Fungal Lysosomes or Spherosomes

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ABSTRACT

Lysosomelike bodies in *Ceratocystis fagacearum, Ceratocystis fimbriata, Fomes annosus, Agaricus campestris, Alternaria tenuis, Piptocephalis virginiana,* and *Mycotypha microspora* reacted positively to the Gomori reaction for acid phosphatase, arylsulphatase, and deoxyribonuclease. These bodies fluoresced golden-yellow under ultraviolet light when stained with Nile blue, were visible in dark field, and appeared to be limited by a single membrane.

When the lysosomelike bodies were injured prior to histochemical reactions in *C. fimbriata,* the reaction time was shortened, indicating the presence of a membrane. A well-defined, single membrane was seen in fine structure around lysosomelike bodies of *C. fagacearum.* Deoxyribonuclease and arylsulphatase were found concentrated in the haustorium of *P. virginiana.*

Lysosomelike bodies play an essential role in the release of ascospores from the asci of *C. fimbriata.* The asci of *C. fimbriata* are unwalled, and there are two "ectoplasts" around the ascospores. It is concluded that fungi have lysosomes quite comparable to animal lysosomes, and it is suggested that the term "spherosome" be used synonymously with lysosome in fungi. Phytopathology 60:216-227.

First recognition of the nature and importance of lysosomes was through the pioneering work of deDuve & Wattiaux (6) and Novikoff (22). Early emphasis was on the autolytic properties of these bodies, but recent studies have shown that lysosomes play multiple roles in synthetic processes in the cell. The varied interpretations of lysosomal function from biochemical and cytochemical studies have made defining lysosomes difficult. There is some agreement now that lysosomes have a single membrane and show a positive staining reaction for acid phosphatase. Gahan (10) has emphasized that more than one acid hydrolytic enzyme should be detectable before a body is called a lysosome. Recently deDuve & Wattiaux (6) expanded the lysosomal concept to include most vacuolar components of the cell. They consider the vacuolar system, which contains hydrolytic enzymes, to constitute an intracellular digestive system comparable to the digestive tract of higher organisms.

It is only recently that attention has been given to lysosomelike bodies in plants. The enzyme constituency and function of such bodies in plants is poorly understood at the moment. Frey-Wyssling et al. (9) described single-membrane-bounded bodies in plants, called them spherosomes, and indicated that they were probably sites of lipid synthesis. Sorokin & Sorokin (30) characterized lysosomelike bodies in *Campanula persicifolia,* and concluded that these bodies are not sites of lipid synthesis. Semodeni (28) found lysosomes of corn to contain all the enzyme necessary for lipid synthesis, and concluded that this points to the possible conversion of these organelles to lipid bodies.

Matile (16, 17), Matile et al. (18, 19, 20), and Semodeni (28) found a number of hydrolytic enzymes associated with plant spherosomes (lysosomes) including protease, RNAase, β-amylase, α-glucosidase, phosphatase, esterase, arylsulphatase-C, and NADH-disphorase. Semodeni (28) found that β-glucuronidase, phosphalipase-C, lipase, and arylsulphatase A and B, all of which are typical enzymes of animal lysosomes (27), were absent in cell-free extracts of corn seedlings. There then may be basic differences in the enzyme composition and perhaps function of animal and plant lysosomes.

Microbodies and lysosomes have not been clearly distinguished in higher plants or fungi. Frederick et al. (8) characterized plant microbodies as having single bounding membranes, a granular to fibrillar matrix of variable electron density, and an intimate association with one or two cisternae of rough endoplasmic reticulum. Frederick & Newcomb (7) used "cytosome" rather than "microbody" as a general term to include all single-membrane-delimited organelles in plants, thus conforming to the usage of Mollenhauer et al. (21) in plants and deDuve & Wattiaux (6) in animals. Matile (17) expanded the lysosomal concept in plants in a manner comparable to that presented by deDuve & Wattiaux (6) for animals. Matile (17) indicated that the aleurone vacuole in pea seeds is a lysosome. He also found digestive vacuoles in corn and tobacco comparable to the cytolysomes of animal cells. Matile and his coworkers (18, 19, 20) concluded that plant lysosomes are quite comparable to animal lysosomes. Berjak (4) drew the same conclusion and presented evidence to indicate that the release of enzymes from plant lysosomes was responsible for the senescence of the old root cap cells in *Zea mays.*

The lysosomal concept has only been superficially explored in fungi (20). Armentrout et al. (1) found lysosomelike bodies (spherosomes) in a number of fungi. Pitt (23) and Pitt & Walker (25) found a particulate localization of acid phosphatase in *Botrytis cinerea* and a number of other fungi. A detailed histochemical study of *B. cinerea* revealed the presence of acid phosphatase, acid deoxyribonuclease II, β-galactosidase, and several esterases localized within particles. Attempts to demonstrate β-D-glucuronidase activity were unsuccessful; arylsulphatase activity was weak. Pitt (23) concluded that the particles in which these

enzymes are localized may be comparable to the lysosomes of animal cells. The structures that Pitt designates as particles are quite comparable in size and distribution to the spherosomes described by Armentrout et al. (1) and Armentrout & Wilson (2).

Because of the potency of their enzymatic packets, it is apparent that lysosomes may play important roles in host-parasite interactions during plant parasitism. Yet, except for the work of Pitt & Combes (24), no attention has been given this subject. Pitt & Combes (24) found that during the infection of *Solanum tuberosum* by *Phytophthora erythroseptica* Pethybridge, there was a swelling and disruption of lysosomelike bodies in the host, accompanied by the release of acid phosphatase and esterase. They demonstrated that infection of the host resulted in the liberation of acid phosphatase from the particulate to the supernatant fluid of cell homogenates. Armentrout & Wilson (2) found an interaction between spherosomes (lysosomes) of the host and parasite during mycoparasitism of *Piptocephalis virginiana* Ledbetter & Mercer on *Mycotypha microspora* Fenner. Spherosomes were seen migrating down the germ tube of the parasite into the haustorium. Histochemical tests revealed a heavy concentration of acid phosphatase at the surface of the haustorium. Within the parasitized host cell the spherosomes (lysosomes) were swollen and their membranes were often disrupted.

It was the purpose of this study to explore further the lysosomal concept in fungi, particularly plant pathogens. This was done through the histochemical and morphological characterization of lysosomes and observation on their behavior during healthy and pathological cellular events.

