Fine Structure of the Host-Parasite Interface of Plasmodiophora brassicae in Cabbage

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ABSTRACT

Under the electron microscope the parasitic stages of *Plasmodiophora brassicae* are surrounded by a plasmodial envelope which appears as a seven-layered boundary approximately 230 Å thick comprising four uniformly spaced electron-dense layers separated by three electron-lucent layers. Densitometric tracings of glutaraldehyde-acrolein-fixed and osmium-uranium-lead-stained plasmodia within the cell and isolated from the host indicated that the inner layers of the envelope were slightly thicker and more heavily stained than the layers adjacent to the host and parasite cytoplasm. Upon extraction of the polar lipids from the envelope prior to heavy metal staining, the stained layers were comprised of globular stain deposits about 30-40 Å across. After complete extraction of lipid with pyridine, the integrity of the individual layers was lost; however, the envelope persisted as a prominent boundary of stain deposits, presumably on proteinaceous components of the membranes. The envelope is viewed as two closely appressed unit membranes, each with a sectional dimension close to that of the host plasma membrane but differing in thickness from the other cytomembranes of the cell and plasmodium. Although unproven, the outer membrane of the envelope was thought to originate through the invagination of the host membrane at the time of plasmodial penetration of the cell. Phytopathology 60:1557-1561.

Additional key words: clubroot, plasmodial envelope.

Unlike many of the obligate parasites among the filamentous fungi, *Plasmodiophora brassicae* Wor. is entirely intracellular and lacks any specialized feeding structures analogous to the haustorium. Rather, the whole plasmodium is immersed in the host cytoplasm and is separated from it only by the plasmodial envelope (12). Compared to the complex haustorial-host boundaries of the rusts and mildews with their sheath membrane, sheath, haustorial wall, and plasma membranes (1), the seven-layered plasmodial envelope is a relatively simple structure. The plasmodial envelope is found only during the actively parasitic or vegetative stages of plasmodial growth, and disappears during sporogenesis to be replaced by the tri-layered plasmodial or sporangial membrane (12) surrounding the protoplast of the plasmodium in the sporulating thallus. Because the envelope constitutes the sole visible boundary separating the actively metabolizing host cell and the rapidly growing parasite, it must play an important role in regulating the exchange of metabolites between the host and the parasite. Recently, we have been able to isolate intact viable plasmodia from infected cabbage cells (5) and thus afford a more critical examination of the parasite's nutrition. In order to understand the potential role of the plasmodial envelope in the nutrition of the parasite and its relation to the host, the ultrastructural details of the host-parasite interface in cabbage cells were compared with the membranes surrounding isolated plasmodia. Comparisons were also made between the plasmodial envelope and various cytomembranes within the host cell and the parasite in order to examine possible ontogenetic relationships between them.

MATERIALS AND METHODS.—Young clubroot galls were taken from *P. brassicae*-infected cabbage, *Brassica oleracea* var. *capitata* L. 'Jersey Queen', 28 days after transplanting 1-week-old seedlings into infested soil (13). At the time of sampling, plasmodia in most cells were growing vegetatively and had not begun sporulating. Half-mm cubes of infected tissue were fixed by one of the following procedures and prepared for observation under the electron microscope. (i) Routinely, tissues were exposed at 21 C to 3% acrolein and 3% glutaraldehyde in 0.05 M phosphate buffer at pH 6.8 for 1.5 hr, followed by 2% buffered osmium tetroxide (pH 6.8) for 2 hr at 4 C. (ii) Alternatively tissues were fixed in 3% acrolein and 2.5% glutaraldehyde as above, but the secondary fixation-staining with osmium was omitted. (iii) Tissues prepared as lipid-extracted controls were first fixed in 2.5% buffered glutaraldehyde (pH 6.8), then placed for 4 hr in large excesses (20 ml) of pyridine, or methanol or ethyl ether methanol, 2:1 (11). After the extraction period, tissues were stained in 2% buffered osmium tetroxide (pH 6.8) for 2 hr. (iv) Tissue pieces were also fixed in 2% unbuffered $KMnO_4$ for 15 min, washed, and dehydrated. All glutaraldehyde-fixed tissues were dehydrated in a graded acetone series, embedded in a mixture of Araldite 6005 and Epon 812, and sectioned on a diamond knife using a Porter-Blum-2 ultramicrotome. Sections mounted on 300-mesh copper grids were stained with Reynold's lead citrate and 2% uranyl acetate and photographed under a JEM-7 electron microscope. As an alternative staining procedure, some tissues were soaked for 12 hr in saturated uranyl acetate at the 70% acetone step of dehydration. Whole

