ULTRASTRUCTURE AND PHYSIOLOGY OF GERMINATION IN *PHYTOPHTHORA PARASITICA*

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE
UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN MICROBIOLOGY
MAY 1970

by

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ABSTRACT

Flagella and cleavage vesicles form during the initial stages of direct sporangial germination in Phytophthora parasitica in the same manner as they do in indirect germination. Later, however, the flagella degenerate and cleavage of the cytoplasm is not complete. Instead, a new wall layer is deposited onto the existing sporangial wall, and this germination wall extends with the germ tube and forms the hyphal wall. Material for the germination wall first appears around vesicular aggregates concentrated at the periphery of the sporangial cytoplasm. As the wall forms the vesicular aggregates become encased in it, and the wall eventually consists of irregular deposits of wall material interspersed with the vesicular aggregates. These are clearly cytoplasmic in nature and different from lomasomes, since they are associated with ribosomes and other small elements of the cytoplasm. Likely to contribute to the wall formation are the endoplasmic reticulum, microbodies, dictyosome-derived vesicles, and possibly polyvesicular bodies and larger vacuoles with fibrillar contents. The basal plug of the sporangium forms via the same mechanism as the germination wall and also contains numerous vesicular inclusions. Fewer vesicular inclusions are found in the sporangial wall, and none have been observed in the walls of the growing hyphae. This difference in wall structure can be explained on the basis of their different growth patterns. Penetration of the sporangial plug is concomitant with prominent lomasomal activity and the cytoplasm at the hyphal tip is characterized

by the presence of numerous vesicles, which are probably derived from dictyosomes.

Ultrastructural studies of cyst germination and inhibition of direct cyst germination confirm the role of cellular organelles in wall formation and hyphal growth. In direct sporangial germination and in elongating hyphal tips, dictyosome-derived vesicles appear to function as a transport system for wall precursor material. In indirect germination, on the other hand, vesicles occur which are also most likely produced by dictyosomes, but their main function is to form the plasmalemma of the developing zoospores in the sporangium. The dictyosome thus probably performs a dual role in Phytophthora: during direct germination it might serve primarily as a transport system for wall material; during indirect germination it appears to function as a membrane donor.

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CHAPTER I

INTRODUCTION

In a physiological sense germination in fungi consists of the transformation from the relatively inactive state of the spore to that of the highly active vegetative thallus. It is usually characterized by the absorption of water, an increase in respiration, and a higher rate of biosynthesis. Morphological changes accompany this transition; the spore generally swells, forms a germ tube, and eventually produces a mature thallus. In recent years, ultrastructure research has added a new dimension to mycology and plant pathology. We can now think of spore germination not only in terms of the shape, size, and mutual arrangement of cells but also as a multitude of minute but significant integrated changes of subcellular components.

This study was initiated to correlate with an ultrastructural study of indirect germination in Phytophthora parasitica by Hohl and Hamamoto (1967). Phytophthora parasitica, strain P 113, proved to be particularly useful for the study of the ultrastructural aspects of germination, since the formation of the sporangia and their mode of germination, either direct or indirect, could be controlled by environmental conditions (Aragaki and Hine, 1963; Aragaki, Mobley, and Hine, 1967). During indirect germination the sporangial cytoplasm is cleaved by a mechanism involving Golgi-derived cleavage vesicles to form biflagellate zoospores which are released from the sporangium. During direct germination the sporangia germinate with one or several germ tubes and no motile stage is formed. Hohl and Hamamoto (1967)

suggested that the Golgi apparatus vesicles might have a dual role in morphogenesis, one involving mainly their membranes, a case observed in the formation of cleavage vesicles during indirect germination, the other their contents, possibly in the form of wall material being transported to a newly forming vegetative wall or to the tip of the growing germ tube in direct germination.

The major aim of this study was to elucidate the ultrastructural aspects of direct germination in Phytophthora parasitica and to compare these aspects with indirect germination. Emphasis will be placed on the type of germinal wall formation, hyphal extension, and the role of cellular organelles in these developmental processes. Although relatively few fungi have been examined, it has been proposed that there are three, basically different mechanisms of vegetative wall formation during spore germination, each type being characteristic of certain groups of fungi (Bartnicki-Garcia, 1969). In one type the wall is produced as an extension of the spore wall, or one of its innermost layers. A second type involves the de novo formation of a vegetative wall under the spore wall. The third type is the de novo formation of a cell wall on a naked protoplast. This occurs during the encystment of the zoospores of aquatic Phycomycetes. The wall composition of Phytophthora parasitica is known to be largely glucan and not chitin or cellulose (Bartnicki-Garcia, 1966), making it possible to determine whether the composition is consistant with the categories of wall formation proposed.

Beside germinal wall formation, wall synthesis will be studied at the germ tube apex. Grove et al. (1967), Girbardt (1969), and Hemmes

and Hohl (1969) have stressed the importance of a vesicular zone at the apex of the hyphal tip. In other fungi, a fibril-free region at the hyphal apex (Strunk, 1963) and mitochondria (Hawker and Abbott, 1963) have been found concentrated at the hyphal tip and implicated in wall formation.

Little is known about the mechanism by which the vegetative wall material is transported and assembled at the cell periphery. Marchant, Peat, and Banbury (1967) have proposed that polyvesicular bodies pass through the plasmalemma to form the lomasomes and that they are uniquely involved in the formation of chitinous walls only. Other studies have implicated the endoplasmic reticulum, dictyosomes, lomasomes, or the plasmalemma in germinal wall formation (Bracker, 1967). Special attention has been given to this problem, since preliminary studies have shown that polyvesicular bodies, lomasomes, and dictyosomes are abundant in the sporangia, cysts, and hyphae of Phytophthora parasitica.

Ho and Hickman (1967) pointed out that secondary zoospore production and hyphal germination of cysts are analagous to indirect and direct germination in sporangia of Phytophthora megasperma var. sojae. The two types of cyst germination are also found in Phytophthora parasitica and makes available a simple, synchronous system for additional study and comparison of the role of cellular organelles in wall formation and hyphal extension.