Evidence is presented that fungi have lysosomes comparable to animal lysosomes, and that fungal spherosomes are the same as lysosomes. Therefore, the term "lysosome" will be used synonymously with spherosomes in this paper.

MATERIALS AND METHODS.—Organisms used for light and electron microscopic observations were: *Basidiobolus ranarum* Eidam; *Ceratocystis fagacearum* (Bretz) Hunt and *Ceratocystis fimbriata* Ell. & Halst.; *Fomes annosus* (Fries) Cooke and *Agaricus campestris* L. ex Fr.; *Alternaria tenuis* Nees.; and for mycoparasitism, *P. virginiana* on *M. microspora*.

For light microscopy, organisms were cultured according to the method of Armentrout et al. (1) by placing blocks of agar with mycelium on sterile, moist petri dishes at 24 C for 24-36 hr. After incubation, the agar block with inoculum was removed from the slides, and a cover slip was placed on the remaining mycelium for microscopic observation. Slides prepared in the above manner were used for phase contrast, bright field, dark field, fluorescence microscopy, and for the demonstration of hydrolytic enzyme activity in the lysosomes.

For fluorescence, the method of Armentrout et al. (1) was employed. After incubation, 0.01% Nile blue (14) was allowed to flow under the cover slip, and slides were observed for fluorescence. Phase contrast, dark field, and bright field observations were made on all the above organisms.

For demonstration of acid phosphatase, acid deoxyribonuclease, and arylsulphatase activity, the following modifications of the Gomori lead sulfide precipitation procedure (15) were employed: To demonstrate acid phosphatase activity, slides were placed in a solution of 0.6 lead nitrate in 500 ml at pH 4.5 to which 50 ml of 0.1 M sodium glycerophosphate were added. After incubation at 37 C for 2-3 hr in this solution, the slides with mycelium were rinsed with distilled water and placed in 0.5% aqueous ammonium sulfide for 3-5 min and observed under bright field. For the demonstration of acid deoxyribonuclease activity, the method of Gomori was modified according to Aronson et al. (3); DNA (calf thymus, Sigma 1 mg/ml) was substituted for sodium glycerophosphate as the substrate. For the demonstration of arylsulphatase activity, a modification of the Gomori technique introduced by Goldfischer (12) was employed. In this procedure, *p*-nitrocatechol sulfate is substituted for sodium glycerophosphate as the substrate. The incubation medium was prepared by dissolving 15-30 mg *p*-nitrocatechol sulfate (σ) in 5.0 ml veronal-acetate buffer at pH 5.5. Controls used for the above enzyme activity demonstrations were (i) slides incubated in reaction medium without substrate; (ii) in ammonium sulfide only; and (iii) a mycelial mat boiled in water and carried through the entire procedure. No reaction for acid phosphatase, arylsulphatase or, deoxyribonuclease was visible in the checks (Fig. 1-D, 2-J).

The structure-linked latency for acid phosphatase activity was tested by the method of Gahan (10). Slides of *C. fimbriata* were incubated for 5, 10, 20, 40, and 60 min in the Gomori medium and observed for enzyme activity. To demonstrate activation of enzymes and decreased latency, different slides with *C. fimbriata* were incubated at 37 C in 0.05 M acetate buffer at pH 5.0 for 1 hr, in 10% Formalin containing 0.9% sodium chloride for 1 hr, and slides were treated with 0.25% Triton-X-100 detergent for 1 hr. All slides prepared for light microscopy were observed and photographed with a Zeiss Photomicroscope.

For electron microscopy, blocks of agar containing mycelium were minced and fixed in 3% glutaraldehyde in phosphate buffer at pH 6.8 for 3-4 hr. The tissue blocks were then washed in pH 6.8 phosphate buffer and postfixed in 2% osmium tetroxide (phosphate buffered to pH 6.8) for 2 hr. The tissue blocks were dehydrated in ethanol and placed in propylene oxide to which Epon mixture was added as the propylene oxide-Epon mixture was drawn off. Finally, the blocks were placed in pure Epon and then into Beem capsules. The embedded tissues were sectioned on an LKB Ultratome with glass knives, stained with uranyl acetate and lead citrate, and examined on a Seimans IA electron microscope.

To demonstrate acid phosphatase activity under the electron microscope, blocks of agar containing mycelium were minced and fixed in 3% glutaraldehyde in phosphate buffer at pH 6.8 for 3-4 hr. The minced tissue was then placed in the Gomori medium with sodium glycerophosphate as a substrate and incubated at 37 C for 24 hr. After incubation, the tissue was rinsed with distilled water, postfixed in 2% osmium tetroxide (phos-

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Fig. 1. Photomicrographs of histochemical reactions for hydrolytic enzymes in hyphae. (All about × 2000.) **A)** Gomori reactions for DNAase. **B)** Acid phosphatase. **C)** Arylsulphatase in *Ceratocystis fimbriata*. **D)** Control (no substrate) for Gomori reaction in *C. fimbriata*. **E, F)** Haustoria of *Piptocephalis virginiana* in *Mycotypha microspora*. Darkening around haustorial lobes (arrows) results from Gomori reaction for arylsulphatase. **G)** Phase-contrast view of lysosomes in *C. fagacearum*. **H)** Gomori reaction for acid phosphatase in *Ceratocystis fagacearum*. **I)** Fluorescence under ultraviolet light of lysosomes treated with Nile blue in *C. fagacearum*. **J)** Gomori reaction for DNAase in *Agaricus campestris*. **K)** Fluorescence under ultraviolet light of lysosomes in *A. campestris* treated with Nile blue. **L)** Gomori reaction for arylsulphatase in *A. campestris*.

phate buffered to pH 6.8) for 2 hr, and washed in buffer prior to preparation for electron microscopy as described above.

Results.—Lysosomes were most abundant in hyphal tip cells; some aspects of their morphology and behavior have been described previously (1, 2). The dimensions and movement of the lysosomes in the various fungi studied were similar. There was a tendency of lysosomes to aggregate in dead and dying cells.

Histochemical evidence.—In *B. ranarum*, *C. fagacearum*, *C. fimbriata*, *F. annosus*, *A. campestris*, and *A. tenuis*, lysosomes were visible in dark field, fluoresced golden-yellow under ultraviolet light when stained with Nile blue, and showed a concentration of acid phosphatase, acid deoxyribonuclease, and arylsulphatase activity. Representative reactions in the various organisms are illustrated in Fig. 1 and 2.