TABLE 1. Thickness in angstroms of various membranes in cabbage cells parasitized by *Plasmodiophora brassicae* measured from electron micrographs

	Membrane						
Fixation	*P. brassicae* M[a]	Cabbage M	*P. brassicae* ER	Cabbage ER	Cabbage PL	PE	PM
Glutaraldehyde osmium	75 ± 7[b]	75 ± 9	89 ± 6	94 ± 7	117 ± 8	225 ± 16	126 ± 12
$KMnO_4$	80 ± 11		78 ± 5	82 ± 9		172 ± 21	

[a] M = Mitochondrion; ER = endoplasmic reticulum; PL = plasmalemma; PE = plasmodial envelope; PM = plasmodial membrane.

[b] Figures represent the mean and standard errors of at least 50 cross sectional measurements.

plasmodia were removed intact from clubroot tissues and were purified on Ficoll density gradients (5). Plasmodia in the isolation medium were mixed with an equal volume of molten 3% water agar at 45 C. Plasmodia were pelleted in the agar by centrifugation for 10 min at 1,000 *g*. The solidified agar was cut in mm cubes which were fixed and stained by the acrolein-glutaraldehyde-osmium procedure given above. Isolated plasmodia were negatively stained by mixing equal volumes of plasmodia with 2% phosphotungstic acid and by drying small drops on carbon-coated copper grids.

Dimensions of various membranes in the host cell and in the parasite were measured by the method of Sjostrand (8) at randomly chosen points on the projected image of the micrograph. The thickness of the membranes was recorded where the membrane was cross sectioned. Densitometric scans were made on enlarged photographic prints of the plasmodial envelope prepared by a number of the above techniques. A 1-cm wide strip of the envelope image was scanned with a Photovolt recording densitometer. The envelope images were brought to the same magnification so that the distances between features in the membrane elements fixed and stained by the various techniques could be compared.

RESULTS.—Under low magnifications, the plasmodial envelope appeared as a densely staining layer about 225 Å thick at the interface of the host and parasite cytoplasm. At higher magnifications using the acrolein-glutaraldehyde fixation followed by osmium tetroxide with a saturated uranyl acetate soak, the envelope could be resolved into four uniformly spaced electron-dense layers separated by three electron-lucent layers (Fig. 1). The envelope has an undulating profile in section with a smooth surface. Only when the envelope is sectioned normal to the plane of its axis are the membrane elements clearly visible. Frequently, the layered images fade into more diffuse zones as the angle of the envelope relative to the direction of the knife cut shifts away from the normal. In general, the appearance of the plasmodial envelope resembles two closely appressed, tri-layered membrane elements separated by an electron-translucent layer. Unlike the envelopes surrounding the mitochondrion (Fig. 1) or the nucleus, the membrane elements of the plasmodial envelope are very uniformly spaced.

When the thickness of the various membranes in the parasitized cells were compared (Table 1), their average cross sectional dimensions compared closely with those for other membranes reported in the literature (6, 7). With the exception of the mitochondrial membranes, the thickness of most membranes fixed with permanganate was slightly less than when the tissues were fixed with glutaraldehyde-acrolein. The mitochondrial membranes of both the host and the parasite were the thinnest, averaging 75 Å across, whereas those of the endoplasmic reticulum were slightly thicker than those of the mitochondrion. The plasmalemma was the thickest membrane in the host cell (average 117 Å). Cells in which the plasmodium was undergoing early cleavage and sporogenesis permitted the thickness of the plasmodial membrane to be compared with that of the plasmodial envelope and other cytomembranes. The plasmodial membrane was very close to that of the plasmalemma in thickness and, when stained with osmium (Fig. 1), produced the characteristic tri-layered image of two electron-dense layers separated by an electron-lucent layer.