The study will be divided into the following parts: (1) The physiology and control of germination, (Chapter III), (2) Electron microscopy of direct sporangial germination, (Chapter IV), (3) Electron

microscopy of encystment and cyst germination, (Chapter V), and

(4) Electron microscopy of inhibition of cyst germination (Chapter VI).

CHAPTER II

MATERIALS AND METHODS

A. Culture Methods: Phytophthora parasitica Dast., reclassified as Phytophthora palmivora Butler by J. Tokunaga (1969), strain P 113, used throughout this study, was provided by Dr. R. B. Hine of the Department of Plant Pathology, University of Hawaii. It represents a single spore isolate and has the advantage that sporangium formation can be uniformly induced by exposing the dark-grown mycelium to light (Aragaki and Hine, 1963).

The fungus was grown in darkness at 31 C on a vegetable-juice agar (10% Campbell Soup Corp. V-8 juice, 0.1% $CaCO_3$, 1.5% agar) in 60 x 15 mm petri dishes. After 3-4 days the cultures were transferred to room temperature under fluorescent light of approximately 500 foot-candles for 24 hrs (temperature at level of plates approximately 28 C). Petri dishes heavily covered with sporangia were flooded with 5 ml of the desired incubation medium. Submerged sporangia were then brushed loose from the hyphae with a transferring loop and transferred to 60 x 15 mm plastic petri dishes for further study.

B. Physiological Studies: For direct germination a 50% papaya extract was made by mixing equal weights of papaya pulp (Carica papaya L.) in the green-yellow stage of ripeness, from the Department of Tropical Agriculture Farm, University of Hawaii, Waimanalo, Hawaii, with 20 mM Sörenson's phosphate buffer in a waring blender for 2 min. This extract was centrifuged at 2500 rpm for 15 min and dialyzed by

claved for 15 min at 120 C and brought back to the original volume with fresh, sterile phosphate buffer. For indirect germination 20 mM phosphate buffer or distilled water was used as the germination solution. The percentage of indirect sporangial germination was determined after 30 min and of direct germination after 5 hrs of incubation.

Zoospores for encystation and cyst germination studies were produced by incubating a sporangial suspension at 28 C for 15 min in distilled water and then decanting twice in order to separate the zoospores from sporangial cases and ungerminated sporangia. The percentage of direct cyst germination was determined 5 hrs after zoospore release, whereas, indirect cyst germination was determined after 10 hrs.

Triplicate measurements were made for each parameter, counting three microscope fields per plate containing a minimum of 50-100 sporangia or cysts per field.

C. Light Microscope Studies: Light microscope photographs were taken with a Zeiss phase microscope using Kodak Plus-X Pan sheet film.

D. Electron Microscope Techniques: Direct Sporangial Germination. Two 60 x 15 mm petri dishes heavily covered with sporangia were flooded with a total of 5 ml of 25% papaya extract (25 g of papaya plus 75 ml of distilled water). The suspension was transferred to 60 x 15 mm plastic petri dishes and incubated at 28 C. Samples were fixed at time 0 and after 1.5, 2.5, and 3.5 hrs of incubation by adding an equal volume of cold, 2.5% phosphate-buffered glutaraldehyde,

pH 7.4, into the suspension. After 0.5 hr of fixation, the suspension was centrifuged at low speeds and the pellets were mixed with an equal volume of 3% agar, cut into small blocks and resuspended in fresh fixative for 0.5 hr. The blocks were then washed in Sörenson's phosphate buffer at pH 7.4 for 0.5 hr. One group of blocks was postfixed for 5 min in 1% Palade's osmium tetroxide (Palade, 1952) at pH 7.4, the other group for 1 hr in cold, 2% unbuffered potassium permanganate.

Cyst Germination. Separate fixation procedures were used for the study of encysting and mature cysts. Suspensions of zoospores in distilled water or 50% papaya extract were fixed 0.5 hr after release from sporangia with an equal volume of 2% glutaraldehyde in 0.5 M sodium cacodylate buffer at pH 7.2. At this time approximately 50% of the cells had encysted. The suspensions were fixed for 1 hr, centrifuged at low speeds, embedded in 3% agar, and washed in cacodylate buffer, pH 7.2, for 0.5 hr. Postfixation was for 1 hr in 1% Palade's osmium tetroxide.

Suspensions of mature cysts in 50% papaya extract or distilled water were fixed with an equal volume of GOP fixative (Schäfer-Danneel, 1967), 1½ and 5 hr after release from sporangia respectively, at a time when germ tube growth had been initiated in both suspensions of cells. The suspensions were fixed for 0.5 hr, centrifuged at low speeds, embedded in 3% agar, and resuspended in fresh fixative for 0.5 hr. All agar blocks were then washed in Sörenson's phosphate buffer at pH 7.4 for 0.5 hr and stained with 0.5% veronal-acetate buffered uranyl acetate, pH 7.2, for 1 hr.

All tissues were dehydrated in ethanol and followed by embedding in epon. Thin sections were poststained in lead citrate (Reynolds, 1963) and viewed with a Hitachi HU-11A electron microscope.

E. Inhibition Studies: Twenty-four hour cultures of sporangia were flooded with 10^{-3} g/ml solutions of Acti-dione (Upjohn Company) and transferred to 60 x 15 mm plastic petri dishes for further study. Suspensions of inhibited cysts in 50% papaya extract were fixed 1, 3, and 5 hrs after zoospore release with an equal volume of GOP fixative (Schäfer-Danneel, 1967). The suspensions were fixed for 1 hr and embedded as the normal, directly germinating cysts.

CHAPTER III

RESULTS

Germination Types: Phytophthora parasitica has the ability to germinate in different ways depending on the incubation medium. Basically, the sporangia germinate in two ways. After inoculation of sporangia into distilled water, release of biflagellate zoospores results in excess of 90% of the sporangia within 30 minutes (indirect sporangial germination, fig. 1-4). In 50% papaya extract, germ tubes are extended in up to 90% of the sporangia after five hours of incubation (direct sporangial germination, fig. 1-3) and no motile stage is formed.