Because of the dark staining quality of lysosomes and lysosomelike bodies, it is difficult to determine the existence of a delimiting membrane in most electron micrographs. The histochemical determination of hydrolases in fine structure revealed a concentration of these enzymes at the surface of lysosomes, and this may explain the resulting difficulty in distinguishing a membrane.

Strong reaction in the particulate fraction was obtained after 20 min with no injury, 5 min with Triton X-100, and 0 time with Formalin or acetate buffer. No strong reaction was obtained in the diffuse fraction with no injury within 60 min after, and in 20 min with, Triton X-100 injury; 10 min with Formalin; and 5 min with acetate buffer (Table 1).

Histochemical Gomori reactions for acid phosphatase were made and examined in thin sections under the electron microscope. Concentrations of these enzymes were greatest at the surface of the lysosomes of *C. fimbriata* (Fig. 3-A).

Lysosomes in mycoparasitism.—Armentrout & Wilson (2) found an interaction of the lysosomes in the host *M. microspora* and the parasite *P. virginiana* during mycoparasitism. They found acid phosphatase concentrated at the surface of the haustorium. We found deoxyribonuclease and arylsulphatase (Fig. 1-E, F) concentrated in the haustorium of *P. virginiana*. This is added evidence that lysosomes are involved in the release of enzymes at the haustorial surface.

Ascospore liberation in C. fimbriata.—Evidence was found that lysosomes play a major role in the liberation of ascospores from the asci in *C. fimbriata*. It has long been recognized that the asci of species of *Ceratocystis* "deliquesce" prior to ascospore discharge out the neck of the perithecium. Through fine structure studies we found a relationship between the behavior of the lysosomes in the ascus and this lytic process.

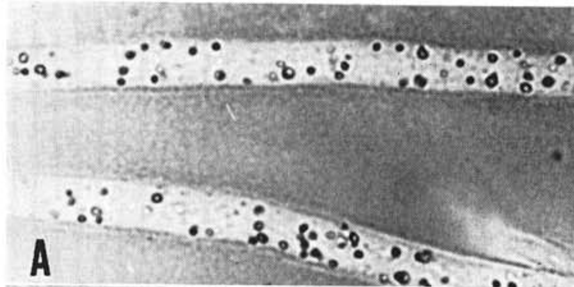
In asci with immature ascospores, the lysosomes are dark-staining and spherical (Fig. 4-A, 5-E, 6-A). As the ascospores mature, there is a concomitant enlargement of the lysosomes and a lightening of their staining reaction (Fig. 5-E). When the outer sheath is formed around the ascospores and the ascospores are fully formed, the lysosomes lose their distinct spherical shape and become more amorphous (Fig. 6-A). Lysosome membranes fuse with vacuoles, presumably forming autophagic vacuoles (Fig. 5-A, C, D) delimited by single membranes. Membrane debris is detectable within these vacuoles (Fig. 5-D).

Associated with the change in lysosomal morphology is a change in the extra-spore cytoplasm and the membrane around the ascus. The glycogen in the cytoplasm disappears and the cytoplasm becomes granular as the membrane surrounding the ascus becomes faint and disappears (Fig. 4-C). The breakdown of the extra-spore contents of the ascus and the ascus membrane is associated with the release of contents from individual lyso-

Table 1. Gomori reactions in *Ceratocystis fimbriata* after different intervals of time following various treatments

Injurious treatments	Incubation time, min 0	5	10	20	40	60
	Particulate fraction					
None	0[a]	+	+	+++	+++	+++
Triton X-100	+	+++	+	+	+	+
Formalin	+++	+++	+	+	+	+
Acetate buffer	+++	+++	+++	+	+	+
	Diffuse fraction					
None	0	+	+	+	+	+
Triton X-100	+	+	+	+++	+++	+++
Formalin	+	+	+++	+++	+++	+++
Acetate buffer	+	+++	+++	+++	+	+++