Post-staining of the glutaraldehyde-acrolein-fixed plasmodium within the cabbage cell with osmium and uranium permitted resolution of the plasmodial envelope into four densely staining layers lying closely spaced by three electron-lucent layers (Fig. 2-a) (12). Densitometric tracings of this image of the plasmodial envelope indicated that the inner two electron-dense layers were slightly thicker and more electron-dense than the layers adjoining both the host and parasite cytoplasm (Fig. 3-a). When tissue which had been fixed in glutaraldehyde and acrolein only was extracted with acetone, then stained with uranium and lead, the membrane elements of the envelope were less well de-

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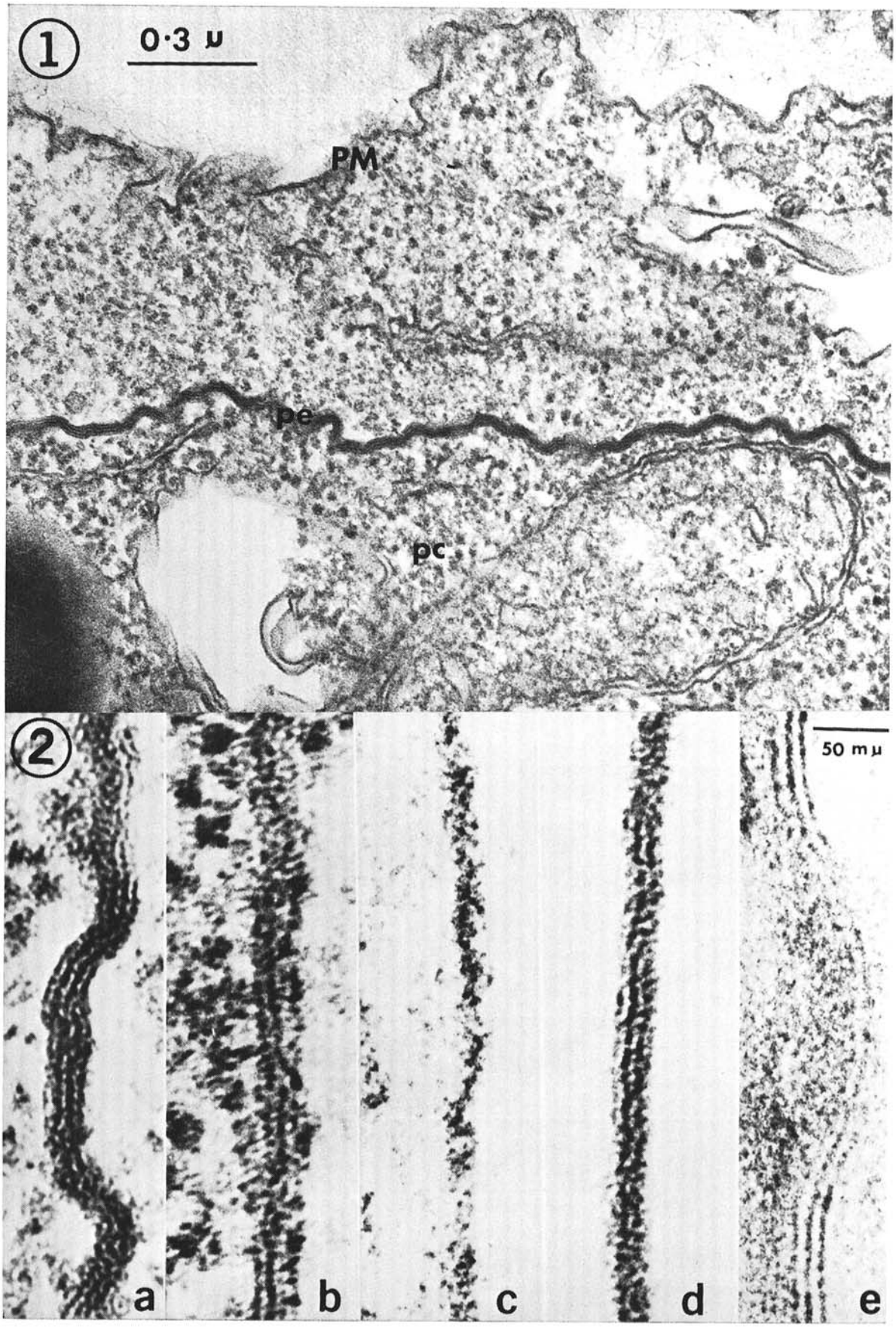
