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Ultrastructural Details in Germinating Sporangiospores of *Rhizopus stolonifer* and *Rhizopus arrhizus*

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Electron microscope examination of sporangiospore sections from *Rhizopus stolonifer* (Ehrenb. ex Fr.) Lind. and *R. arrhizus* Fischer revealed details on intracellular organization not previously reported. Aldehyde fixation followed by chrome-osmium postfixation permitted clear depiction of ribosomes hitherto unrevealed in these cells. Mitochondria were diversiform. Spore wall structures in the two species were generally similar, but outer contours differed sufficiently to permit easy species identification in examination of sections. The spores of both species abounded in cytosomes, corresponding in size, shape, and heavy-metal "stain" affinities to spherosomes in cells of higher plants. The osmiophilic response of these spherosome-like inclusions was intensified by treatment of sections with thiocarbohydrazide solution and subsequent application of aqueous osmium tetroxide, which strengthens an assumption that they are lipid-rich. The margins of the spherosome-like inclusions in lead citrate-stained sections included dense particles, about 60 A across, whose crystalline-like arrangements suggested that protein as well as lipid was present. Frequent and close associations between the spherosome-like inclusions and various cell membranes suggested that such bodies participate in membrane elaboration during germination.

Rhizopus species are mucoraceous fungi which usually reproduce asexually, forming spores within a sporangium. The enveloping sporangial membrane ruptures when the spores mature, thus releasing them. Several species commonly spoil stored processed foods and fresh fruits and vegetables.

Electron micrographs of *Rhizopus* sporangiospores have appeared in reports by Hawker and Abbot (10) and by Nečas, Havelková, and Soudek (14). Those workers established that the mature sporangiospore in the two species examined, *R. sexualis* and *R. nigricans* Ehrenb. (syn. *R. stolonifer* Lind.), contains several nuclei, classically organized mitochondria, and vesicles and granules of various sizes and densities. A thick, ridged outer wall encloses the protoplast. During germination, an inner wall forms and extends around the emerging hypha. Neither group of workers used fixation treatments which would permit them to visualize ribosomes.

The present study confirms the previous observations on *R. stolonifer* and extends electron microscope observations to spores of yet another species, *R. arrhizus*. The use of chrome-osmium postfixation made it possible to demonstrate ribosomes in these spores for the first time. Newly described are ultrastructural and cytochemical features of spherosome-like inclusions, whose associations with membranous cell components were also noted.

MATERIALS AND METHODS

Collection and germination of spores. Sporangiospores from 2-week-old cultures of *R. stolonifer* or *R. arrhizus* growing on V-8 juice-agar (17) were harvested according to procedures previously described (1). Germination occurred in a medium (5) consisting of 10 g of glucose, 4.5 g of KH_2PO_4, 1.0 g of NH_4Cl, and 0.5 g of $MgSO_4 \cdot 7H_2O$, dissolved in 1 liter of distilled, deionized water, brought to *p*H 6.0 with NaOH, and then autoclaved. When suspensions of 10^6 to 10^7 spores per ml were held for 6 to 7 hr at 25 C in this medium, while aerated on a shaker, about 50% developed germ tubes up to several spore-diameters long, and about 40% more showed some degree of swelling.

Preparation of spores for electron microscopy. Spores were fixed in 4% formaldehyde or 6.25% glutaraldehyde in 0.1 M phosphate buffer at *p*H 7.0 to 7.2 and were postfixed in either 1% $KMnO_4$ in phosphate buffer or in modified Dalton's fixative (3). In this modification, equal parts of aqueous 2% osmium tetroxide and aqueous 2% potassium dichromate (*p*H 6.8) were mixed immediately before use. No sodium chloride was added. Spores were left for 1 hr at room temperature in the permanganate fixative and for 12 to 18 hr at 5 to 7 C in the chrome-

osmium mixture. Details of the dehydrating, embedding, and sectioning procedures were reported previously (1). Usual procedures for staining sections included application of Reynolds' lead citrate (15) for 10 min, or uranyl acetate (saturated solution in methanol) for 2 min, followed by lead citrate for 10 min.

