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Electron Microscopy of the Infection and Subsequent Development of Soybean Nodule Cells

D. J. GOODCHILD AND F. J. BERGERSEN

Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization, Canberra, Australia

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ABSTRACT

GOODCHILD, D. J. (Commonwealth Scientific and Industrial Research Organization, Canberra, Australia), AND F. J. BERGERSEN. Electron microscopy of the infection and subsequent development of soybean nodule cells. J. Bacteriol. **92**:204–213. 1966—Electron microscopy of thin sections of the developing central tissue cells of young soybean root nodules has shown that infection is initiated by a few infection threads which penetrate cells of the young central tissue. Extension growth of the threads may be a result of pressure developed from the growth of the bacteria within the threads. Release of bacteria from a thread is preceded by the development on an infection thread of a bulge with a cellulose-free membrane-bounded extension; bacteria move from this into the host cells by an endocytotic process and remain enclosed in an infection vacuole which is bounded by a membrane of host-cell origin. Multiplication of the intracellular bacteria takes place within these vacuoles. Until the host cell becomes filled with bacteria, the vacuoles separate into discrete units at each division. Later, division of the bacteria occurs within each vacuole, thus leading to the mature structure of the central tissue cells in which several bacteria are enclosed within each membrane-bounded unit.

The fine structures of the central tissue cells of soybean root nodules were first described by Bergersen and Briggs (2), who showed that the bacteroid form of *Rhizobium japonicum* in these cells was enclosed in groups within an enclosing membrane of host-cell origin. The supposed derivation of these structures was described in diagrammatic form from electron micrographs which were not presented.

The infection of soybean nodule cells has now been re-examined by use of improved methods and has been shown to be similar to the endocytotic processes described by Holter (5), in which particles are first adsorbed to the outer surface of the boundary membrane of a cell and then become enclosed within the cell in a vacuole formed from an invagination of this membrane.

MATERIALS AND METHODS

Nodulated soybeans, var. Shelby, were grown from inoculated seed as previously described (1), and nodule tissue was sampled at intervals, timed from the first day on which nodules could be detected by eye on the root surface. Cubes of tissue of side no greater than 1 mm were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (*p*H 7.0) for 12 to 16 hr at 4 C. The tissue was then washed in buffer with four changes over a period of 2 hr and with 2.0% osmium tetroxide in 0.1 M phosphate buffer for 2 hr, and was again washed with buffer as above before being dehydrated through 25, 50, 70, and 100% acetone. The embedding polymer catalyst (DMP-30) was then added as a 0.5% solution in 100% acetone, and the tissue was left in it for 30 min before washing with two changes in 100% acetone. A 50% solution in acetone of the monomer (Epon) was then added for 2 hr, followed by Epon alone for 24 hr at 45 C. After this period of impregnation, the tissue was placed in gelatin capsules, and Epon containing 0.6% DMP-30 was added. Polymerization was continued for 3 days at 60 C.

Sections were cut with an LKB Ultrotome with glass knives and were stained with saturated uranyl acetate in 50% ethyl alcohol followed by Karnovsky's lead hydroxide stain (6). Sections were examined in a Siemens Elmiskop I electron microscope.

RESULTS

Infection threads. In soybean root nodules, infection threads are relatively rare. After initial penetration of central host-tissue cells and release of bacteria from the threads, further proliferation of the infection is accomplished by division of infected host cells. In the material examined in this work, infection threads were observed only in a limited number of sections from a few tissue blocks from nodules aged 1 to 2 days.

These blocks were repeatedly sectioned, and the information obtained is illustrated in Fig. 1–6. Several sections were obtained in which the passage of a thread could be followed right across a host cell. One of these is shown in Fig. 1. The chief features were the greater thickness of the thread cellulose than of the host cell wall, and the convoluted nature of the host membrane surrounding the thread. The bacteria within these young infection threads were tightly packed and showed evidence of distortion consistent with the existence of considerable end-to-end pressure as the bacteria grew in length and divided. The relationship between the tip of an infection thread and the host cell wall is clearly shown in Fig. 2, and in Fig. 3 a section through a part of a thread crossing a cell wall is shown. Both of these figures strongly suggest that the infection thread is thrusting through the cell wall rather than growing in a less forceful way through pre-existing cell wall pits. In all cases, the thread was clearly an invagination of the outer surface of the host cell, with the cellulose layers and the enclosing membranes of the thread being continuous with those of the wall.

Release of bacteria from the infection thread. Release of bacteria from the infection threads was observed to follow closely after the penetration of a host cell by a thread. The events were as follows. A lateral cellulose-enclosed bulge developed on the thread and became filled with bacteria. The bulge then extended, but cellulose was no longer deposited (Fig. 4). Bacteria became attached to the host membrane surrounding this bulge (Fig. 5), and the membrane folded around each bacterium as it moved into the cytoplasm of the host cell in a process which seemed to be identical with endocytosis (5), although taking place from within the cellulose-free portion of the infection thread (Fig. 5 and 6). The entire bacterial content of the cellulose-free bulge from the infection thread appeared to move into the host cell in this manner, leaving empty membrane sacs such as that shown in Fig. 4.

