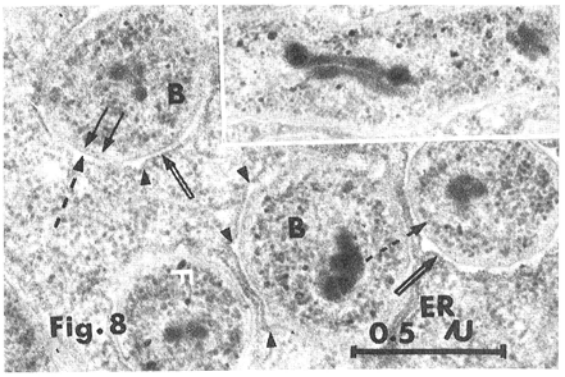

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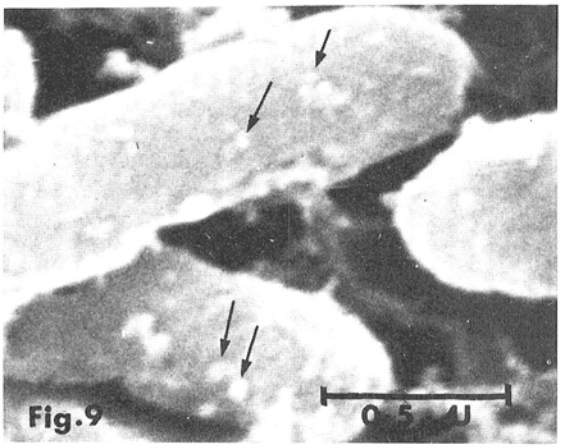


Fig. 9

containing bacteroids, were three-to-four times bigger than the latter (Fig. 6, arrows). The latter were somewhat vacuolated, bacteroid-free parenchymatous cells (Fig. 6, broken arrows). Ventilation appeared to be served by the large intercellular spaces (Fig. 12, 17).

Bacteroidal cells.—The Rhizobium-infected cells (bacteroidal cells) followed through a sequence of development. They were arbitrarily categorized into early, intermediate and advanced stages for the sake of convenience. These three stages were defined as follows: at the early stage, each rhizobial bacteroid was enclosed in a membrane envelope; at the intermediate stage, two to several bacteroids were enclosed in a membrane envelope; and at the advanced stage, the bacteroids were not enclosed in a membrane envelope because at this stage the envelopes had become disorganized. All three developmental stages were observed to be present in all root nodules irrespective of nodule size.

Bacteroidal cells at the early stage of infection formed a layer approximately three-to-four cells in thickness on the inner side of the cortical tissue cells. In these cells, each bacteroid was enclosed in a membrane envelope believed to originate from the host plasma membrane (5, 7, 11). The membrane envelope showed projections in the cross-sectional view in thin sections (Fig. 8, arrowheads). These are also seen as projections in the scanning micrographs (Fig. 9, arrows). At this stage, the host cells were rich in cellular contents (Fig. 7, 8), and the number of rhizobia in each cell was relatively low, compared to the intermediate and advanced stages of infection.

Bacteroidal cells at the intermediate stage of infection were located toward the center of the nodule immediately next to the early stage cells, and constituted about 70% of the central tissue cells. The other 30% was equally shared by the cells at the early stage and the advanced stage. At the intermediate stage, each membrane envelope normally contained several bacteroids. This was evident both in the sectioning (Fig. 10, 15) and in scanning micrographs (Fig. 11, 14). This phenomenon could suggest that the host cells apparently ceased their cell division and reduced their cellular activity while the bacteroids continued to divide. At this stage, numerous membrane envelopes containing bacteroids occupied most of the cellular space, therefore the cellular organelles such as mitochondria, endoplasmic reticula, etc. were localized in the intermembrane spaces between the membrane envelopes (Fig. 10, 13, arrows). Rough endoplasmic reticulum was present, and sometimes was intimately associated with the membrane envelope of bacteroids (Fig. 15). The rhizobial infection threads believed to be transient in nature (7) were repeatedly observed in the bacteroidal cells of the intermediate stage (Fig. 14, 15).