[a] 0 = No Gomori reaction; +++ = strong reaction.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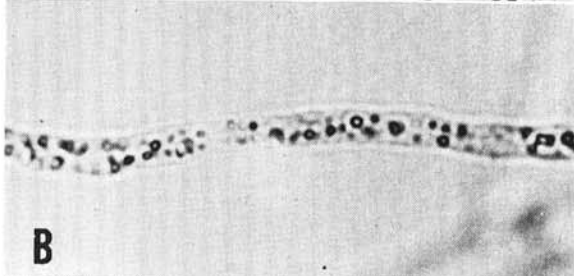
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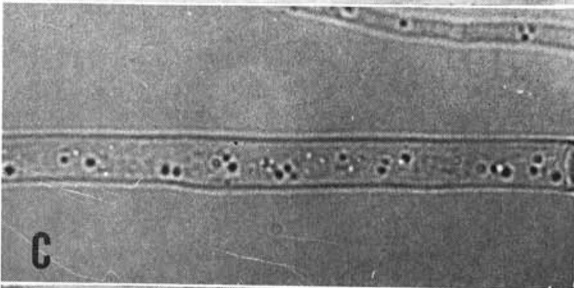
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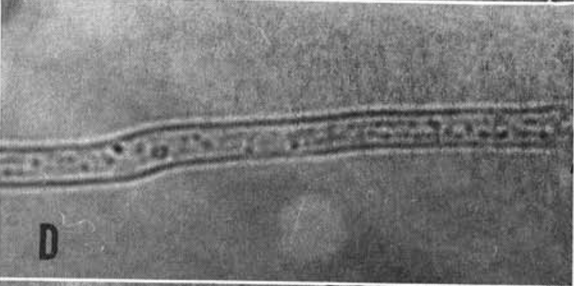
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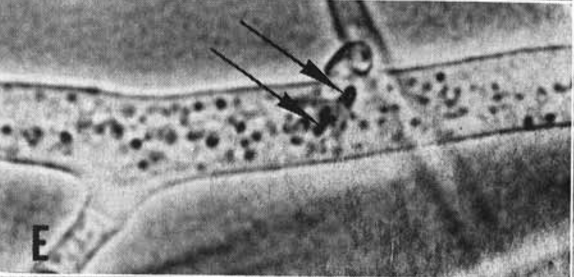
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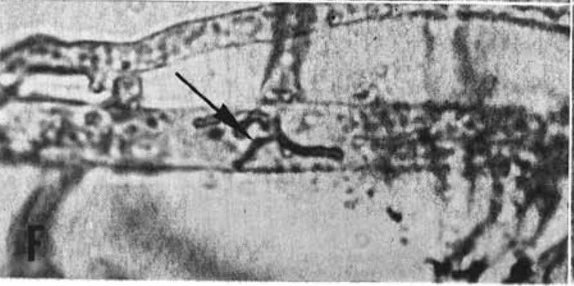
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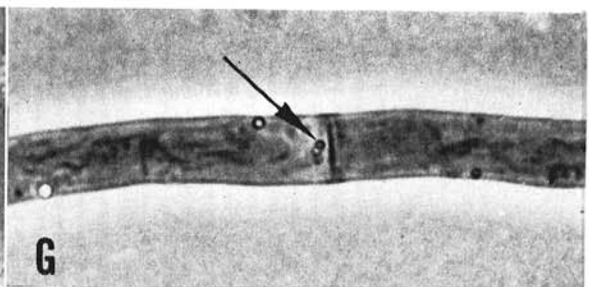
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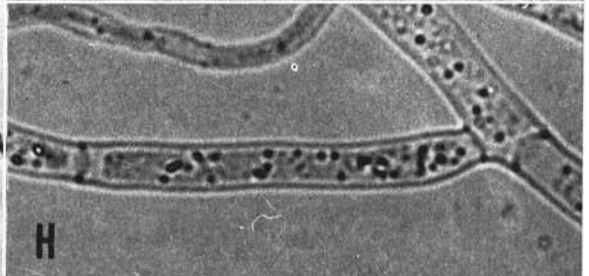
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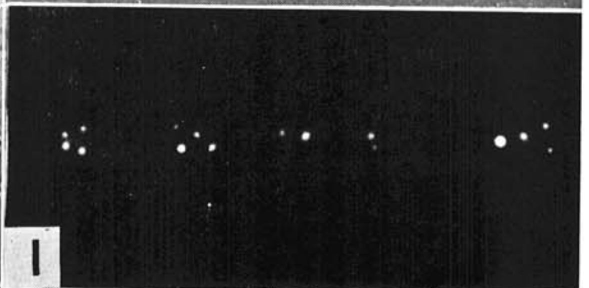
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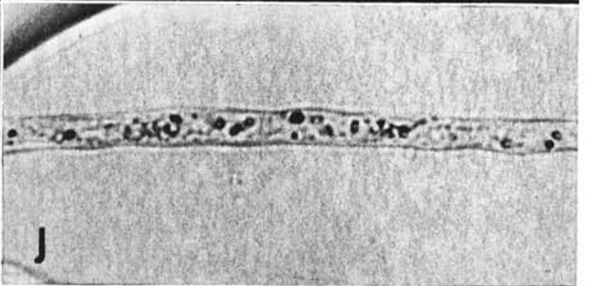
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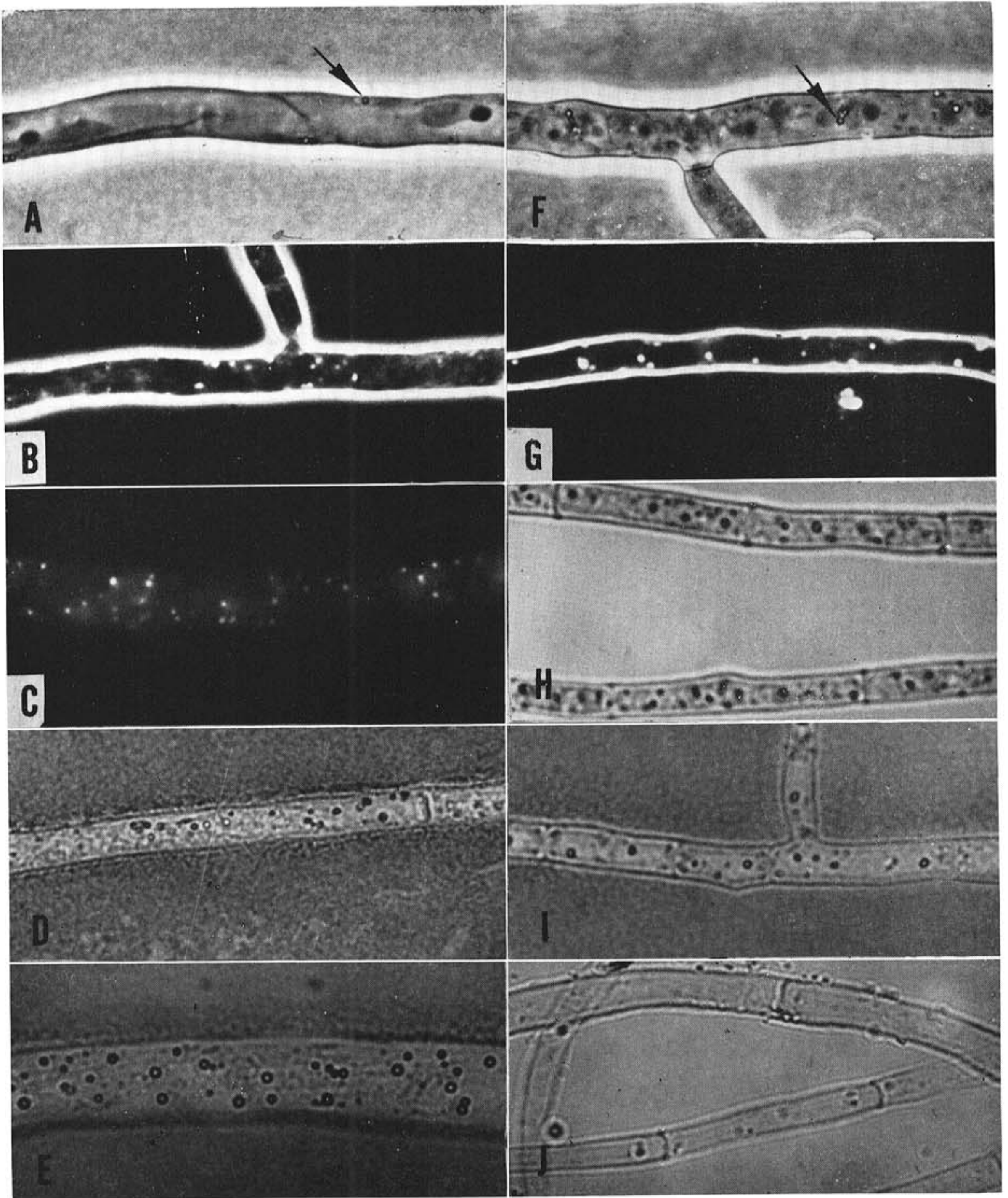