Osmium tetroxide staining. Special staining for enhancement of lipid-rich components in chrome-osmium-fixed sections of spores required slight modification of the OTO method described by Seligman and co-workers (16). Sections mounted on uncoated nickel grids were immersed for 0.5 to 1 hr in warm (40 to 45 C) aqueous 0.5% thiocarbohydrazide. They were washed several times in warm 50% ethyl alcohol and once in distilled water before being stained by immersion for 10 to 15 min in aqueous 1% osmium tetroxide. The ethyl alcohol rinses freed the treated sections from a precipitate often found when only distilled-water rinses were used.

Acid phosphatase reaction. A method described by Ericsson and Trump (6) for demonstrating acid phosphatase activity was used on formaldehyde-treated spores. Spores were incubated with the glycerophosphate substrate for 1 hr at 32 C. They were then treated as prescribed and postfixed with chrome-osmium. The dehydrating and other preparative procedures were carried out as usual. All sections were examined with RCA EMU-3 microscopes.

Chilling treatment. Spores which had been incubated for 6 hr at 25 C in liquid medium were transferred to chilled fresh medium in cotton-plugged flasks. These were held on a shaker for 4 days in a room maintained at 0 C ± 2 C. About 10% of the chilled spores retained their ability to form normal colonies according to plating tests done at that time.

RESULTS AND DISCUSSION

General observations. Nuclear and mitochondrial outlines, cell wall details, and various cell inclusions were clearly seen in aldehyde-permanganate-fixed sections after lead citrate staining (Fig. 1, 3a, and 4). The relatively poor contrast of organelles against ground plasms in chrome-osmium-fixed material was improved by staining sections with uranyl acetate followed by lead citrate (Fig. 3b).

The numerous nuclear outlines were circular, oval, or sometimes very elongate, but not as irregular in shape in germinating as in nongerminating spores. Mitochondria were dispersed throughout the sections and often showed contorted, lobed profiles. Cristae were closely parallel and frequently extended almost completely across a mitochondrion. Except for minor differences, the intracellular organization of the *R. arrhizus* spore closely resembled that of *R. stolonifer* (Fig. 1a and 3a). *R. arrhizus* spores were smaller than the *R. stolonifer* spores (about 5 μ for nongerminating spores of *R. arrhizus*, compared with about 7.5 μ for this strain of *R. stolonifer*). Average numbers of nuclei and mitochondria were greater in *R. stolonifer* spores, but the shapes assumed by those organelles were very similar in the two species.

One detail of spore ultrastructure not previously emphasized in studies of these fungi is the appearance of ribosomes. After chrome-osmium postfixation, it was possible to observe dense, approximately round, cytoplasmic particles which lay apparently free throughout the cells (Fig. 2a). An average diameter for the largest ones was approximately 170 A, which is in the size range ascribed to ribosomal particles from many other kinds of cells. Endoplasmic reticulum strands were sparse in these spores and ribosomal associations with endoplasmic reticulum were not observed. The great abundance of the ribosomal particles at all stages of germination and also in nongerminating spores contributed to the difficulty of distinguishing organelle outlines clearly after chrome-osmium postfixation.

Walls and plasmalemmas in both species were delineated equally well with both fixation treatments (Fig. 1, 2, and 3). The wall present before germination (Fig. 1b), referred to as the outer wall (ow), exhibited two distinctly different zones or layers. In *R. stolonifer*, an electron-opaque outer layer displayed numerous prominences (Fig. 1a and 2b) representing the ridges first pictured by Hawker and Abbott (10). Below this layer was a light, thicker zone, strewn with electron-dense areas. The wall structure in *R. arrhizus* was quite similar, except that the prominences of the outer opaque layer were less abrupt. Thus, in *R. arrhizus*, the spore contours were more gently curved and this made it possible to distinguish the spores of the two species in sectioned material.

As is shown in Fig. 1b, the absence of an inner wall was clearly evident in the ungerminated spore. An inner wall appeared only in germinating spores (Fig. 1a, 2a, 2b, 3, and 4) and surrounded the emerging tube, as Hawker and Abbott also noted. It was composed of a single zone of moderately electron-dense material which sometimes appeared fibrillar (Fig. 2b). Infoldings of the plasmalemma, which lay directly below the inner wall, were seen occasionally (Fig. 2a). At such places, membranous material occupied the space between the inner wall and the plasmalemma.