Bacteroidal cells at the advanced stage of infection were situated in the core of the root nodule. At this stage, the degeneration of host cellular organization was evidenced by the dissolution of the plasma membrane and other cellular membranes of the host cell and the membrane envelopes of bacteroids (Fig. 16, 17). Cellular organelles generally degenerated and were hardly identifiable.

The interstitial cells at this stage had a large central vacuole and a thin layer of cytoplasm, except for some fully deteriorated cells which were observed only after the complete degeneration of the bacteroidal cells.

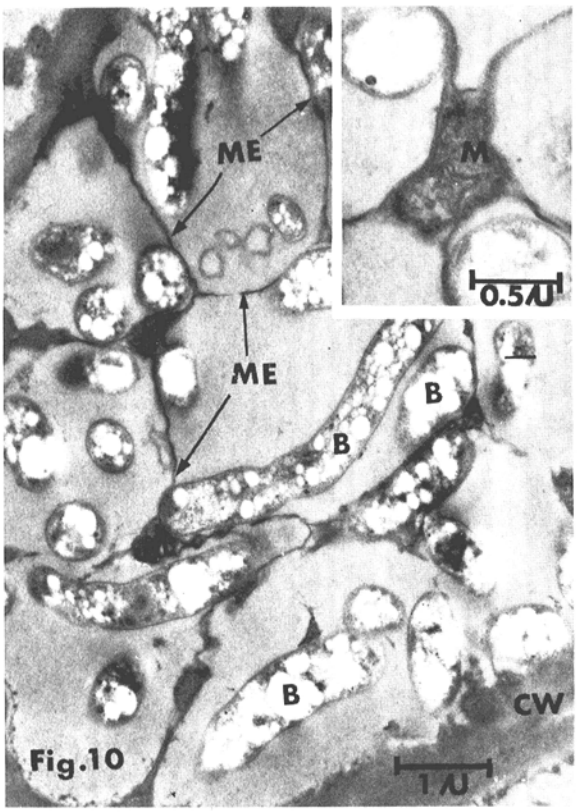
Bacteroids.—The scanning electron micrographs showed that the bacteroids were rod-shaped with a rather uniform size, regardless of the stage of rhizobial infection (Fig. 7, 14, 16). However, in thin sections, the bacteroids were seen to have various sizes and shapes (Fig. 14, 15). Therefore, it became obvious that the size and shape variation in sections represented the different orientation of bacteroids relative to the cutting plane (Fig. 10, 12, 15). Consequently, the misinterpretation arrived from TEM was eliminated with the help of SEM.

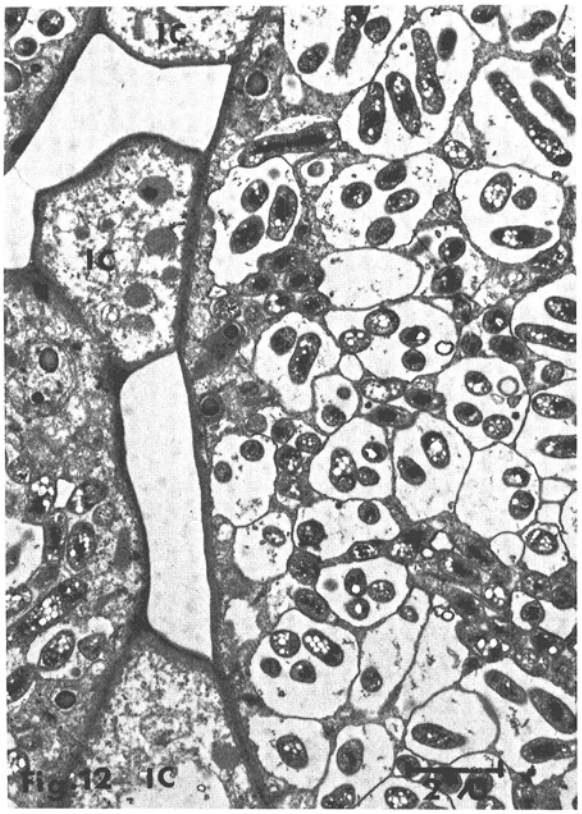
The bacteroids in cells at the early stage of infection showed little vesiculation, numerous ribosomes, and thick strands of nuclear materials in the longitudinal view [Fig. 8 (inset)]. In cross-sectional view, however, they were seen as several rounded spots (Fig. 8). There were a few electron-dense β-hydroxybutyric acid particles (5, 6, 7).