Fig. 2. Photomicrographs of histochemical reactions for hydrolytic enzymes in hyphae. (All about × 2,000.) **A)** Phase-contrast view of lysosomes (arrow). **B)** Dark field view of lysosomes. **C)** Fluorescence under ultraviolet light of lysosomes in *Fomes annosus* treated with Nile blue. **D)** Gomori reaction of DNAase in *F. annosus*. **E)** Gomori reaction for DNAase in *Basidiobolus ranarum*. **F)** Phase contrast view of lysosomes (arrow) of *Alternaria tenuis*. **G)** Dark field view of lysosomes in *A. tenuis*. **H)** Gomori reaction for arylsulphatase in *A. tenuis*. **I)** Gomori reaction for DNAase in *A. tenuis*. **J)** *A. tenuis* control for Gomori reaction (no substrate).

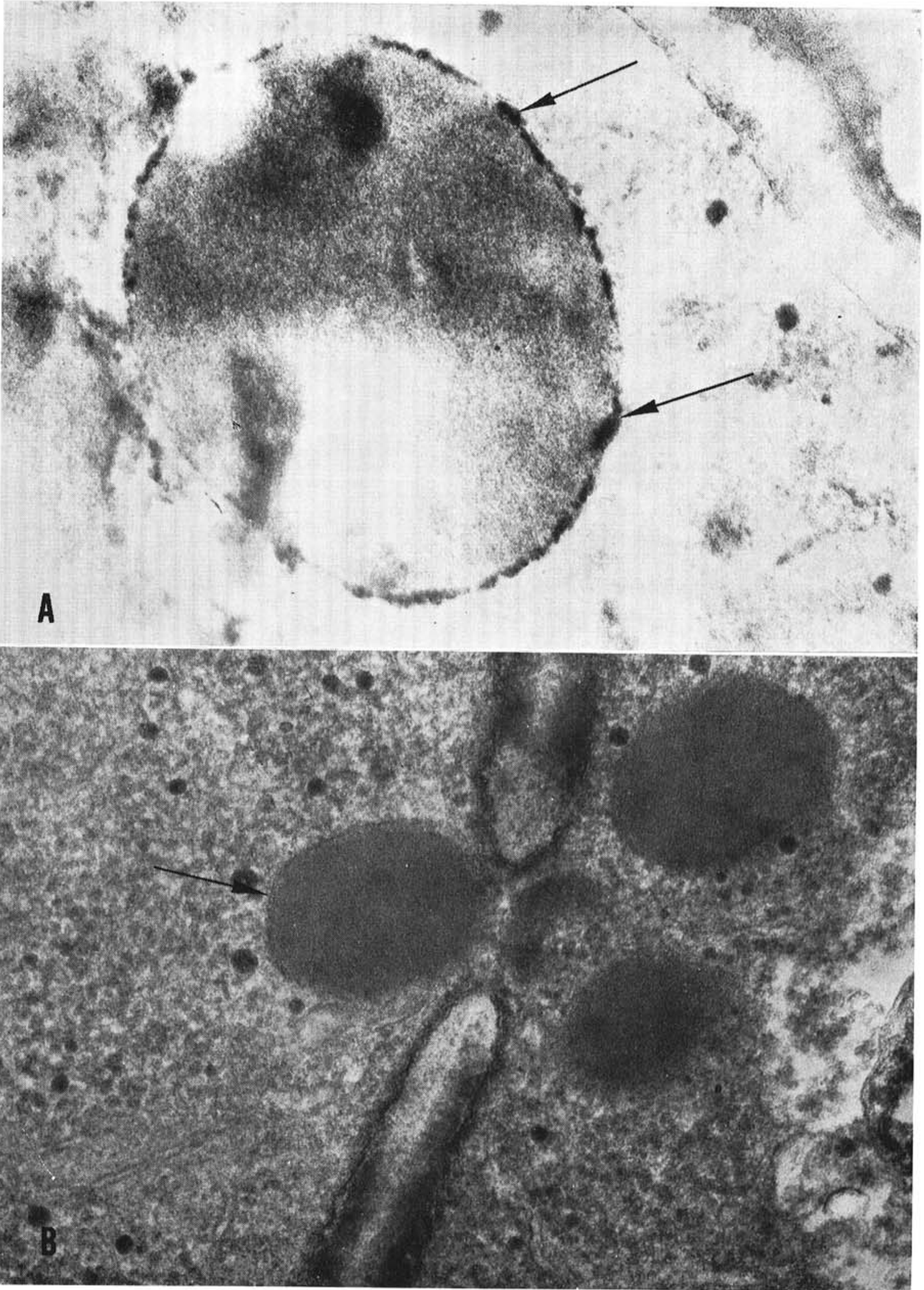

Fig. 3. A) Electron micrograph of hypha showing Gomori reaction for acid phosphatase in *Ceratocystis fimbriata*. Note indications of a concentration of enzyme at the surface of the lysosome (about × 120,000). **B)** Lysosome at a septal pore of *C. fagacearum*. Note membrane (arrow). (About × 40,000)