Zoospores released into an aqueous medium round up and encyst within one hour. The cysts, too, may germinate by more than one means. Cyst germination can be controlled by placing the cysts in distilled water to produce secondary zoospores (indirect cyst germination, figs. 1-6, 1-7, 1-8), or 50% papaya extract to produce germ tubes (direct cyst germination, figs. 1-9, 1-10). In both types of germination germ tubes are produced initially. Approximately 90% of the zoospores in papaya extract produce germ tubes having an average length of 40 μ at 2 hrs after release from the sporangium (fig. 1-9). However, germ tubes are not extended from cysts in distilled water until 5 hrs of incubation. At this time, these cysts extend a short germ tube which attains an average length of 6 μ (fig. 1-6). Within two hours approximately 30% of these cysts produce a single secondary zoospore, which exits through the short

germ tube, leaving empty cyst cases in the medium (fig. 1-8). The temporal sequence of the germination types is graphically illustrated in figures 1-21 and 1-22.

Physiology and Control: Density of sporangia, culture temperature, sporangial age, and pH were factors considered for optimal percentages of spore germination. Density. The density of sporangia or cysts in the incubation medium, within the range tested, does not have an effect on the percentage or type of sporangial germination (fig. 1-23). Indirect germination averaged 95% and direct germination 85% in cultures with sporangial densities ranging from 40-320 sporangia per sq. mm. The percentage of cyst germination was also over 90% regardless of cell density. Temperature. Temperature optimum is 28 C for both indirect and direct germination, in agreement with the results of Aragaki et al. (1967). Sporangial Age. Approximately 90% of the sporangia on culture plates germinate indirectly or directly up until 144 hrs. At this time there is a sharp drop to 25% germination and a subsequent decline (fig. 1-25). pH. The pH has little effect on indirect germination in the range from pH 5.5 to 8.0, but the cultures have a maximum of 95% at pH 7.0 and minima of 90% at the extremes tested, i.e., pH 5.5 and 8.0. The extent of direct germination changes little over the pH range tested (fig. 1-24).

The optimal conditions for sporangial or cyst germination as determined are: cultures 24-48 hrs old, incubated at 28 C at pH 7.0, with a sporangial density of approximately 160-240 sporangia/sq. mm.

The percentage of direct germination, 51% after 11 hrs reported by Aragaki et al. (1967), was greatly enhanced by modification of the 25% papaya extract. These modifications included dialyzation of a well-blended 50% aqueous papaya extract, boiling the dialyzate, and adjustment of the pH with Sörenson's phosphate buffer to a final pH of 7.0. These steps increased the extent of direct germination to 80% within 3 hrs (fig. 1-26).

An artificial medium for direct germination was devised. A large papaya (5 x 4 in) has the following major components by weight: moisture 86.80%, total carbohydrate 12%, and protein 0.39% (Wenkam and Miller, 1965). An artificial incubation medium was prepared containing 12% sucrose, 0.39% Caseamino acids, and 0.03% calcium ions in 20 mM Sorenson's phosphate buffer at pH 7.0. Results are shown in fig. 1-27. The only difference noted from sporangia germinating in papaya extract was the lack of hyphal branching (fig. 1-18). Sucrose alone will retard release of zoospores, but will not induce direct germination by itself (fig. 1-20).

A further analysis of the components of the artificial medium showed that sucrose was not a specific component. Glucose, trehalose, or galactose were approximately equal to sucrose and maltose or dextrin even better carbohydrate components in stimulating extensive direct germination (fig. 1-28). Likewise, several single amino acids, serine and tyrosine, stimulated much more extensive direct germination than others (fig. 1-29). A third variable of importance is the pH of the incubation medium. The percentage and rate of germ tube production increased as the pH approached that of a papaya extract, pH 6.0, (fig. 1-30).

Fig. 1-1 Diagram of various germination types of Phytophthora parasitica drawn to scale at approximately X 400. Ultrastructural studies were made of direct sporangial germination, direct cyst germination, and indirect cyst germination.