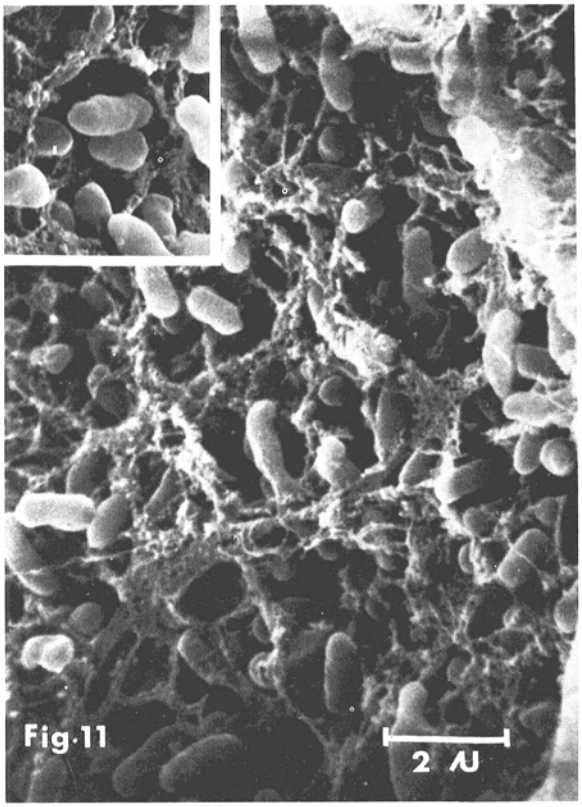
At the intermediate stage, the bacteroids contained numerous electron-transparent vesicles of various sizes (Fig. 10). The nuclear materials remained in the center of the bacteroids, and were more fibrous (Fig. 13). The bacteroids at the advanced stage remained the same as in the intermediate stage of infection (Fig. 16, 17).

DISCUSSION.—From the gross-structural organization, the results have clearly demonstrated that the degeneration of bacteroidal cells in soybean root nodule starts from the center of a nodule. This observation is in agreement with that of Mosse on clover root nodule (11). However, the pattern of degeneration of the two is different. This is attributable to the difference in rhizobia and host species, because development of root nodule varies with host and rhizobia species (1, 10, 12). For example, a clover root nodule is club-shaped, with its growing region situated at the tip of the nodule. Bac-

Fig. 3-9. 3) A close up view of the surface of a root nodule. **4)** A micrograph showing the empty cortical cells of a root nodule. **5)** A thin section of glutaraldehyde-fixed cortical cells. (Their scanning view is in Fig. 2-B). They are parenchymatous cells containing proplastids. M = mitochondrion, CW = cell wall, V = vacuole, Pd = proplastids. **6)** A portion of central tissue consisting of bacteroidal cells (arrows) filled with rhizobia and rhizobia-free interstitial cells (broken arrows). **7)** A bacteroidal cell in the early stage of rhizobial infection. A portion of a cell contains only a few bacteroids (B) while most of the remaining space is occupied by cellular organelles. **8)** A portion of a bacteroidal cell in the early stage of rhizobial infection showing cross-sectional view of bacteroids (B). The inset is a longitudinal section of a bacteroid. The bacteroid has a plasma membrane (arrows), cell wall (broken arrows), and membrane envelope (hollow arrows). Each bacteroid is embedded in the cytoplasm with numerous ribosomes and endoplasmic reticula (ER). Note: The membrane envelope has projections (arrowheads). Glutaraldehyde-fixed preparation. **9)** A high magnification scanning electron micrograph of a bacteroidal cell in the early stage of rhizobial infection showing particles of the membrane envelope of the bacteroids (arrows). The size of the particles closely approximates those of the projections seen in Fig. 8 (arrow).