Development of the intracellular infection. These events led to groups of bacteria, each individual of which was surrounded by a membrane-enclosed vacuole, within the cells of the central tissue. These bacteria became distributed within the progeny of these cells as cell division proceeded, a process observed in light microscope observation of thicker sections (2). Further development of the infection within these cells took place by multiplication of this initial inoculum. Initially, the host membrane enveloping each bacterium divided with it (Fig. 7), resulting in the host cytoplasm becoming completely occupied by bacteria, each of which was enclosed in its own membrane envelope (Fig. 8). Further bacterial multiplication followed, but the membrane envelopes no longer divided. Instead, division of the bacteria within the envelope (Fig. 9 and 10) resulted in at first two bacteria and later several (four to eight) within each envelope, which is the chief characteristic of the mature structure of the cells of the central tissue of nitrogen-fixing soybean nodules (Fig. 11 and 12).

Other features. The cytoplasm of the host cells initially contained all the features of young plant cells. Ribosomes and endoplasmic reticulum were especially prominent (Fig. 1–8) until the final stages of development were reached, at which time the background cytoplasm became featureless (Fig. 11 and 12). Proplastids were frequently seen in sections (Fig. 3), and mitochondria were invariably present, becoming confined to the host cell perphery and being concentrated adjacent to intercellular spaces (Fig. 8) and empty interstitial cells (Fig. 12) which may act in ventilating the central tissue. The nucleus was a prominent feature of the young host cells (Fig. 3 and 4), and bacteria released from infection threads were frequently grouped adjacent to the nucleus; however, no ultrastructural connections such as proposed by Mosse (9) were seen.

Some changes in the bacteria occurred during these developments. There was a progressive increase in the amount of electron-translucent material, first at the poles and then more generally within each bacterium. This material is probably β-hydroxybutyric acid, which is present in bacteroids isolated from soybean nodules to the extent of up to 30% of the dry weight (Appleby, *personal communication*). A further change in the bacteria was a thickening of the filamentous material within the nuclear regions (compare Fig. 6, 8, and 10).

DISCUSSION

The transient presence of infection threads in soybean nodules contrasts with the situation in nodules of other species of legume in which their fine structure has been described, and in which the remains of infection threads may still be found after all the subsequent development of the intracellular infection has taken place. Although this led to difficulty in finding sections of soybean nodules with threads, it was an advantage in that it ensured that events were being observed and not structures residual from past events. A notable difference with soybean infection threads was the absence of densely staining matrix ma-