Fig. 1-2. 1) Electron micrograph of a cabbage cell infected with *Plasmodiophora brassicae* showing the prominent multilayered plasmodial envelope (pe); parasite cytoplasm (pc); host plasma membrane (PM). 2) Electron micrographs of the plasmodial envelope of *Plasmodiophora brassicae*. Gall tissue fixed in glutaraldehyde-acrolein and stained with osmium, uranium, and lead (a). Gall tissue fixed in glutaraldehyde-acrolein, then extracted with acetone and stained with uranium and lead (b). Gall tissue fixed in glutaraldehyde-acrolein, then extracted with pyridine and stained with uranium and lead (c). Isolated plasmodia fixed and stained as in A (d). Envelope of isolated plasmodia negatively stained with phosphotungstic acid (e).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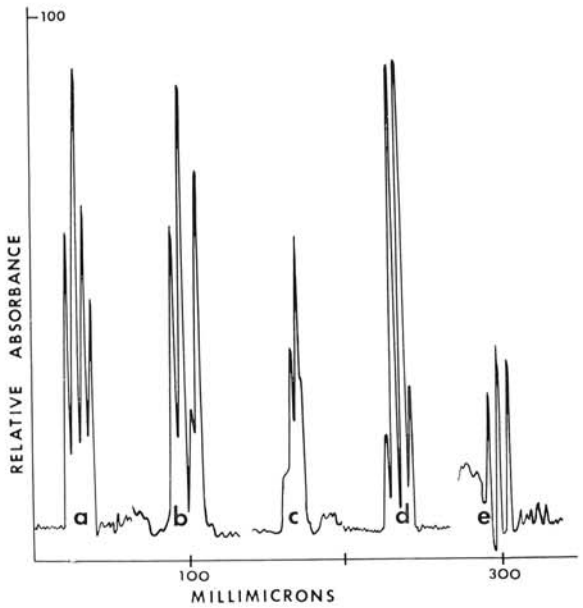