Thus, germinating sporangiospores of *R. stolonifer* and *R. arrhizus* were found to be amenable to electron microscope examination after either of two fixation treatments in wide use for both plant and animal material. It was expected that the spores of two closely related species would resemble each other in many aspects of subcellular organization. The details

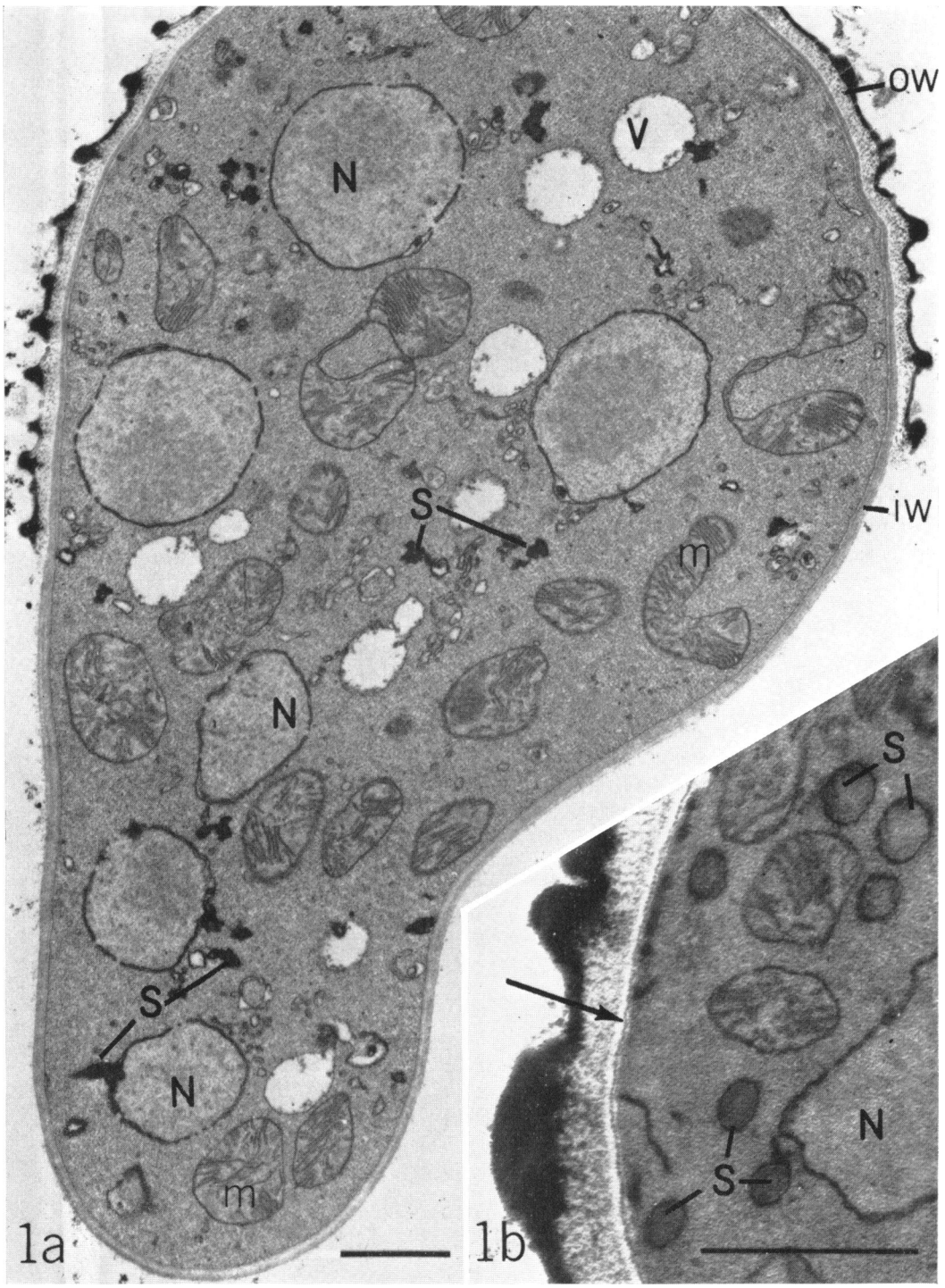

FIG. 1a. *Section through germinating sporangiospore of Rhizopus stolonifer showing nuclei (N), mitochondria (m), vacuoles (V), and dense inclusions (S), some of which appear to be membrane-associated (arrows). Outer wall (ow) has broken; new inner wall (iw) surrounds protoplast. $KMnO_4$-postfixed, lead citrate-stained.*