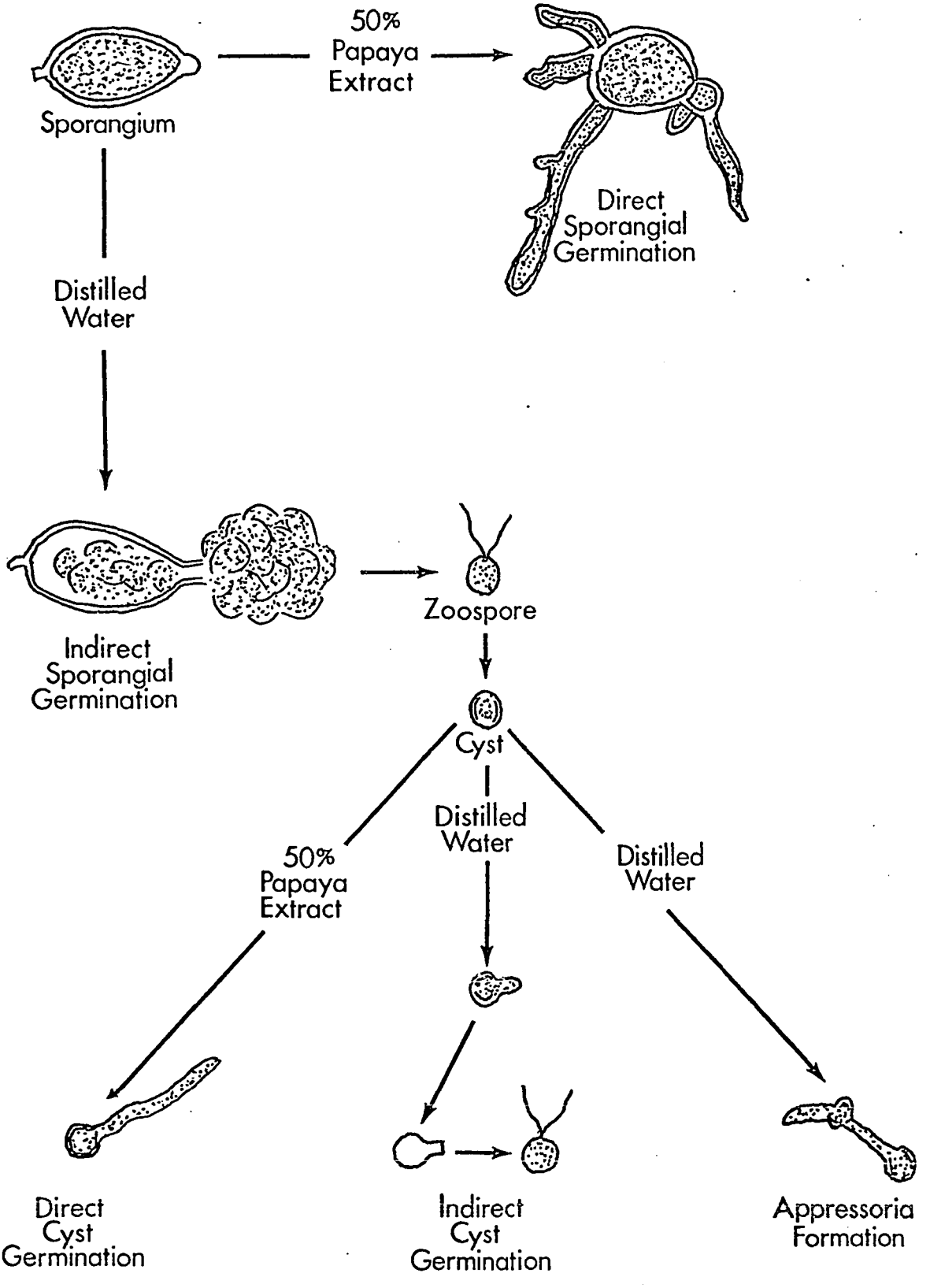
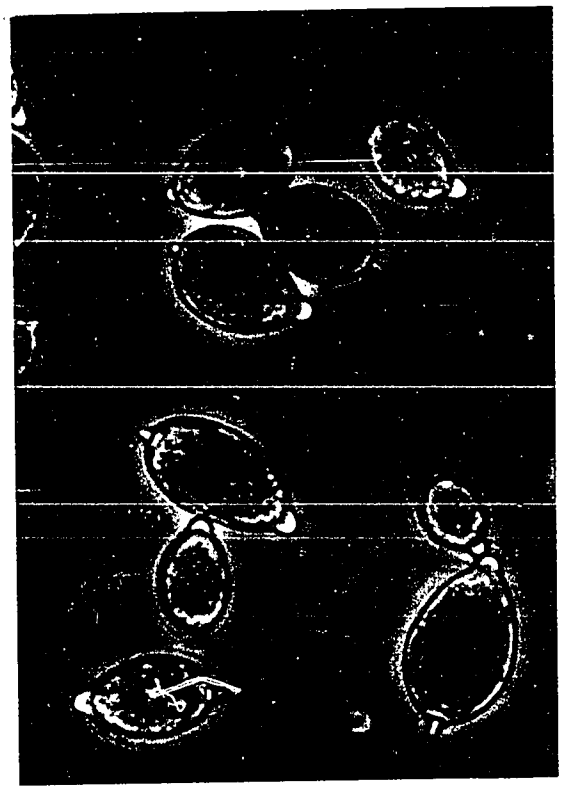

50%
Papaya
Extract
Sporangium
Direct
Sporangial
Germination
Distilled
Water
Indirect
Sporangial
Germination
Zoospore
Cyst
Distilled
Water
50%
Papaya
Extract
Distilled
Water
Direct
Cyst
Germination
Indirect
Cyst
Germination
Appressoria
Formation

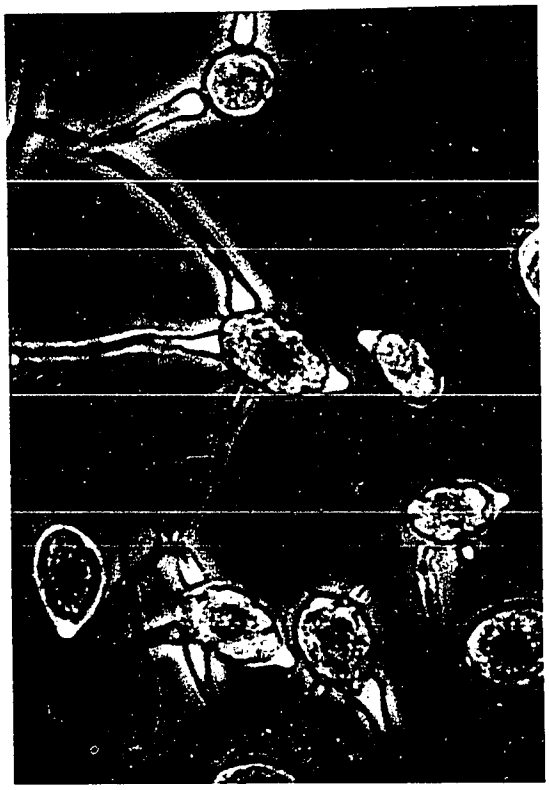
Fig. 1-2 Fully-expanded sporangia of Phytophthora parasitica prior to initiation of germination. X 400

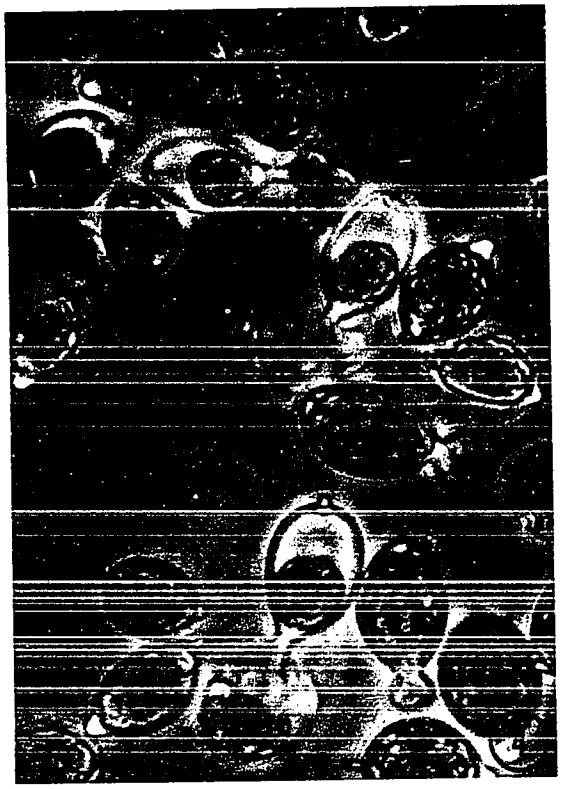
Fig. 1-3 Extension of germ tubes (direct sporangial germination) occurring in 50% papaya extract after 3 hrs of incubation. X 400