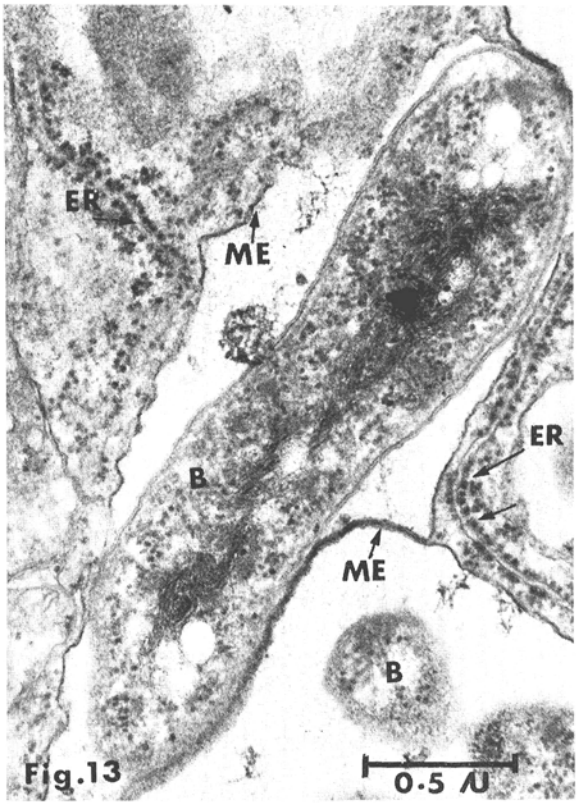
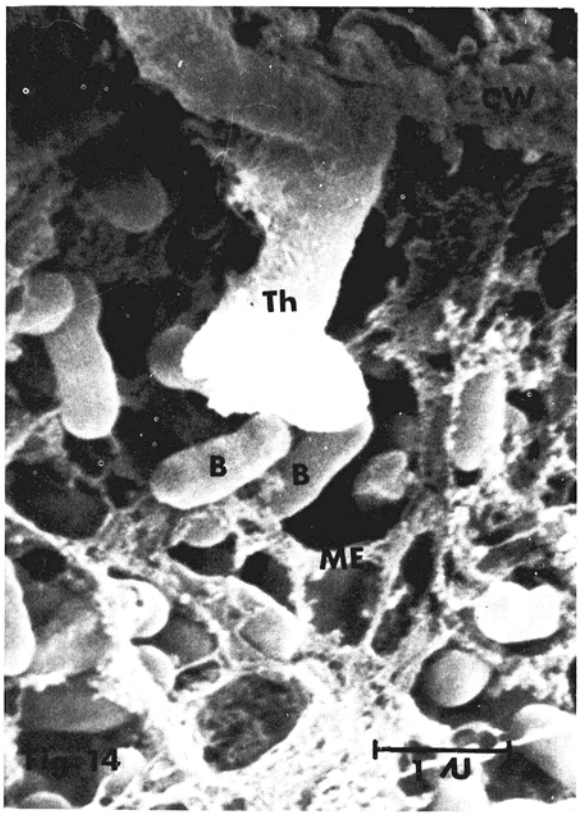
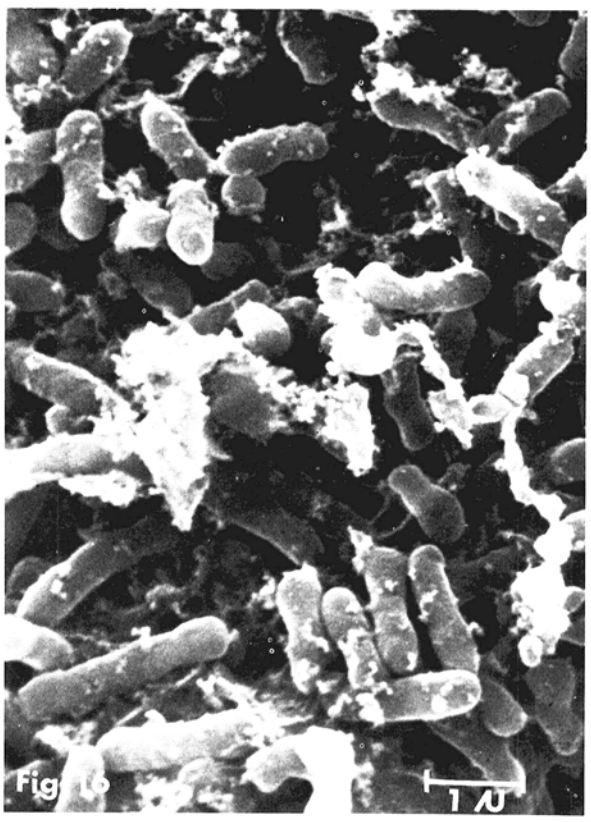
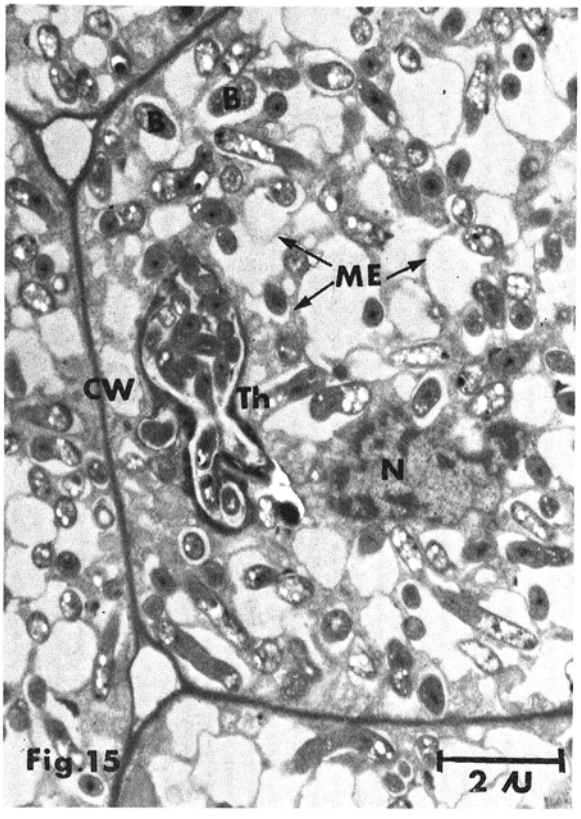