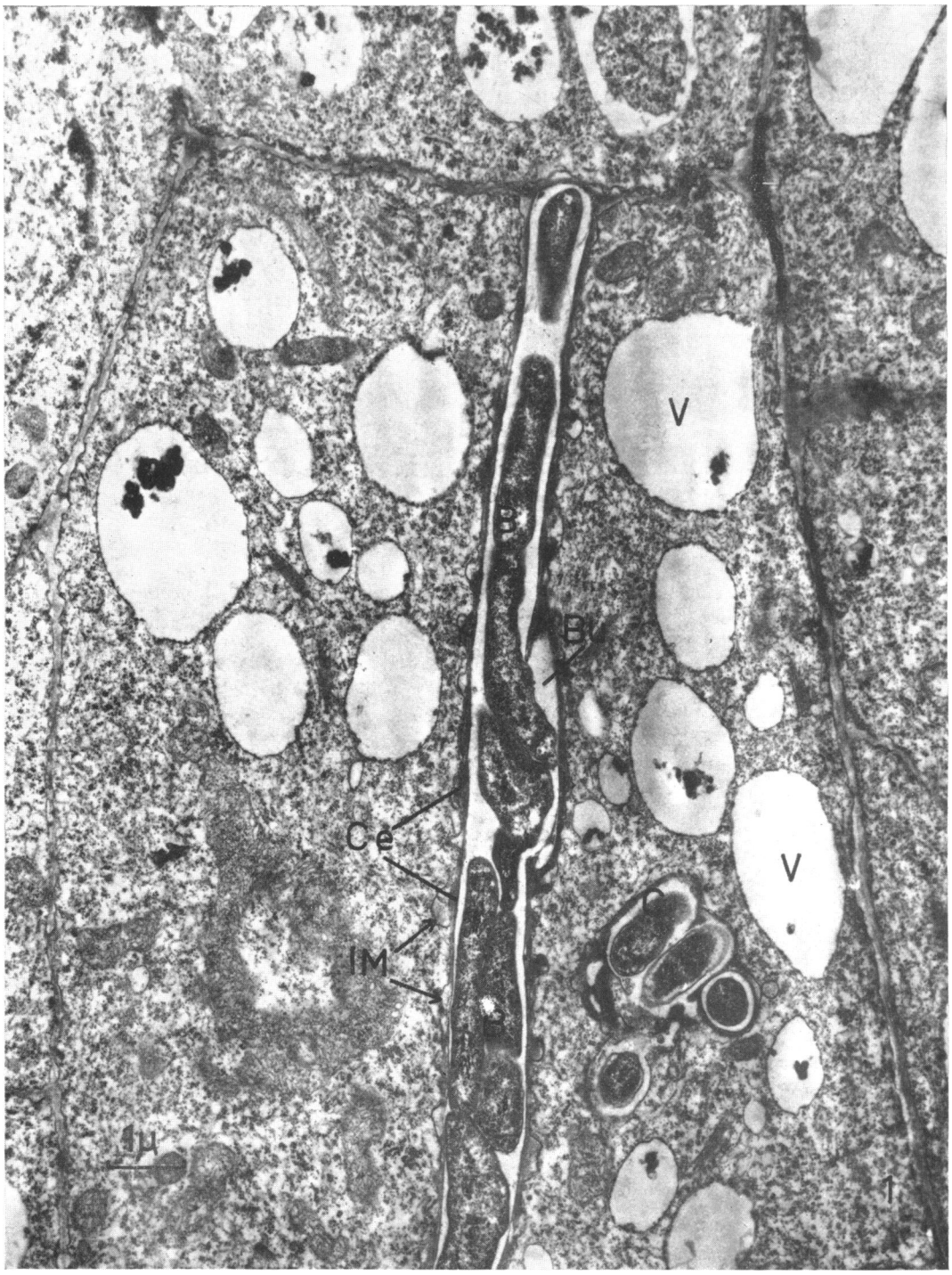

FIG. 1. *Infection thread crossing a central tissue cell of a nodule aged 1 to 2 days. The bulge (Bu) was seen in other sections of this cell to be connected in another plane to the group of bacteria (C) which is partially enclosed by cellulose and partially by a cellulose-free membrane. (IM) Infection thread membrane. (Ce) Infection thread cellulose. (V) Vacuole. (B) Bacterium.*