Fig. 1-4 Release of zoospores (indirect sporangial germination) occurring in distilled water after 10 minutes. X 400

Fig. 1-5 Encysted zoospores formed after one hour of incubation in distilled water. X 500







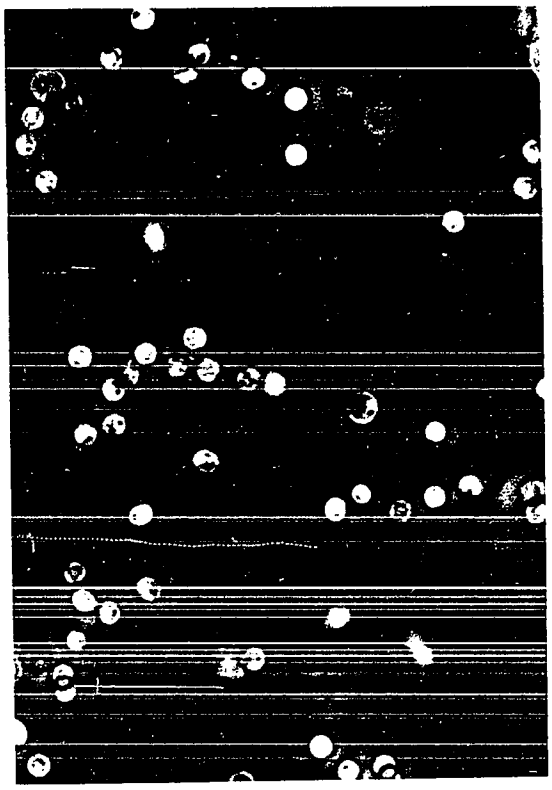
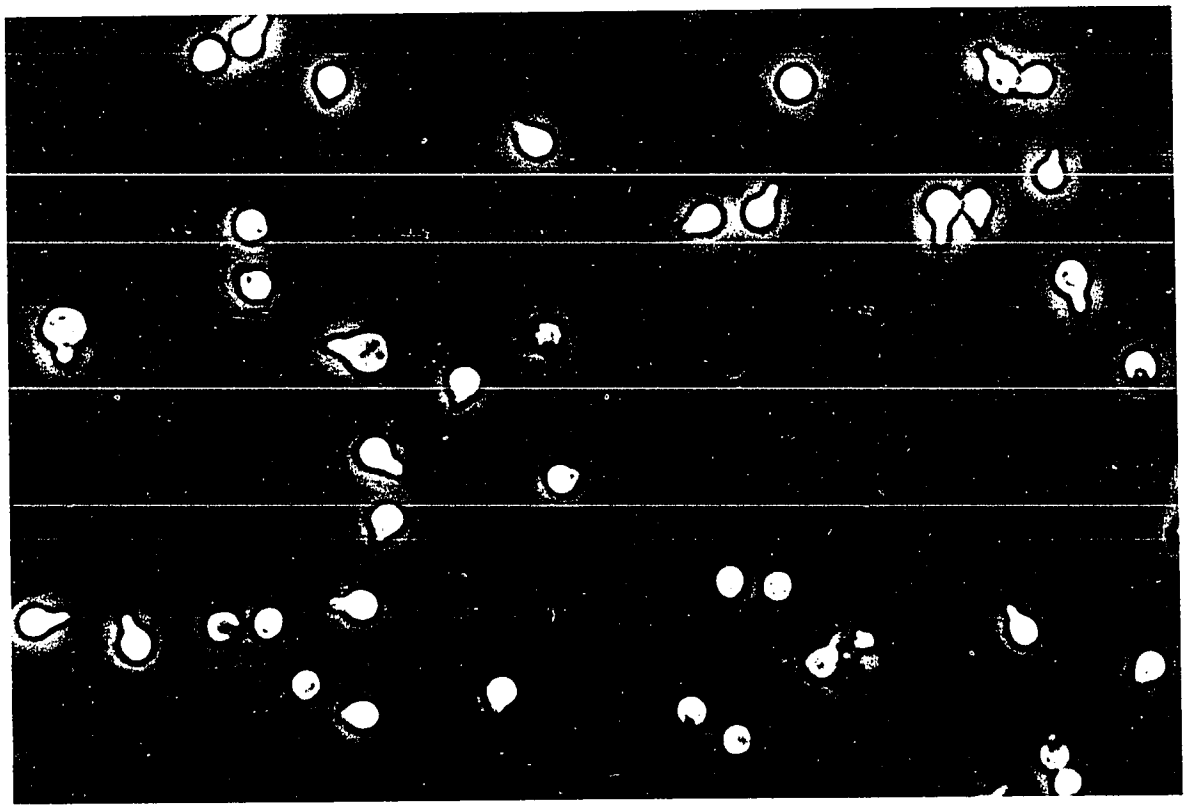
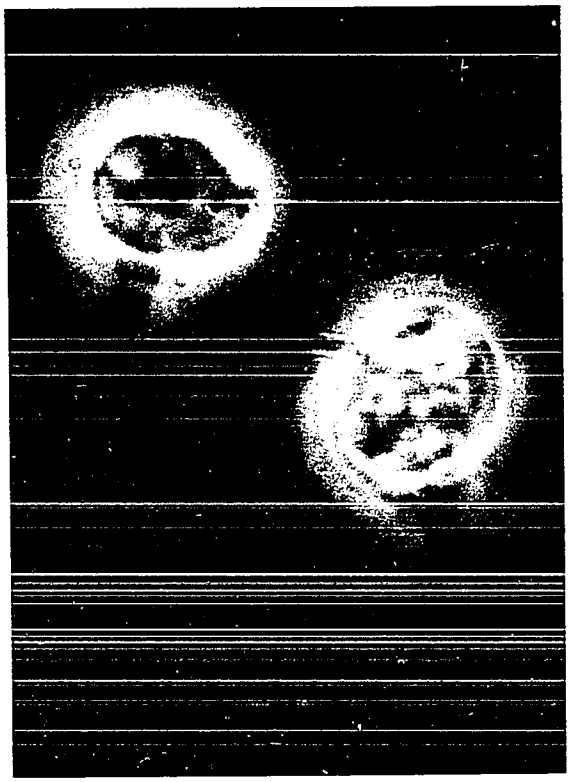