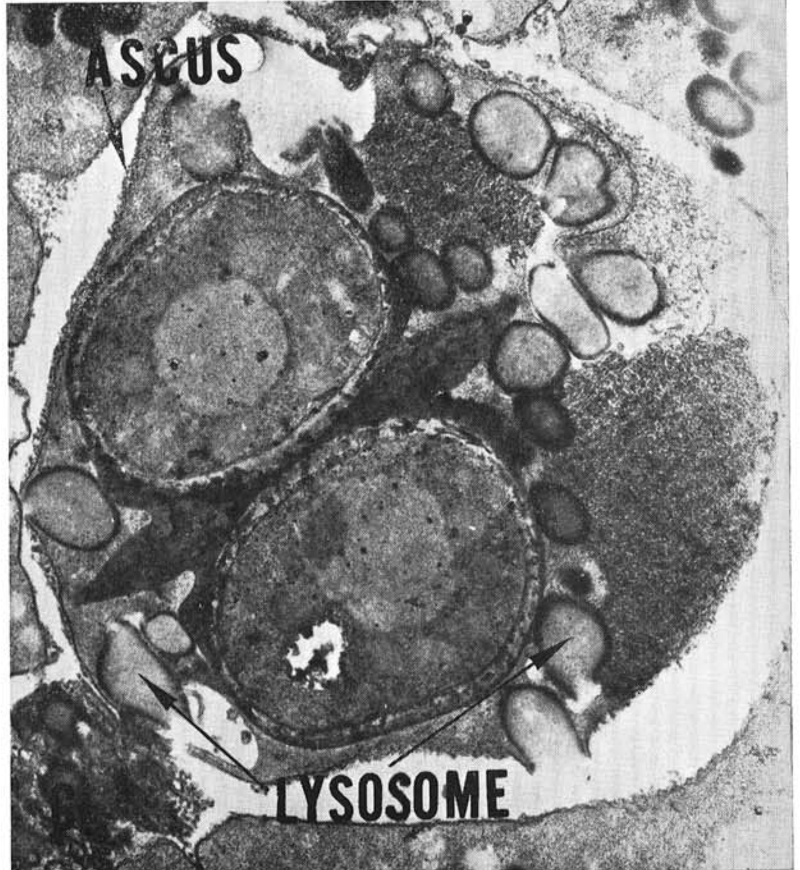

Fig. 4. Centrum of *Ceratocystis fimbriata.* **A)** Asci, ascogenous cells, and ascospores. Note unwalled cells (protoplasts). (About × 6,000) **B)** Membranes of two adjacent asci. (About × 40,000) **C)** Ascus with mature ascospores. Note brim arrangement of spores and "deliquescence" (lysis) of ascus membrane associated with lysosomes. (× 15,000)

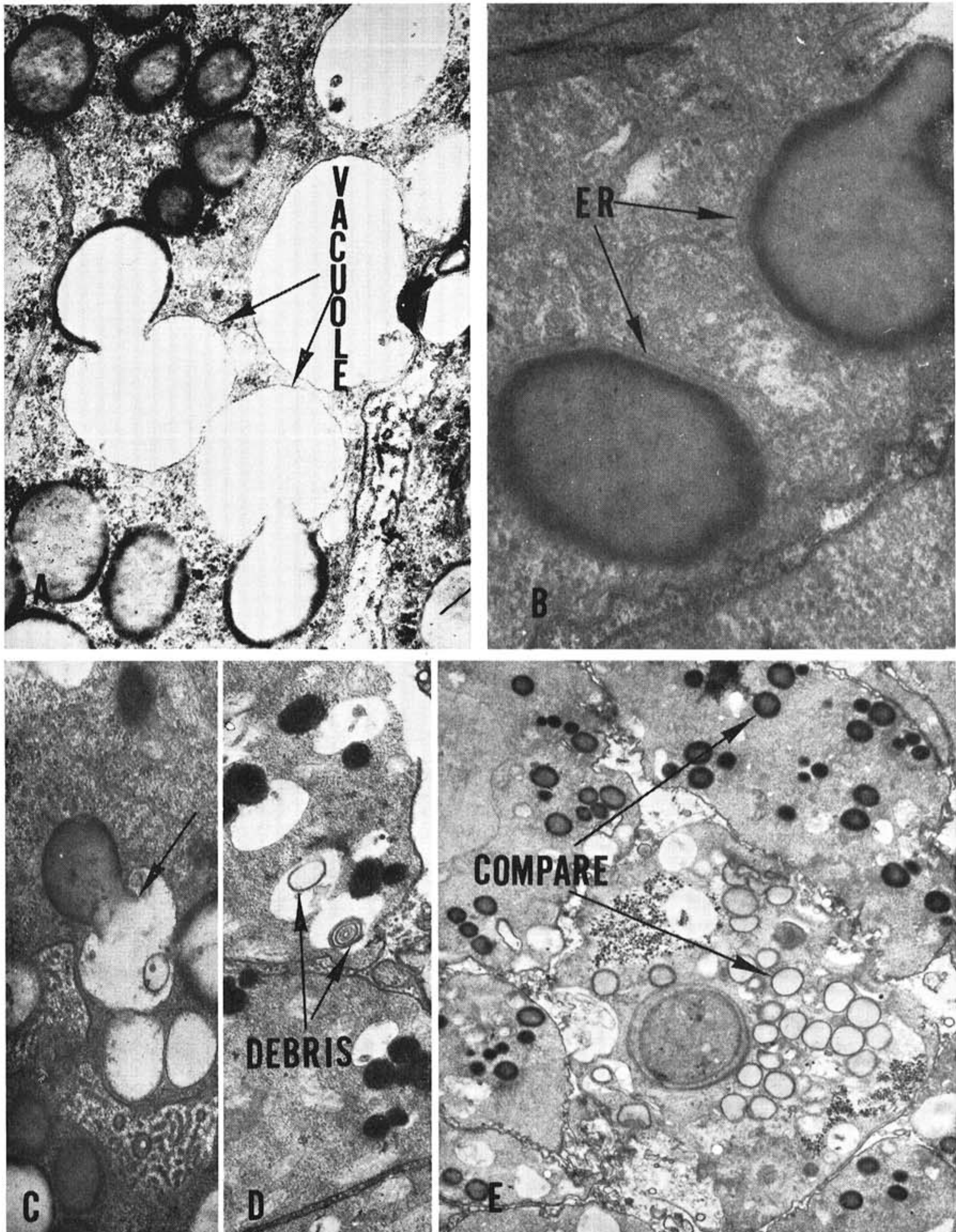

Fig. 5. Lysosomes and autophagic vacuoles in the centrum of *Ceratocystis fimbriata*. **A)** Lysosomes fusing with vacuoles apparently forming autophagic vacuoles (arrows) (About × 16,000) **B)** Endoplasmic reticulum associated with lysosomes. (About × 40,000) **C)** Lysosome "rupturing" into autophagic vacuole. (About × 14,000) **D)** Membrane debris in autophagic vacuoles (arrows). (About × 8,000) **E)** Lysosomes in ascus with maturing ascospores are lighter staining than lysosomes in young ascus. (About × 6,000)