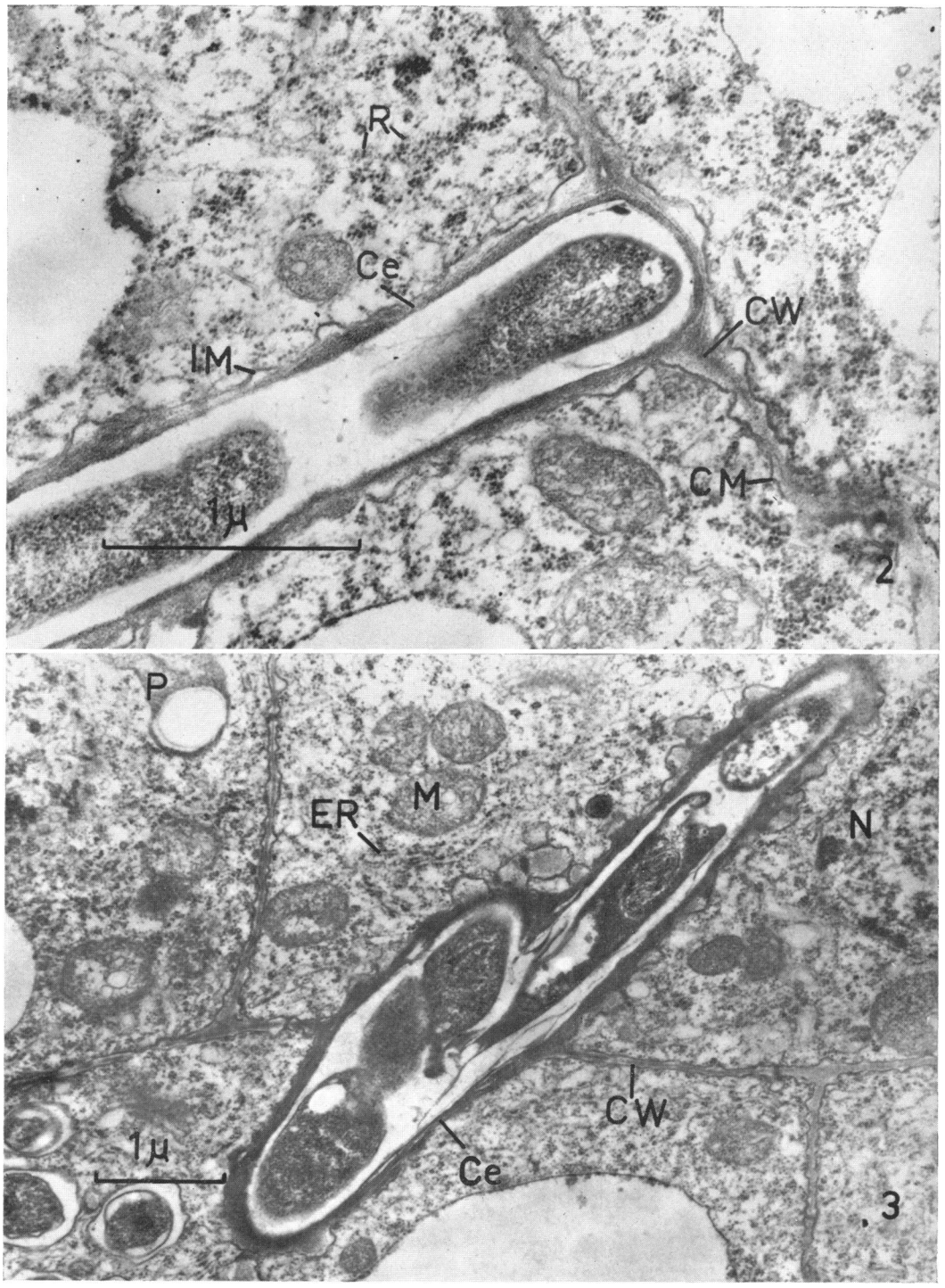

FIG. 2. *Higher magnification of the tip of the thread shown in Fig. 1. (Ce) Infection thread cellulose. (IM) Infection thread membrane. (CM) Host cell membrane. (CW) Host cell wall. (R) Ribosomes. The continuity of cell membrane and infection thread membrane is seen, and the relationship of the cellulose of the thread and the host cell wall suggest that the tip of the thread is thrusting into the neighboring cell.*

FIG. 3. *Section through a portion of an infection thread as it has crossed the boundary between two host cells of a nodule aged 1 to 2 days. The orientation of the cell walls suggests that the thread has forced a passage from top to bottom. (M) Mitochondria. (P) Proplastid. (N) Host nucleus. (CW) Cell wall. (Ce) Infection thread cellulose. (ER) Endoplasmic reticulum.*

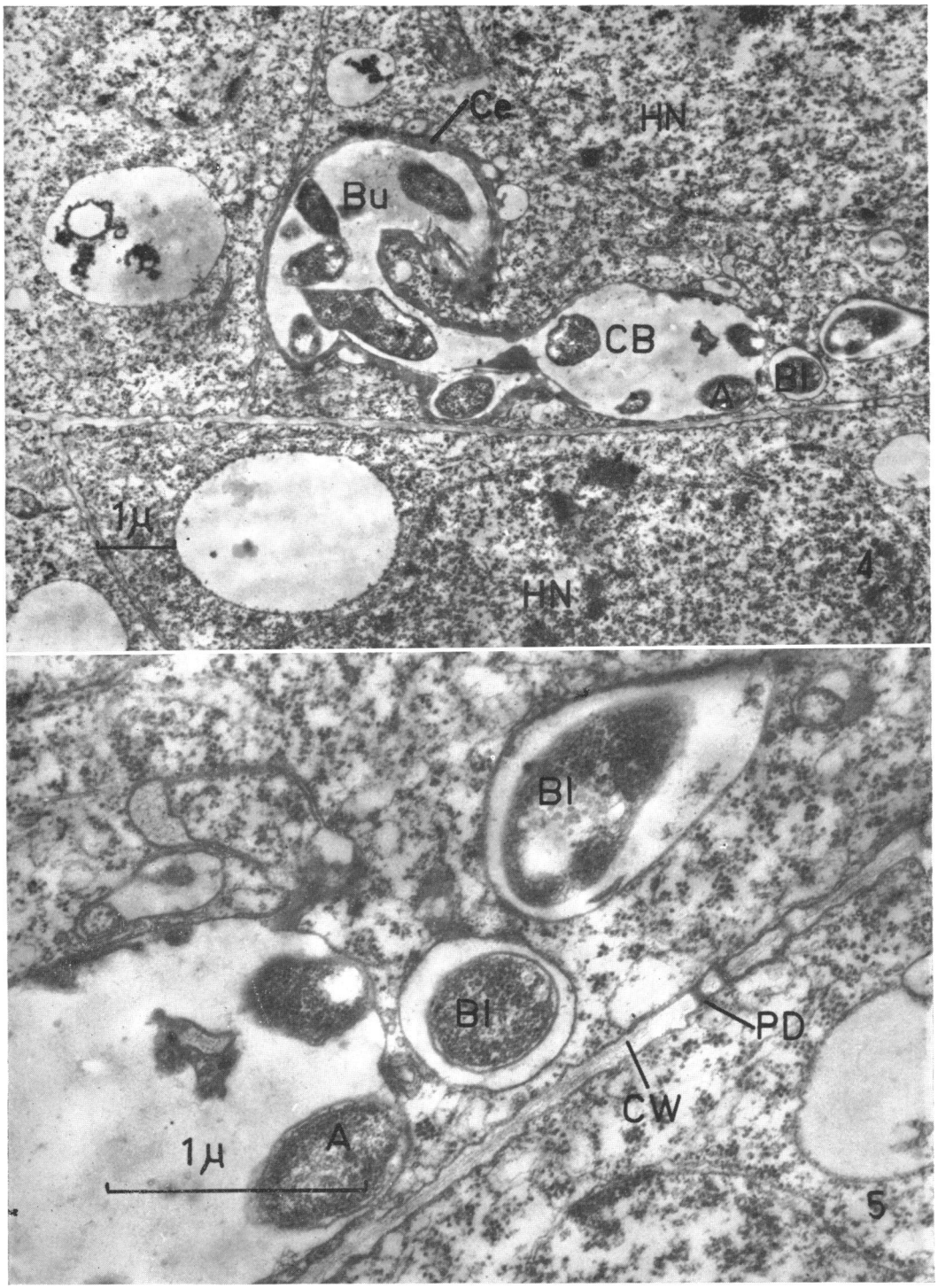

FIG. 4. *Release of bacteria from the infection thread. A cellulose-lined bulge (Bu) has developed, and this has developed a cellulose-free bulge (CB). The bacteria within the latter appear to move into the cytoplasm by endocytosis after first becoming applied to the inner surface of the membrane lining the bulge (A). (HN) Host nucleus. (BI) Bacterium within the ingestion vacuole. (Ce) Infection thread cellulose.*