Fig. 1-6 Short germ tubes extended from cysts after five hours of incubation in distilled water (indirect cyst germination). X 600

Fig. 1-7 Release of secondary zoospores. X 1,000

Fig. 1-8 Empty cyst cases remaining after secondary zoospore release. X 2,000





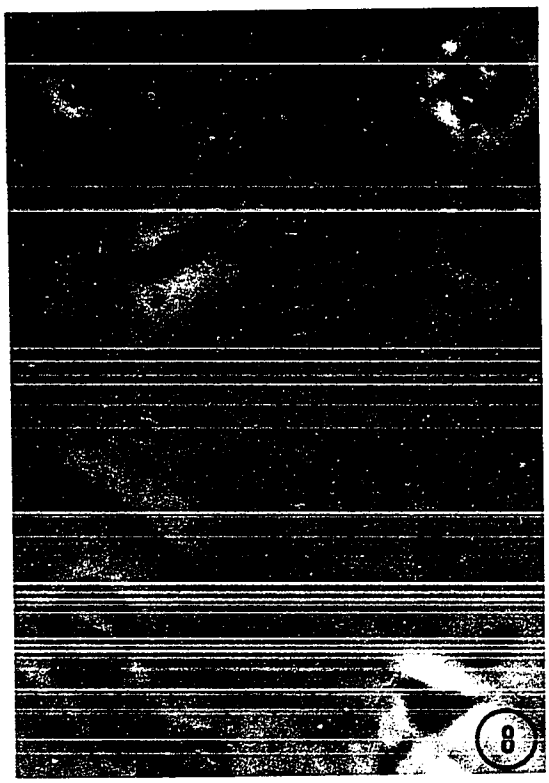

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Fig. 1-9 Direct cyst germination after 3 hrs of incubation.
X 400

Fig. 1-10 Direct cyst germination after 5 hrs of incubation.
X 600

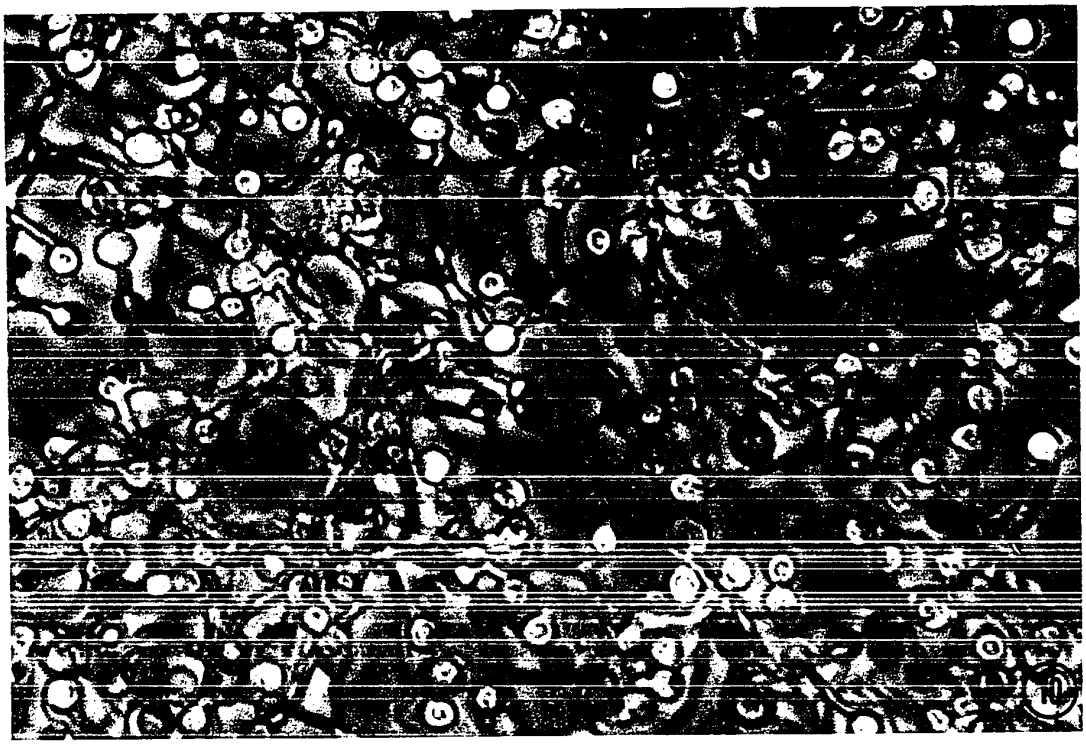


Fig. 1-11 Appressoria formation. A small percentage of cystsproduce appressoria in distilled water. X 600

Fig. 1-12 Higher magnification of appressoria formation. X 1,400

Fig. 1-13 Sporangia incubated in 50% papaya extract buffered to pH 7.0 after 3 hrs of incubation. X 200

Fig. 1-14 Sporangia incubated in an artificial extract containing sucrose, amino acids, and calcium ions buffered to pH 7.0 after 3 hrs of incubation. X 200

Fig. 1-15 Sporangia incubated in dialyzed, autoclaved papaya extract, pH 7.0 after 3 hrs of incubation. X 200

Fig. 1-16 Sporangia incubated in 12% aqueous sucrose buffered to pH 7.0 after 3 hrs of incubation. X 200