FIG. 1b. *Portion of nongerminating sporangiospore, characterized by absence of inner wall (arrow) between outer wall and protoplast. Note numerous dense inclusions (S), several mitochondria, and part of a nucleus. $KMnO_4$-postfixed, lead citrate-stained. All line markers denote distance of 1 μ, unless otherwise indicated.*

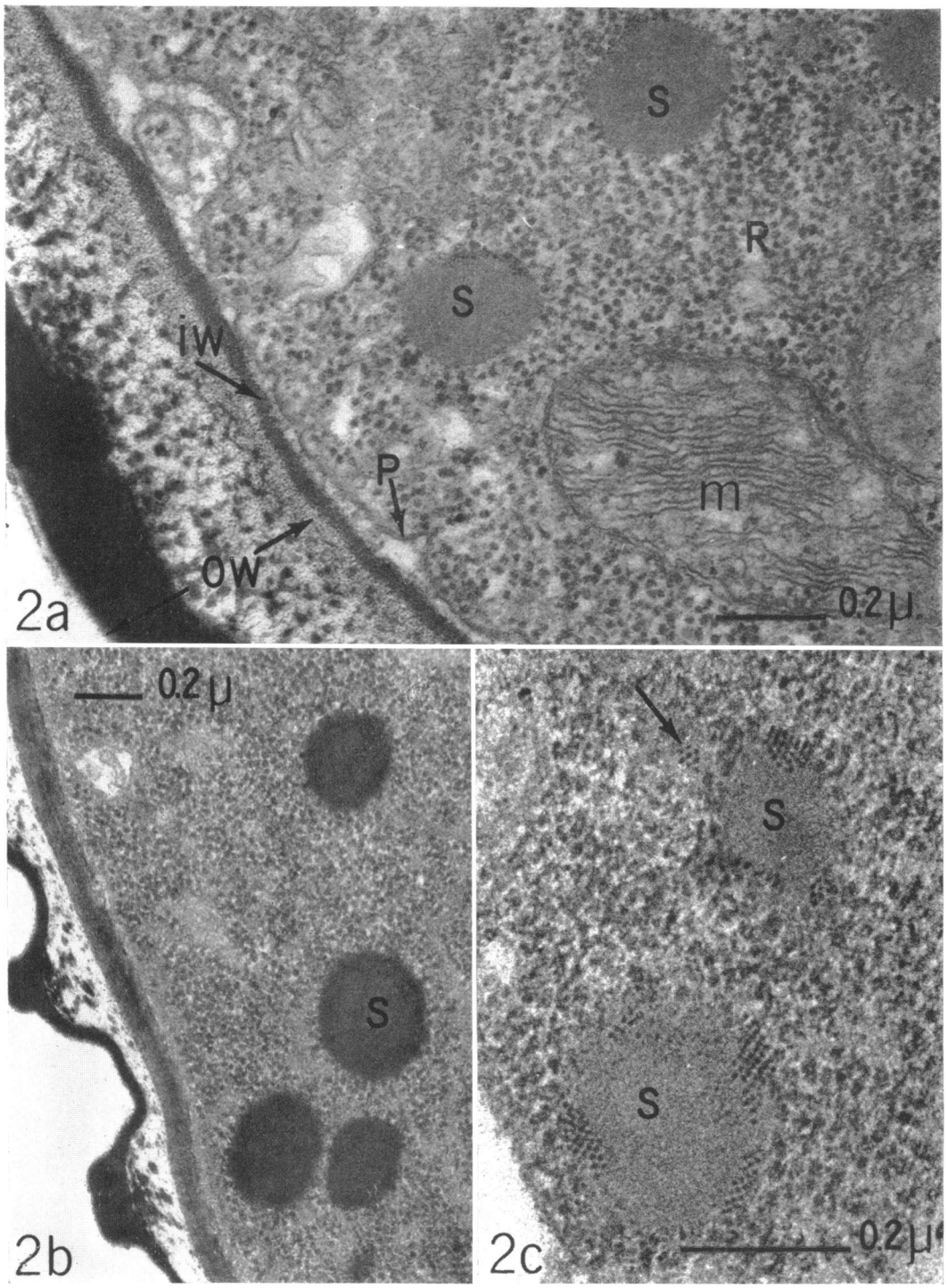

FIG. 2a. *Portion of germinating Rhizopus stolonifer sporangiospore postfixed in chrome-osmium, showing details of inner (iw) and outer wall (ow) structures, plasmalemma (P), dense inclusions (S), and mitochondria (m). Ribosomal particles (R) appear freely distributed in hyaloplasm. Uranyl acetate, lead citrate staining.*

FIG. 2b. *Portion of cell section that was osmium tetroxide-treated. S inclusions appear dark, unbounded. Wall prominences typical for R. stolonifer spore.*

FIG. 2c. *S inclusions in chrome-osmium-fixed cell, showing regularly arranged dense particles at margins. Lead citrate-stained.*