Fig. 3. Densitometric tracings through electron micrographic images of the plasmodial envelope of *Plasmodiophora brassicae* prepared as Gall tissue fixed in glutaraldehyde-acrolein and stained with osmium, uranium, and lead (a). Gall tissue fixed in glutaraldehyde-acrolein, then extracted with acetone and stained with uranium and lead (b). Gall tissue fixed in glutaraldehyde-acrolein, then extracted with pyridine and stained with uranium and lead (c). Isolated plasmodia fixed and stained as in A (d). Envelope of isolated plasmodia negatively stained with phosphotungstic acid (e).

fined. In image, the dark layers appeared to consist of loosely associated rows of globose stain deposits (Fig. 2-b). Although densitometric tracings of the envelope have similar dimensions to the osmium-stained envelope, the ranking of the membrane elements was less uniform (Fig. 3-b).

Following the post-fixation lipid extraction of infected tissue by methanol-chloroform or by pyridine, the thickness and distinctness of the envelope image was considerably reduced (Fig. 2-c, 3-c). No longer were the membrane layers of the envelope visible; rather, the host-parasite boundary consisted of a loosely aggregated layer of stain deposits.

In cross section, the outer boundary of isolated plasmodia resembled closely that of plasmodia within host tissue (Fig. 2-d). More distinguishable were the two inner dense layers of the envelope (Fig. 3-d). Upon isolation from the host cell, the plasmodia became spherical; consequently the plasmodial envelope no longer maintained its undulating profile seen in sectioned cabbage cells (Fig. 1, 2-a).

When isolated fragments of the envelope were dried in phosphotungstic acid, occasionally negative images were obtained which resembled the edge-on profile of the seven-layered envelope. Always this image was obtained near the edge of a sheet envelope or where folds in the envelope occurred (Fig. 2-e). Tracings of the negatively stained envelope provided measurements of distances between the layers which corresponded closely to the expected distance in the stained and sectioned envelope (Fig. 2-e, 3-e).

Discussion.—While it is premature to speculate on the relation of membrane ultrastructure to function until biological membrane theory becomes more firmly established (9), the visualization of the plasmodial envelope as two closely appressed unit membranes leads to speculation on its origin. The absence of an envelope surrounding the free living spore forms of *P. brassicae,* i.e., resting sporangium and primary and secondary zoospores (12, *unpublished data*), indicates that the plasmodial envelope is unique to the parasitic stages of the plasmodium within the host cytoplasm. It is possible that the envelope arises through the invagination of the host plasmalemma as the parasite first enters the cell. The similarity in cross-sectional dimensions of the two membranes making up the envelope with those of the host plasma membrane also suggests that the outer membrane could be of host origin. As we have discussed previously (12), the integrity of the outer membrane of the envelope appears to be under host regulation, since at the time of sporulation in the clubroot gall the breakdown of the outer membrane coincides with the death of the host cell.

The marked asymmetry of the inner dense layer of the outer membrane of the envelope was similar to that observed by Thomson (10) for the plasmalemma of oranges after staining with osmium, uranium, and lead. The plasma membranes of numerous plant species are known to stain more intensely in the layer adjacent to the cell wall. During the process of invagination of the host plasma membrane by the invading parasite, the outermost thick layer of the plasma membrane would become the inner dense layer appressed to the plasmodial membrane, and, thus, could conform to our densitometric tracings across the envelope. The close physical association between the plasmodial membrane (inner membrane) and the outer membrane (host plasma membrane) may confer a stability on the outer membrane which permits the isolation from the host cell plasmodia with their envelopes intact. Bracker (1) has suggested that for most of the haustorial parasites "the sheath membrane appears to be a specialized portion of the host plasma membrane". As has been reported by Ehrlich & Ehrlich (3) for *Puccinia graminis tritici* and by Bracker for *Erysiphe graminis,* the sheath membranes around the haustoria are thicker and in *E. graminis* the sheath membrane is able to withstand stresses which destroy other cytomembranes (2). Such appears to be the case with the plasmodial envelope also. The thickened pair of inner dense layers of the envelope may confer a physical stability on the delicate plasmodium which aids in its isolation from the host protoplast (5).

Our experiments on extraction of the plasmodial envelope with various solvents prior to heavy metal staining points to the importance of lipids in maintaining the integrity of the typical tri-layered structure of the membrane elements. It is interesting to

note that even after extraction of both the polar and nonpolar lipids by pyridine or chloroform methanol, a relatively well-stained boundary between the host and parasite cytoplasm remained. Though the lamellar organization of the envelope was gone after lipid extraction, the heavy staining of the boundary suggests that the envelope contains a considerable portion of protein with [redacted] for heavy metals (6). This selective staining of the envelope after lipid extraction (Fig. 2-c) contrasted with the absence of staining of other cytomembranes both in the host and in the parasite (11, Fig. 6-c). Thus, based on their cross-sectional thickness and their staining properties with heavy metals, the membranes of the plasmodial envelope appear to be different from those of the mitochondria, plastids, nucleus, and endoplasmic reticulum. A vast diversity of function and morphogenetic flexibility has been associated with cellular membranes which at one time in their ontogeny may have had a common origin (4). Thus it is not unreasonable to assume that in the plasmodial envelope, membranes of the host and parasite have come together to form a structure which is unique yet vital to the survival of the parasitic stages of *P. brassicae*.

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