Fig. 10-13. 10) A portion of a thin section of glutaraldehyde-fixed central tissue showing a bacteroidal cell in the intermediate stage of rhizobial infection in which several bacteroids (B) are enclosed in a membrane envelope (ME). Note: mitochondria (M) are randomly distributed among the inter-membrane spaces of membrane envelopes (arrows). Also see inset for higher magnification. CW = cell wall. **11)** A scanning view of a portion of a bacteroidal cell in the intermediate stage of rhizobial infection showing several bacteroids in a membrane envelope. Inset shows a clear-cut membrane envelope containing five bacteroids. **12)** Portions of bacteroidal cells in the intermediate stage and portions of interstitial cells (IC). Note: interstitial cells are parenchymatous and not empty cells. **13)** A high magnification micrograph of part of a bacteroidal cell in the intermediate stage showing the intimate association of endoplasmic reticula (ER) with the membrane envelopes (ME). B = bacteroid.







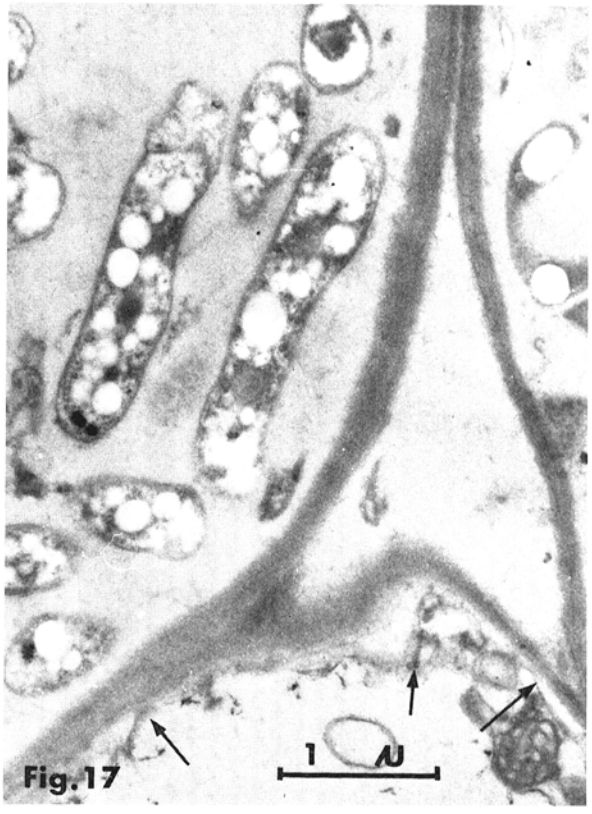


Fig. 14-17. 14) A scanning micrograph showing a residual infection thread (Th) found in the bacteroidal cell at the intermediate stage. CW = cell wall, ME = membrane envelope, B = bacteroid. **15)** A comparable view of Fig. 12 as seen in thin sectioned preparation. Th = infection thread, B = bacteroid, N = nucleus, CW = cell wall, ME = membrane envelope. **16)** A scanning electron micrograph showing a portion of a bacteroidal cell in the advanced stage of rhizobial infection, and in which the membrane envelopes of bacteroids have deteriorated. **17)** Part of a bacteroidal cell in the advanced stage of rhizobial infection. Note: the plasma membrane and the membrane envelopes of bacteroids are lacking in the bacteroidal cells, while the plasma membranes (arrows) of the interstitial cells remain intact.

teroidal tissue of different infection stages (i.e., early, intermediate and advanced) is arranged in segments, with the advanced stage closest to the neck of the nodule (11). On the other hand, a soybean nodule is rounded, with its growing region laid around the nodule except at the neck area. Bacteroidal tissue of different infection stages is arranged in concentric zones with the advanced stage at the center of the nodule.

From the advent of the rhizobial infection to the intermediate stage, the host cells and the bacteroids maintain an orderly organization of cellular membranes. The membrane envelopes of the bacteroids are unmistakably present in the early and intermediate stages of bacteroidal cells as observed in SEM and TEM (Fig. 7-8, 10-15). This observation is in agreement with Dart and Mercer (5), who suggested that the membrane envelope might be invariably present around *Rhizobium* in a nitrogen-fixing nodule. Apparently, in order to allow exchange of materials between the host cytoplasm and the bacteroids, the