FIG. 5. *Higher magnification of the infection thread bulge shown in Fig. 4. (A) Bacterium applied to the inner surface of the infection thread membrane. (BI) Bacteria within ingestion vacuoles. (CW) Host cell wall with plasmadesmata (PD).*

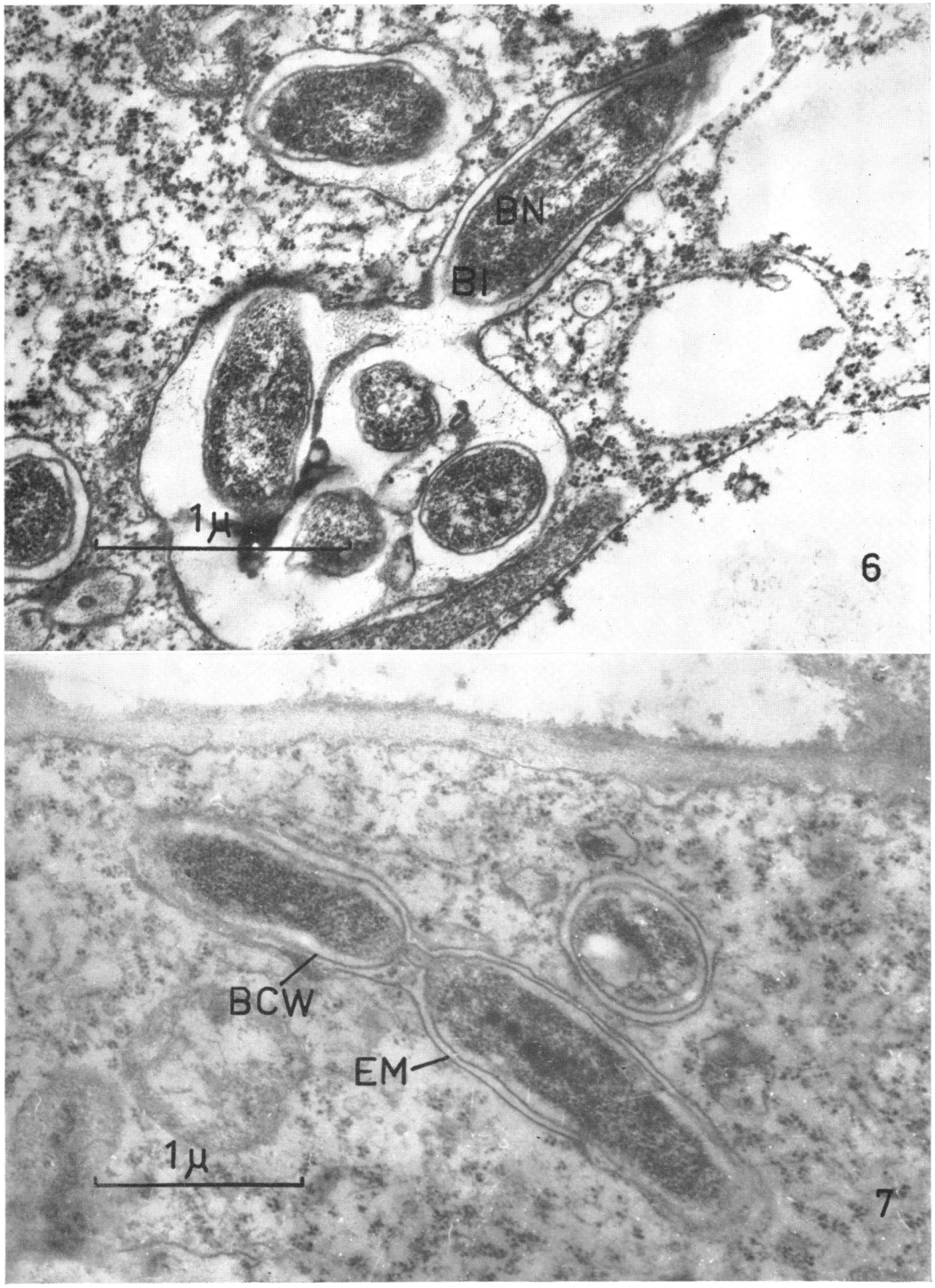

FIG. 6. *Cellulose-free infection thread bulge with a bacterium (BI) moving into the cytoplasm by endocytosis. (BN) Bacterial nuclear region.*

FIG. 7. *Early stage (5 to 6 days) of the development of infection. A bacterium dividing in the cytoplasm and the enclosing membrane envelope in the process of dividing with it. (EM) Enclosing membrane. (BCW) Bacterial cell wall.*