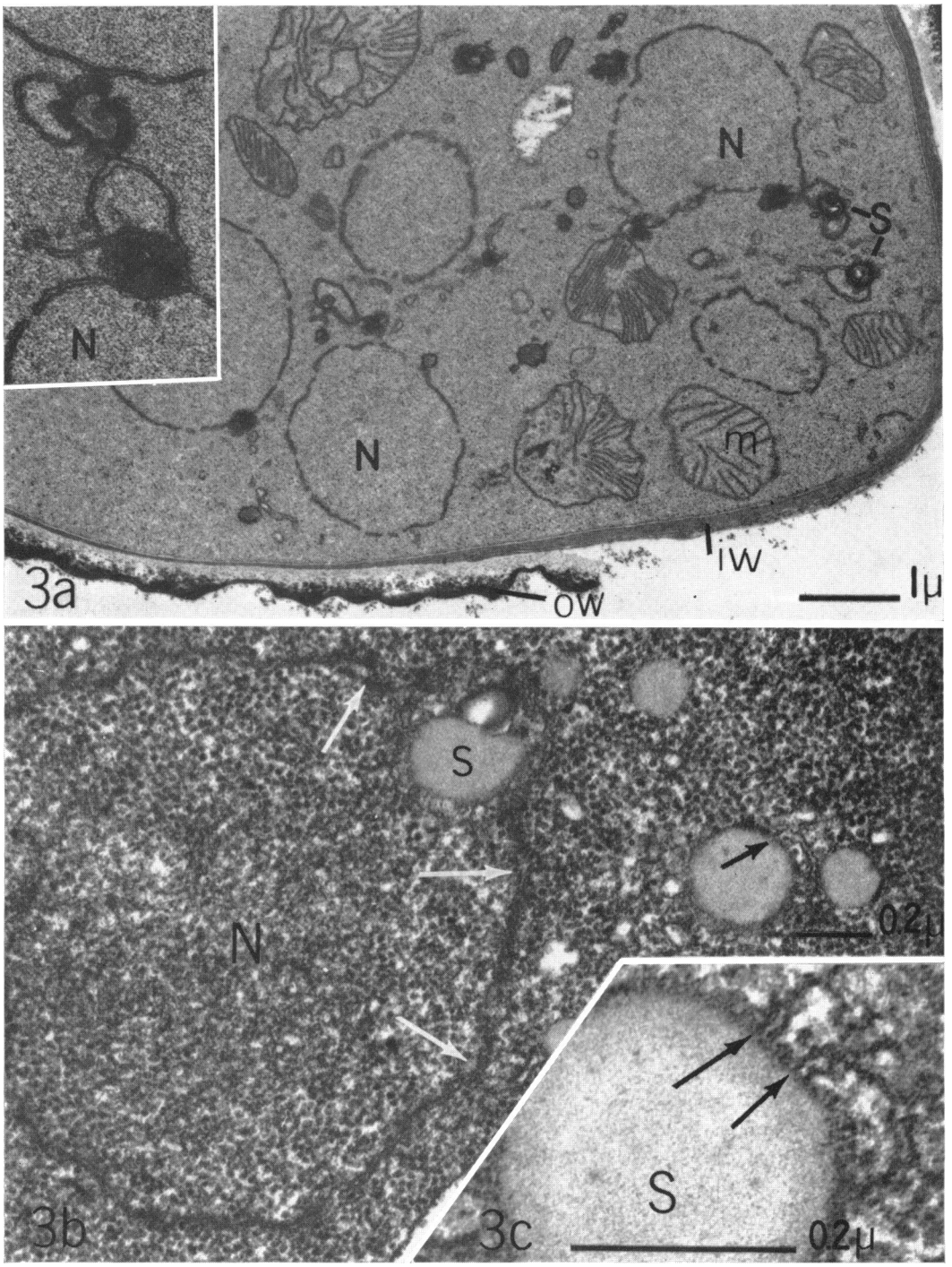

FIG. 3a. *Portion of Rhizopus arrhizus germling showing mitochondria (m), nuclei (N), outer and inner wall layers (ow, iw), and S inclusions, some associated with nuclear envelopes and other membranes. Also note S associations in inset, above left. Outer-wall contour characteristic for this species. $KMnO_4$-postfixed, lead citrate-stained.*

FIG. 3b. *S inclusions and nucleus in R. arrhizus spore postfixed with chrome-osmium. White arrows indicate nuclear envelope. Note similarity of S inclusion associations with nuclei in this and Fig. 3a.*

FIG. 3c. *Enlarged view of S inclusion from section shown in 3b. Black arrows point to membrane segments at margins of S inclusions. Uranyl acetate, lead citrate staining.*