host and parasite must adequately maintain their cellular organization and cellular membrane system. Therefore, during the early and intermediate stages, the rhizobia and the host cells share a somewhat balanced host-parasite relationship, for which the rhizobia withdraw their nutrients from the host cells and fix nitrogen in return. A balanced host parasite relationship is commonly observed in the early stages of plant diseases induced by obligate parasites. For example, it may exist during the early stages of powdery mildew infection (4).

At the advanced stage of infection, the host cellular membranes and the membrane envelopes of the bacteroids deteriorate completely, leaving rhizobia in a ghost cell (Fig. 16, 17). The lack of a membrane envelope indicates a lack of symbiotic nitrogen-fixing ability. Disorganization and deterioration of host cellular membranes are known to occur in an infected cell before its death (9). Thus, the complete degeneration of the host cellular organization indicates that the symbiotic relationship between the rhizobium and the host cell may no longer exist.

A larger nodule is usually older, and so it contains a relatively high proportion of bacteroidal cells in the advanced parasitic stage. Therefore, the efficiency of nitrogen fixation in a root nodule should probably not be judged from the nodule size alone. Rather, it should be judged on the basis of either (i) total surface area of membrane envelopes per bacteroidal cell, or (ii) ratio of surface area between membrane envelope and bacteroids for efficiency per rhizobial bacteroid.

Interstitial cells were easily differentiated from bacteroidal cells because they had no bacteroids. Since interstitial cells are not empty cells, they are most unlikely to serve as a ventilation system for the root nodules, as previously claimed (7). On the other hand, the central tissue cells are a mass of loosely organized cells having numerous large intercellular spaces among them. Therefore, the ventilation of the cells may be provided mainly by the intercellular spaces. The interstitial cells, however, may serve in diffusion and translocation of materials to and from the bacteroidal cells.

The infection threads of soybean rhizobia (*R. japonicum*), which have been described as transient in nature (7), were repeatedly observed in the bacteroidal cells in the intermediate stage with SEM (Fig. 14) and TEM (Fig. 15). This suggests that the remains of these infection threads probably persist in situ after completion of infection. Thus, the fate of infection threads of soybean rhizobia (*R. japonicum*) is comparable to those of *R. trifolii* (11). It may be noted that the infection threads in the soybean root nodule were relatively few in number compared to those in root nodule of Alsike clover induced by *R. trifolii* (J. C. Tu, *unpublished*).

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