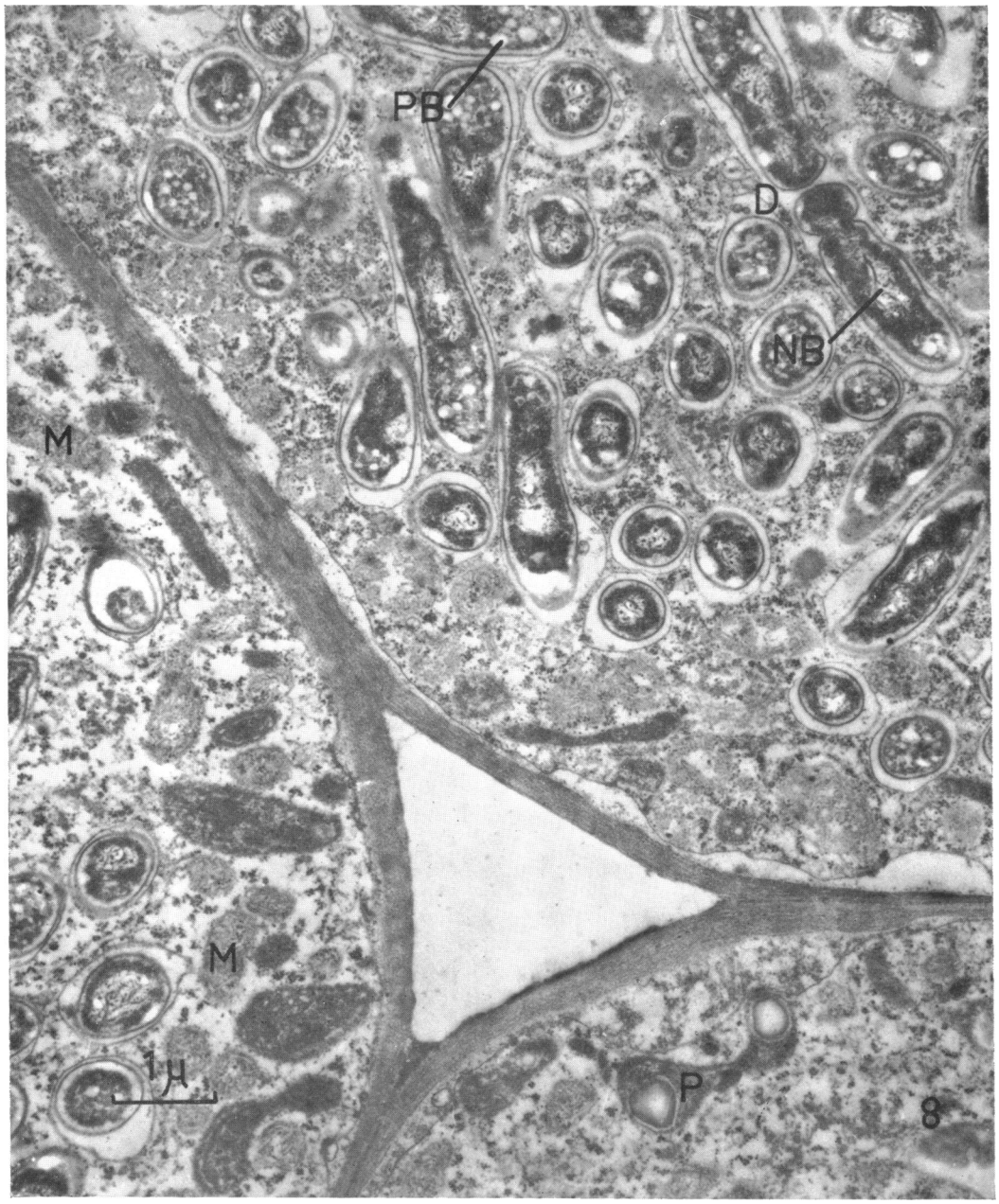

FIG. 8. *Later stage (5 to 7 days). The host-cell cytoplasm is filled with bacteria, each within its own membrane envelope. Division of bacteria within the envelopes is just commencing (D). (M) Mitochondria. (P) Proplastid. (PB) Electron-translucent granules, probably poly-β-hydroxybutyric acid. (NB) Bacterial nuclear filaments.*

terial surrounding the bacteria as reported in *Medicago sativa* (8), in *M. tribuloides*, and in several clover species (3, 9). In soybean infection threads, some matrix material was present, but it took the form of a lightly stained background with some evidence of fibrillar structure (Fig. 2). The thread shown in Fig. 1, and those observed in other similar sections, show remarkable packing of the bacteria, which are obviously in a state of vigorous growth and division. Since this growth results in considerable growth in length of a chain of bacteria, with no appreciable