13


14


15

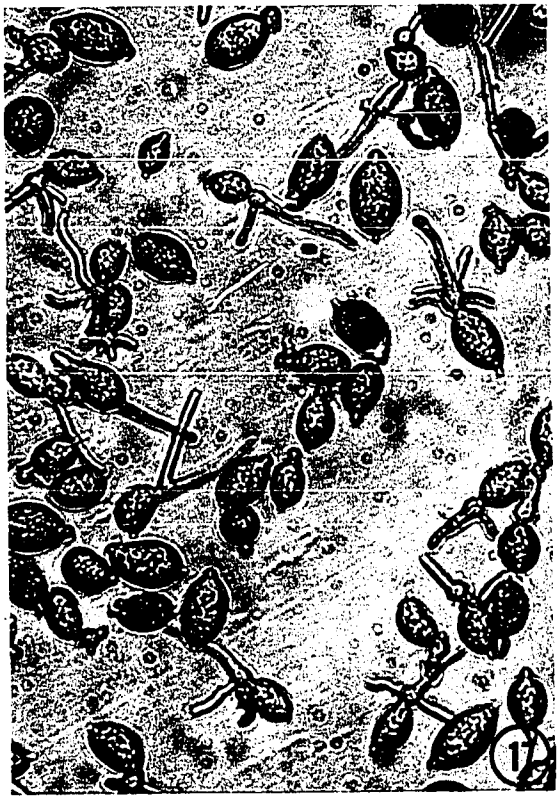

16

Fig. 1-17 Sporangia incubated in 50% papaya extract buffered to pH 7.0 after 5 hrs of incubation. X 200

Fig. 1-18 Sporangia after 5 hrs of incubation in an artificial extract containing sucrose, amino acids, and calcium ions buffered to pH 7.0. Note the absence of branching when compared to the 50% boiled papaya extract (fig. 1-19). X 200

Fig. 1-19 Sporangia after 5 hrs of incubation in dialyzed, autoclaved papaya extract, pH 7.0. X 200

Fig. 1-20 Sporangia after 5 hrs of incubation in 12% aqueous sucrose buffered to pH 7.0. Note empty sporangial cases and plasmolysis of several sporangia. X 200



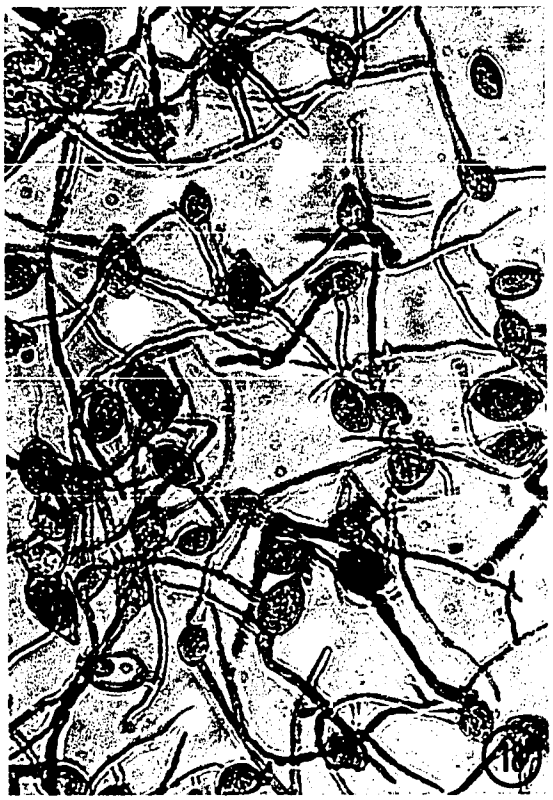


Fig. 1-21 The kinetics of indirect sporangial germination, encystation of zoospores, and direct cyst germination occurring in 50% dialyzed, autoclaved papaya extract at pH 7.0. Each point represents an average of nine separate plate counts of 200 sporangia each with an accuracy of + or - 5%.