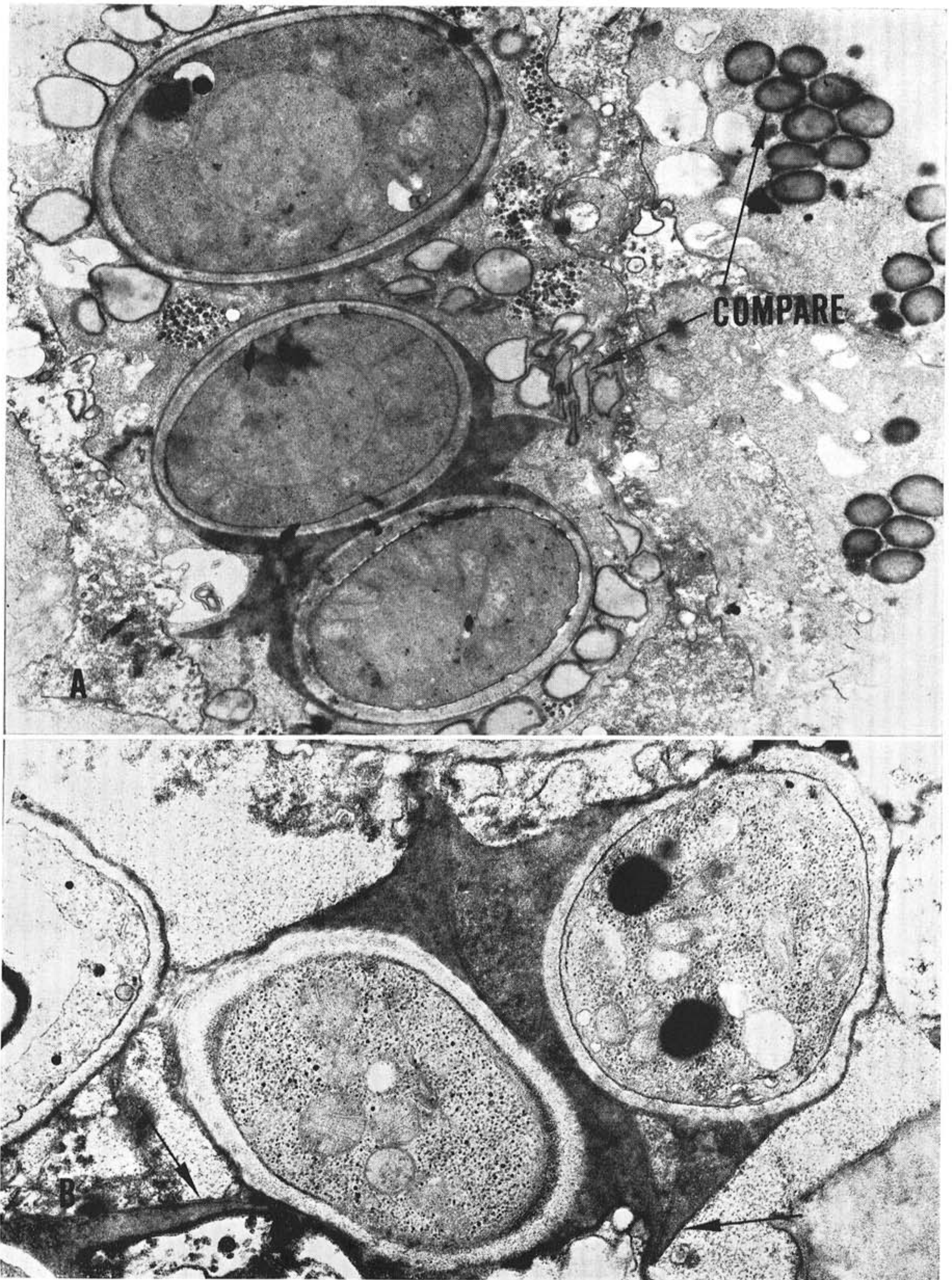

Fig. 6. Mature ascospores of *Ceratocystis fimbriata* in deliquescing asci. **A)** Note amorphous shape of lysosomes in lysing ascus. (About $\times$ 15,000) **B)** Mature ascospores with ascus completely digested. Note membrane around sheath of ascospores (arrow). (About $\times$ 20,000)