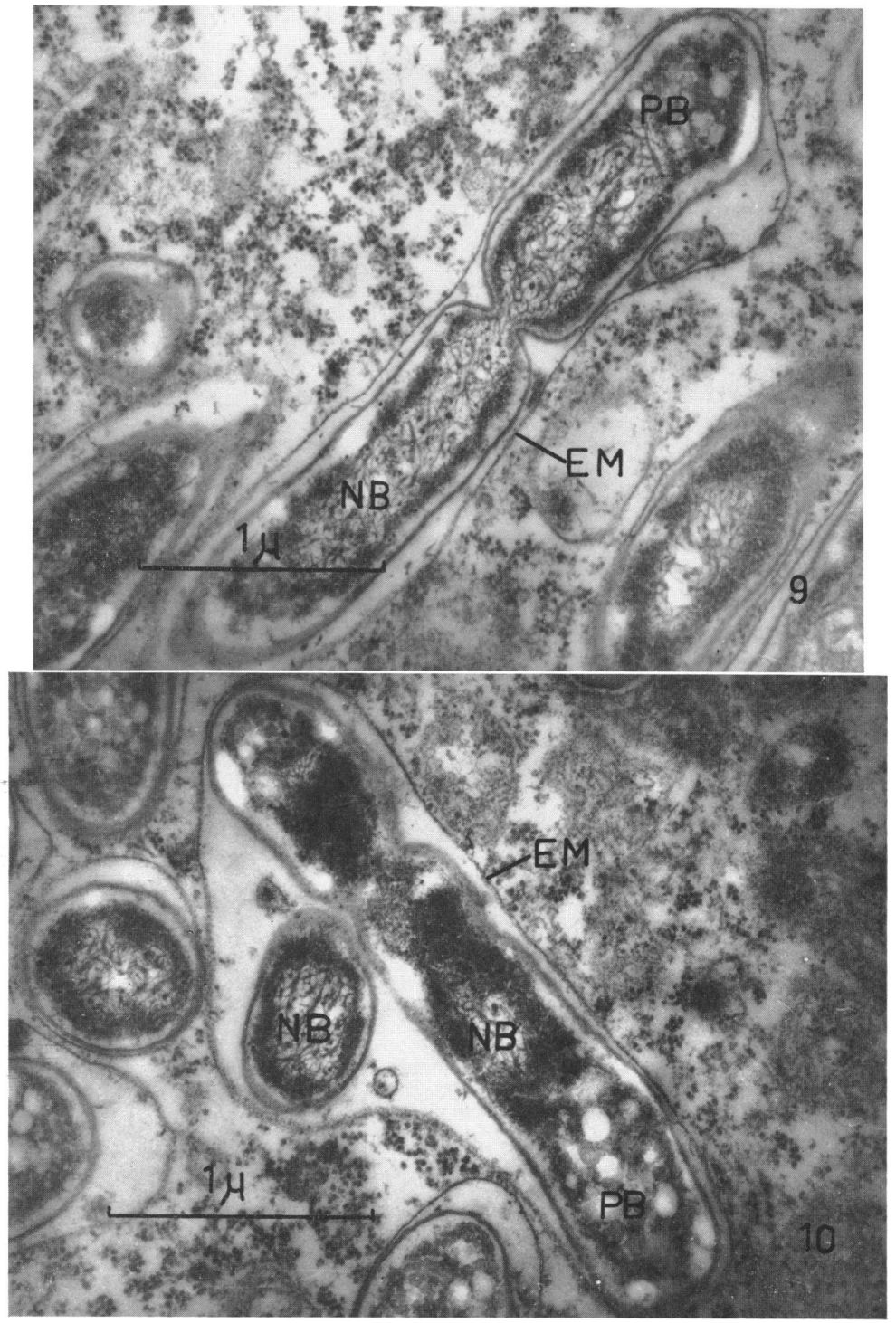

FIG. 9 and 10. *Bacterial division within the membrane envelope (EM). (PB) Electron-translucent material, probably granules of poly-β-hydroxybutyric acid. (NB) Thickened nuclear filaments.*