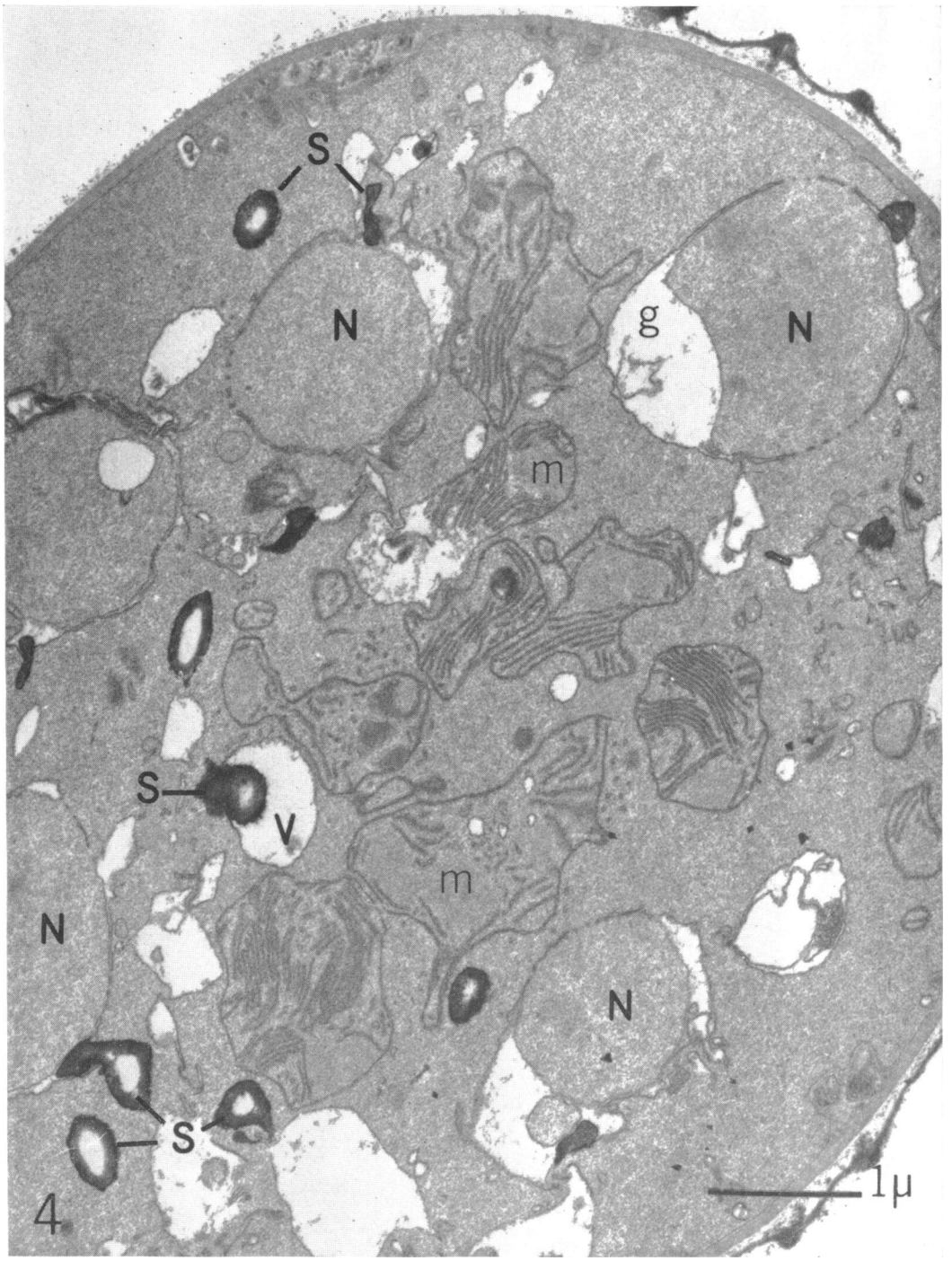

FIG. 4. *Section of germinating Rhizophus stolonifer spore, injured by chilling at 0 C. Note wide gaps (g) between the two membrane layers surrounding nuclei (N), agglomerated and deformed mitochondria (m), and membrane-associated dense inclusions (S). $KMnO_4$-postfixed, lead citrate-stained.*

of wall structure and previously described organelles agreed with the reports on *Rhizopus* by other workers, and new information has been added about ribosomal particles. The evident complexity of the spore walls, shown by both fixations (Fig. 2b and 3a), leaves unanswered many questions concerning the location and distribution of melanin, chitin, lipid, etc., in the several zones or layers.

Cytosomes in Rhizopus spores. Dense inclusions and clear vacuoles abounded in these cells. Particularly noticeable in permanganate-fixed material were dense, crenulated inclusions (denoted "S," for spherosome-like), which appeared to be membrane-bounded (Fig. 1a, 1b, 3a, and 4). In chrome-osmium-fixed material, in contrast, boundaries of the more nearly round bodies, representing the same inclusions, appeared to be lacking (Fig. 2a, 2b, 2c). In many respects, the inclusions fit the descriptions given for spherosomes of higher plant cells (4, 7, 8). The diameters of many of the S inclusions in *Rhizopus* spores fell in the range 0.2 to 0.6 μ. This is within the size range reported (7, 8) for spherosomes (0.2 to 1.3 μ) and also for certain plant cytosomes said to resemble animal microbodies (12). In their report on plant microbodies, Mollenhauer and co-workers (12) mentioned, but did not illustrate, the presence of microbodies in *Rhizopus* material which they had examined; whether the material included spores was not stated.

It is believed that spherosomes contain both lipid and protein (7). S inclusions responded similarly to fixations and were assumed to be similar in composition. To test for the presumed high lipid content of S inclusions, we used a modification of a method (16) which required treatment of osmium-fixed sections with thiocarbohydrazide and subsequent staining with aqueous osmium tetroxide. Figure 2b shows a typical result. The osmiophilic reaction of the S inclusions was intensified by the osmium tetroxide staining, although the details of other cell components were less clear. The intensified osmiophilic response was considered added evidence that the bodies in question are rich in lipid.