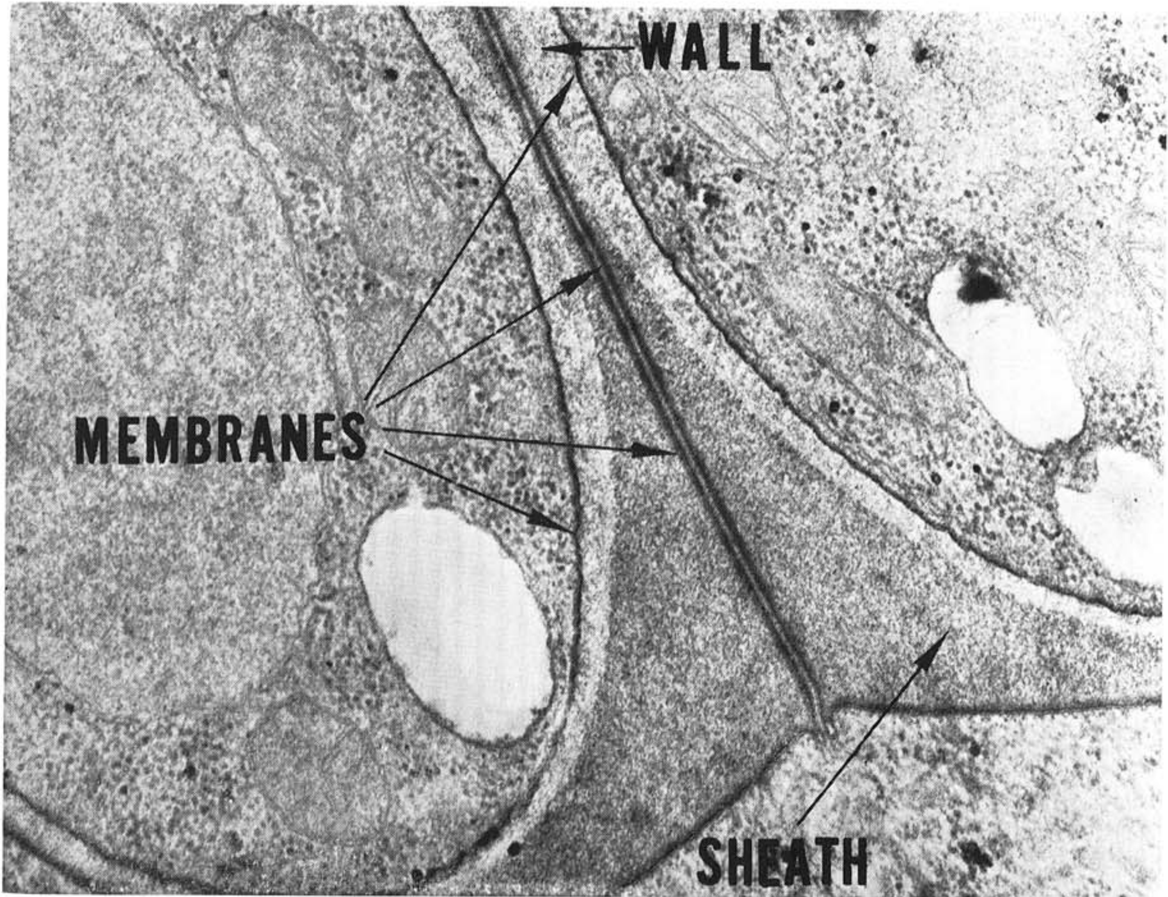

Fig. 7. Two developing ascospores of *Ceratocystis fimbriata*. Note membrane around the outer sheath of the ascospore in addition to membrane around protoplasts. (About × 40,000)

somes, since the greatest effect is often near the lysosome. Occasionally, a close association between lysosomes and the endoplasmic reticulum was detectable (Fig. 5-B).

The asci of *C. fimbriata* are unwalled and delimited by a single membrane (Fig. 4-B). The hat-shaped ascospores of *C. fimbriata* have a dark-staining sheath outside the spore wall (Fig. 6-A, B, C). We found a membrane surrounding the sheath and the entire spore (Fig. 6-B, 7).

DISCUSSION.—Gahan (10) defines a lysosome as a single-membrane-bounded organelle that contains more than one acid hydrolytic enzyme. The presence of at least three acid hydrolytic enzymes in these organelles satisfies the multi-enzyme requirement of his definition. Membranes can be seen around these bodies in *C. fagacearum* (Fig. 3-B) and *M. microspora* (2).

There is confusion over the terms used to designate single-membrane-bounded organelles (cytosomes) in higher plants and fungi. It is desirable at this time to follow the terminology outlined for animal cells (6), at least until more fundamental differences are found between animal and plant single-membrane-delimited organelles, including lysosomes and microbodies. The contention by Frederick et al. (8) that "It is not unreasonable to assume that plant microbodies will be found to contain a changing enzyme complement that includes both hydrolases and oxidases at one stage or another during cellular differentiation", if true, would make a distinction between lysosomes and microbodies difficult or perhaps impossible. Exhaustive studies of microbodies (21) and lysosomes in the same higher plant and fungus are needed.

Although there has been little detailed study of lysosomelike bodies in plants, Gahan (11) states the presence of gross differences between the hydrolase-containing particles in plants and animal cells. He proposes that the term "lysosome" should be reserved for the particles present in animal cells, "spherosome" for the particles present in plant cells. It is impossible at this stage to know whether fungal hydrolase-containing bodies are more like those of higher plants or animals. However, the finding of at least three acid hydrolytic enzymes concentrated in these bodies, and the finding that the enzyme activity is structurally linked, would seem to provide ample justification for calling these bodies lysosomes in the fungi studied.

The time of the Gomori reaction was shortened by previously injuring the cell. This, according to Gahan (10), may result from injury to the lysosomal mem-

brane, thus allowing reagents to penetrate more rapidly. The more diffuse reaction of the enzymes in the injured cells further indicated membrane injury (Table 1). Thus, these data provide additional evidence for the existence of a membrane around these lysosomelike bodies.

It appears in some cases that microbodies and lysosomes have been confused. "Microbodies" are illustrated in *Phytophthora parasitica* as containing "myelin figures" typical of lysosomes (13). Fixation of tissue with potassium permangenate destroys lysosomal contents, and this may explain why this organelle has been overlooked by many investigators, as may be the case in *Gilbertella persicaria,* where "lypid bodies" could be the remnants of lysosomes (5).

Certain cellular inclusions in higher plants and fungi have been termed "lipid bodies", "fat bodies", "granules", "osmiophyllic bodies", etc. The dense staining of these bodies in preparations for electron micrographs makes deliberations over the presence or absence of a membrane difficult, and there is, therefore, confusion over the relationship of such bodies to lysosomes and microbodies. Sorokin (29) found that spherosomes (lysosomes) contained mostly phospholipids and very little neutral lipid, while most reserve oil droplets were of neutral lipid. Illustrations by some authors of "lipid bodies" suggest organelles comparable to our concept of a lysosome. A clearer distinction between lipid inclusions in the cell and lysosomes is needed, and the role of lysosomes in lipid synthesis needs further study.

Lysosomes play important roles in various aspects of animal diseases (6). Yet, only slight consideration has been given their involvement in plant diseases and their control. Lysosomes may be involved in the penetration of plant host cells as they are in animal cells (26). In some cases, lytic responses of host tissue to invasion may be the direct result of the release of hydrolases from host lysosomes (24). Lysosomes may be involved in host resistance and the detoxification of biocides.

The lysosomes in the asci of *C. fimbriata* appear to provide a mechanism for the liberation of the ascospores from the ascus. Through the release of their enzymes they cause the digestion of the surrounding cytoplasm and ascus membrane. The digested extraspore material constitutes the sticky matrix in which the ascospores are embedded when they are extruded from the perithecium.

Lysosomes may play a role in other fungal fructifications through the digestion of nonspore materials and the subsequent release of both sexual and asexual spores. Lysosomes may be involved in autodigestion of cell walls in the formation of anastomoses, rapid lysis of certain basidiomycetous fructifications, and fungi dependent upon the digestion of host tissue for the release of their spores (e.g., *Plasmodiophora brassicae*) could have a "release mechanism" comparable to that found in *C. fimbriata.*

The physiological activity, if any, of the outer spore membrane in *C. fimbriata* needs investigation. If this is a physiologically active membrane, materials that enter the ascospore must then pass through two "ectoplasts", and the ascospores are essentially protoplasts which contain a walled uninucleate cell.

It is recognized that a clear link between the lysosomelike bodies in ultrastructure and those seen with light microscopy was not established in this study. However, the fusion of the bodies in ultrastructure with vacuoles and their association with lytic processes indicates that they are lysosomes. Also, acid phosphatase was found concentrated in the bodies as seen in electron micrographs.

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