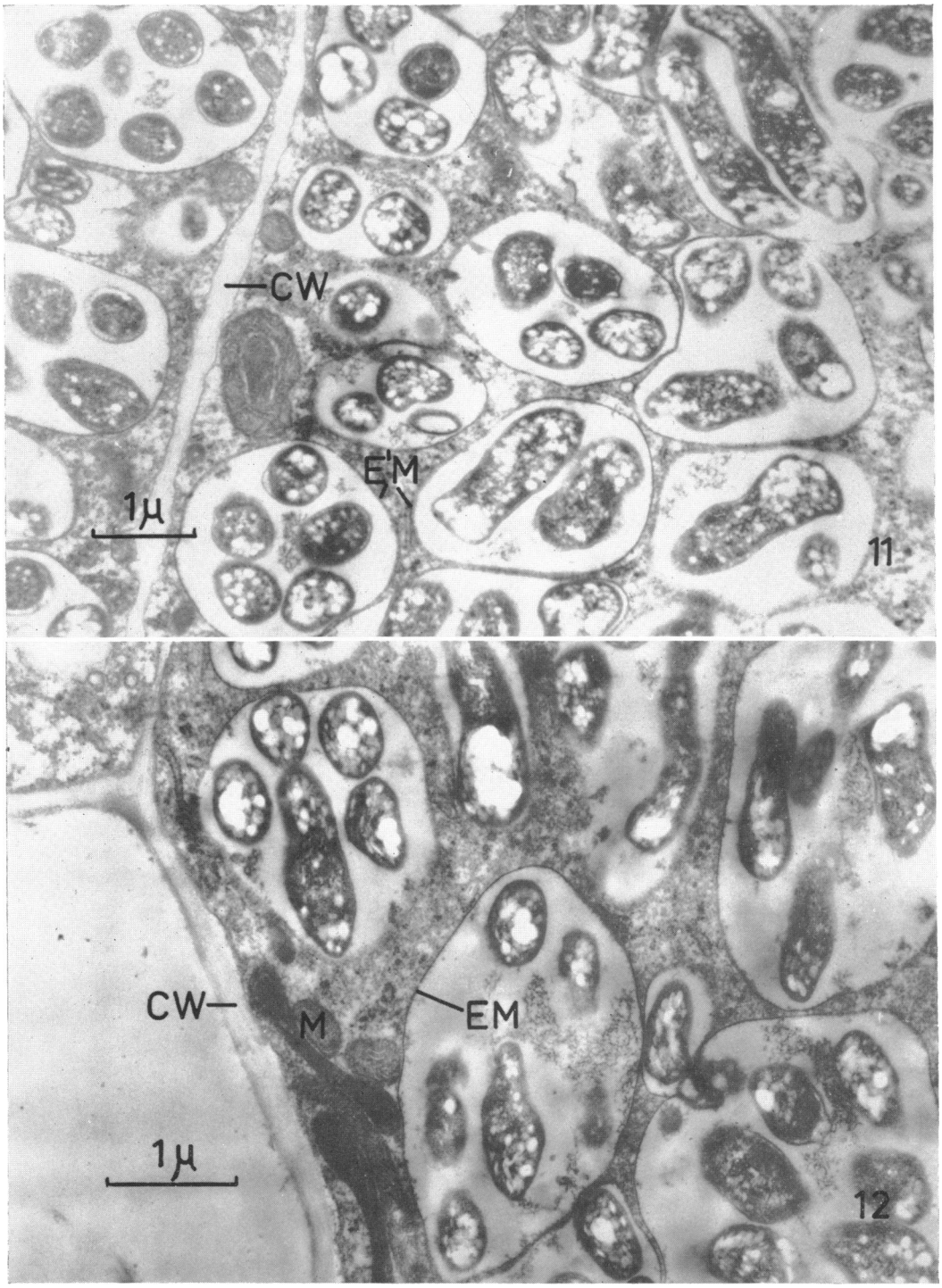

FIG. 11. *Development of the infection almost completed; the bacteria are enclosed in groups of two to six within their membrane envelopes (EM). Prominent electron-translucent granules within the bacteria are probably poly-β-hydroxybutyric acid. (CW) Host cell wall.*

FIG. 12. *Mature nodule cell with large membrane envelopes (EM) containing four to six bacteroids. No further bacterial growth occurs. Mitochondria (M) are concentrated at the periphery adjacent to an empty cell. The host cytoplasm is virtually featureless. (CW) Host cell wall.*

growth in width, and since the sides of the bacteria are in general closely applied to the inner surface of the threads, it follows that considerable force must be exerted upon the closed tip of the infection thread. This could be responsible for the extension growth of the thread as a whole in conjunction with considerations of auxin synthesis, as discussed by Kefford, Brockwell, and Zwar (7). Further indications of this are seen in the obvious displacement in the direction of orientation of the cell wall adjacent to a penetrating thread seen in Fig. 2.

As observed earlier, the infection thread is a true invagination of the surface of the host cell, and subsequent development of the infection is analogous to the endocytosis of particles at the interior extremity of invaginations of the surface of *Amoeba proteus* (5). This type of infection is widespread in infections of cells in which intracellular bacterial growth takes place. Subsequent growth occurs within the infection vacuole, which is surrounded by a membrane initially derived from the host-cell cytoplasmic membrane (Bergersen, *to be published*).

Studies of the development of root nodules of legumes have led to two interpretations of events following penetration of the infection thread into the host cell. Dart and Mercer (3) suggested that membrane envelopes are synthesized about the bacteria after release from the matrix of the infection thread; this is also the basis of the interpretations of Jordan, Grinyer, and Coulter (8). On the other hand, Bergersen and Briggs (2) and Dixon (4) interpreted the process as being basically endocytotic. Close examination of the micrographs presented by all of these authors, however, may show more details consistent with the endocytotic interpretation. For example, Fig. 4 of Dart and Mercer (3) shows the bacteria emerging from the matrix to be enclosed in the thread membrane, and the following figure, although showing no well-stained membrane about the bacteria, shows a sharp interface which may well be an imperfectly preserved membrane rather than the site of deposition of a new membrane. The evidence presented in the present paper clearly shows the endocytotic nature of the process in the soybean nodule.

The results presented differ in one important aspect from earlier work (2), in which it was suggested that a connection between infection thread membrane and membrane envelopes was maintained as small tubules in the host cytoplasm. No evidence was found for this; instead, the enclosing membranes form detached units as bacterial multiplication proceeds, new membrane envelopes becoming discrete and severing all connections with the parent vesicle, until the final stages are reached and the bacteria multiply within an envelope.

ACKNOWLEDGMENT

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