An interesting feature of S inclusion ultrastructure was brought out by lead citrate staining. Lead citrate in alkaline solution is commonly used in electron microscopy of biological subjects to enhance contrast. The effect is attributed to the higher affinity of certain cell components for the heavy metal ions. Figure 2c shows, at relatively high magnification (125,000 $\times$), the appearance of two S inclusions in a lead citrate-stained, osmium-fixed spore section. Against a background of ribosomal particles, the bodies appear moderately dense and homogeneous, except for some small (50 to 60 A) particles on and near their margins. These particles, arranged in crystalline-like patterns, lie in the size range possible for globular protein particles having molecular weights from 20,000 to 80,000 (8). The presence of these particles, visible after the lead citrate treatment, implies that S inclusions have a protein component, possibly enzymatic. What seem to be surface coatings or marginal accumulations of these particles on the S inclusions in osmium-fixed cells were likely responsible for their membrane-bounded appearance in the permanganate-fixed cells. Such an effect, apparent bounding by a membrane, has been demonstrated at interfaces of oil-gelatin mixtures after $KMnO_4$ treatment (8). Carasso and Favard (2), in their study of the "vitelline plaquettes" (yolk deposits) in *Planorbis corneus* eggs, observed crystalloid structures which they interpreted as being composed of protein granules, about 60 A in diameter and with 80 A intergranular spacing. Although Mollenhauer and co-workers (12) saw dense material in the cytosomes which they designated plant microbodies, they found no crystalline inclusions.

The indication that S inclusions contain protein, coupled with their other similarities to spherosomes and microbodies, raised the question of whether S inclusions also are sites of enzyme activity. A modification of Ericsson and Trump's method (6) was used to test for acid phosphatase activity. Fine granulation accumulated on the margins of S inclusions and was considered presumptive evidence of activity. However, the demonstration that regularly spaced, dense granules occupied those locations after application of lead citrate alone raises some doubt about the reputed specificity of a response to the lead-containing glycerophosphate substrate in the Gomori reaction. Tests for other enzymes have not been made.

Mollenhauer and co-workers (12) noted that microbodies were resistant to damaging treatments given cells. In our experience, S inclusions also remained apparently unaltered when germinating spores were damaged by γ radiation (*unpublished data*) or chilling. As an illustration of the effect of chilling, Fig. 4 shows S inclusions, some associated with nuclei and vacuoles, in a germinating spore left 4 days in liquid medium at 0 C. Mitochondria are aggregated and deformed, and the nuclei show degenerative changes, but the dense inclusions appear unaffected.

The above comparisons, based on both morphological and cytochemical data, make it clear that S inclusions are cytosomes having properties in common with both microbodies and spherosomes, but corresponding exactly with neither.

Many workers have noted inclusions present in fungal cells (9, 10, 13, 19, 20), but little has been said about their probable uses. Also unclear are the functions of microbodies and spherosomes (7, 8), although it has been argued that spherosomes may function catabolically because they possess acid phosphatase and other lytic enzymes (18, 21). In germinating *Rhizopus* spores examined in the present study, S inclusions were associated frequently with nuclear and vacuolar membranes (Fig. 1a, 3, and 4) and less often with the plasmalemma or mitochondrial membranes. Many sections contained S inclusions surrounded by, and in contact with, loops or strands of unidentified membranes (Fig. 3). No associations between membranes and dense inclusions were observed in sections of ungerminated spores, although many dense inclusions were present in them (Fig. 1b). Possibly the bodies first exhibit functional activity during germination. The numerous instances in which S inclusions in the spores of both *Rhizopus* species were found to be associated with nuclear and other membranes make it tempting to propose for them a functional role in membrane formation in growing cells with rapidly expanding membrane requirements. Hyde and Walkinshaw (11), speculating on the significance of large osmiophilic bodies which were always associated with a large vacuole and vesicular membrane system in germlings of *Lenzites saepiaria*, suggested that "this entire complex may represent the mobilization of nutrients for the emerging germ tube." In the *Rhizopus* spore, vacuoles enlarge as germination proceeds. Also, during germination, the nuclei replicate, the spore becomes larger, and the mitochondria increase in size and number. Thus, even before the germ tube appears, the spore must have among its growth requirements an urgent need for structural units for membrane assembly. The information about the sequence of events during germination is still too fragmentary to tell whether the membrane-associated inclusions will be used up, or will persist, acting only as templates or sites for the assembly. It might be alternatively proposed that S inclusions are being produced by the membranous components with which they are associated. However, this would require an explanation for their formation at diverse sites. The reverse proposal recognizes that membrane elaboration should require the same materials, protein and lipid, to be brought together, whatever the site. The relative abundance of the inclusions in the spores should encourage attempts to isolate and further characterize them.

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