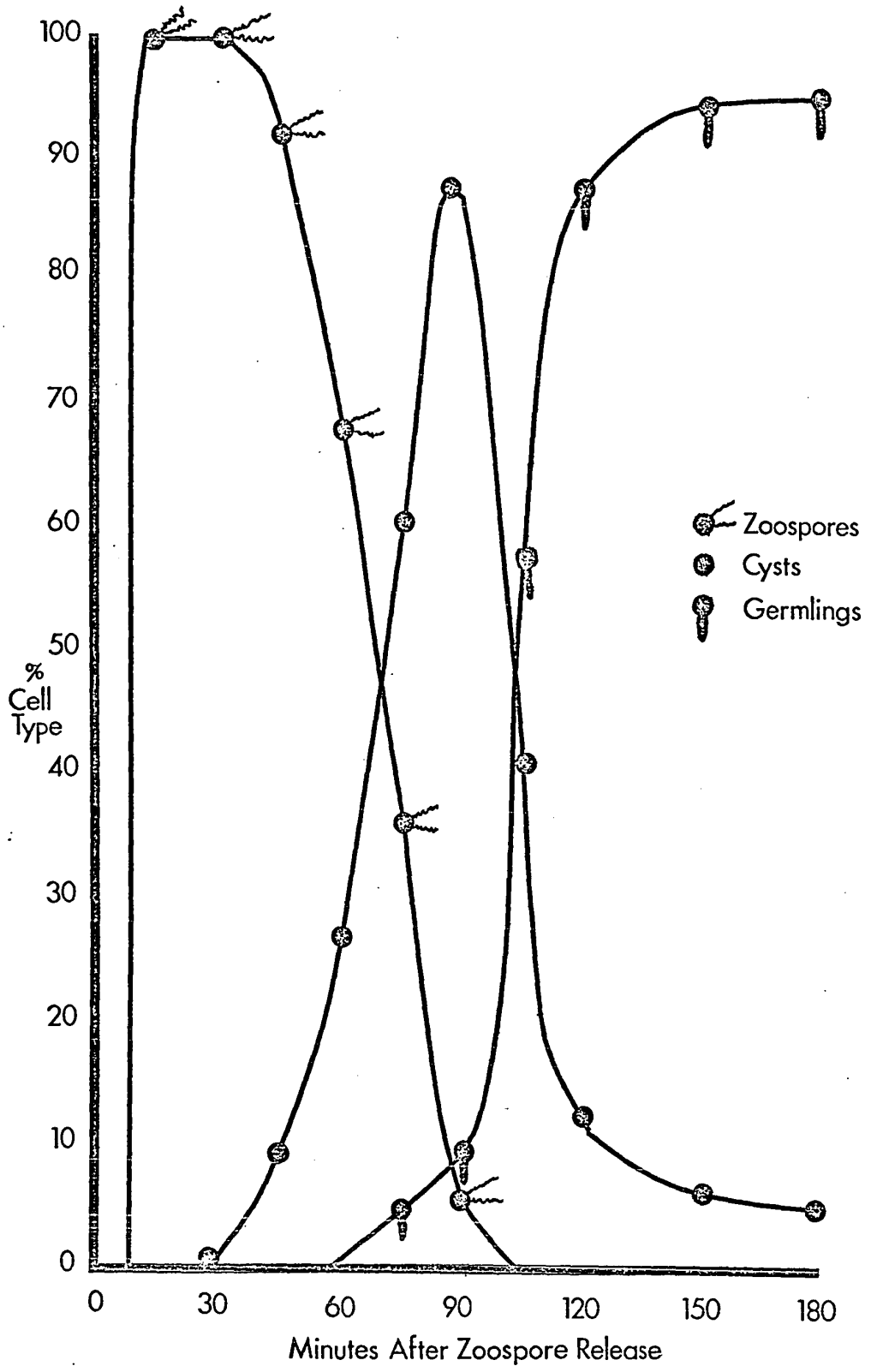

100
90
80
70
60
50
40
30
20
10
0
% Cell Type
0
30
60
90
120
150
180
Minutes After Zoospore Release
Zoospores
Cysts
Germlings

Fig. 1-22 The kinetics of indirect sporangial germination, encystation of zoospores, and secondary zoospore production (indirect cyst germination) occurring in distilled water at 28 C. The germlings indicated have short germ tubes approximately 6 μ long and eventually release the secondary zoospores. Each point represents an average of nine separate plate counts of 200 sporangia each with an accuracy of + or - 5%.

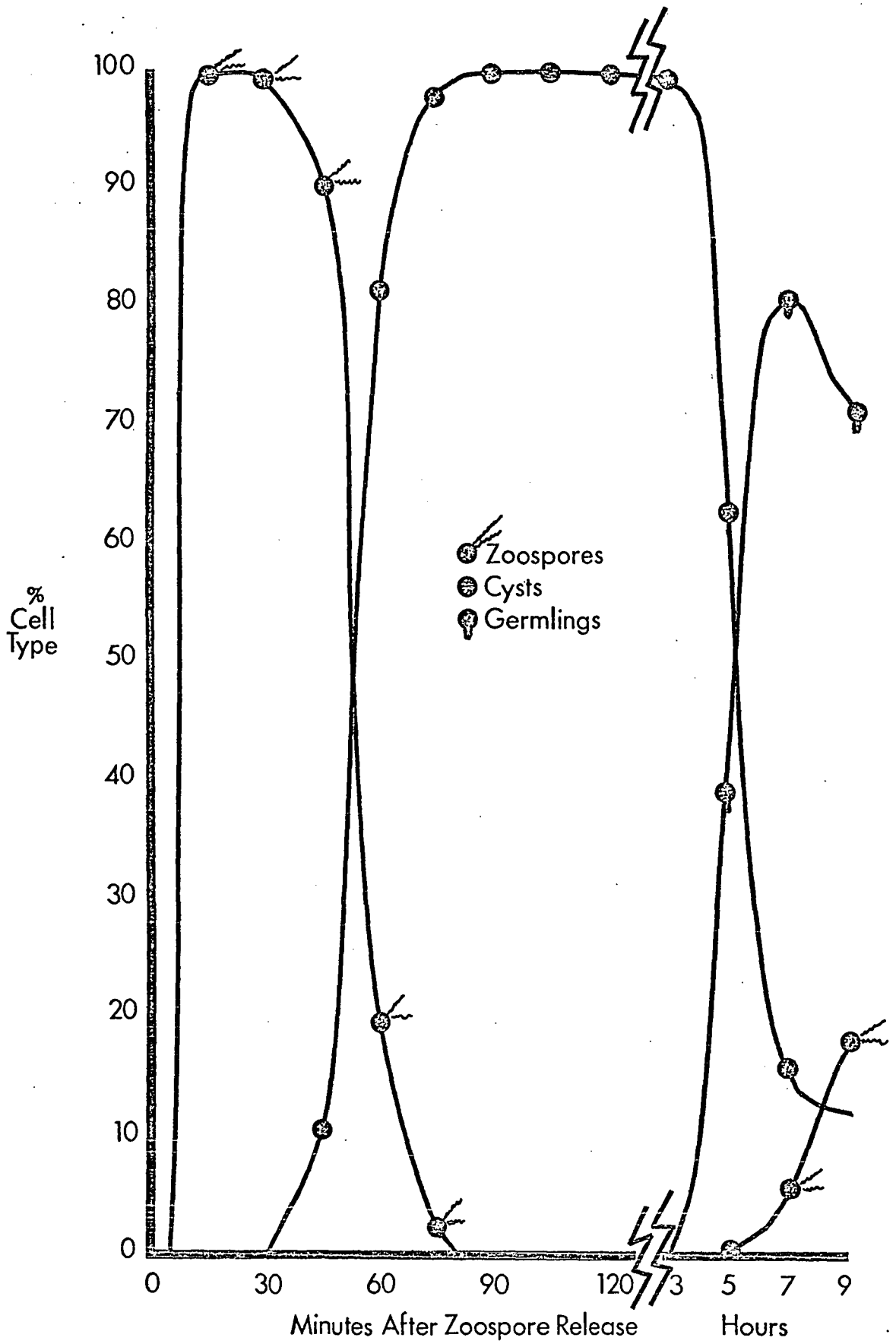

100
90
80
70
60
50
40
30
20
10
0
% Cell Type
Zoospores
Cysts
Germlings
0
30
60
90
120
3
5
7
9
Minutes After Zoospore Release
Hours

Fig. 1-23 Effect of sporangial density on direct and indirect sporangial germination. Direct germination was in 50% dialyzed papaya extract and indirect germination in distilled water at 28 C. Each point represents an average of nine separate plate counts of 200 sporangia each with an accuracy of + or - 5%.

Fig. 1-24 Effect of pH on direct and indirect sporangial germination. Incubation conditions were the same as described in fig. 1-23, but the pH was varied by changine the ratio of components of the Sörenson's phosphate buffer. Each point represents an average of nine separate plate counts of 200 sporangia each with an accuracy of + or - 5%.

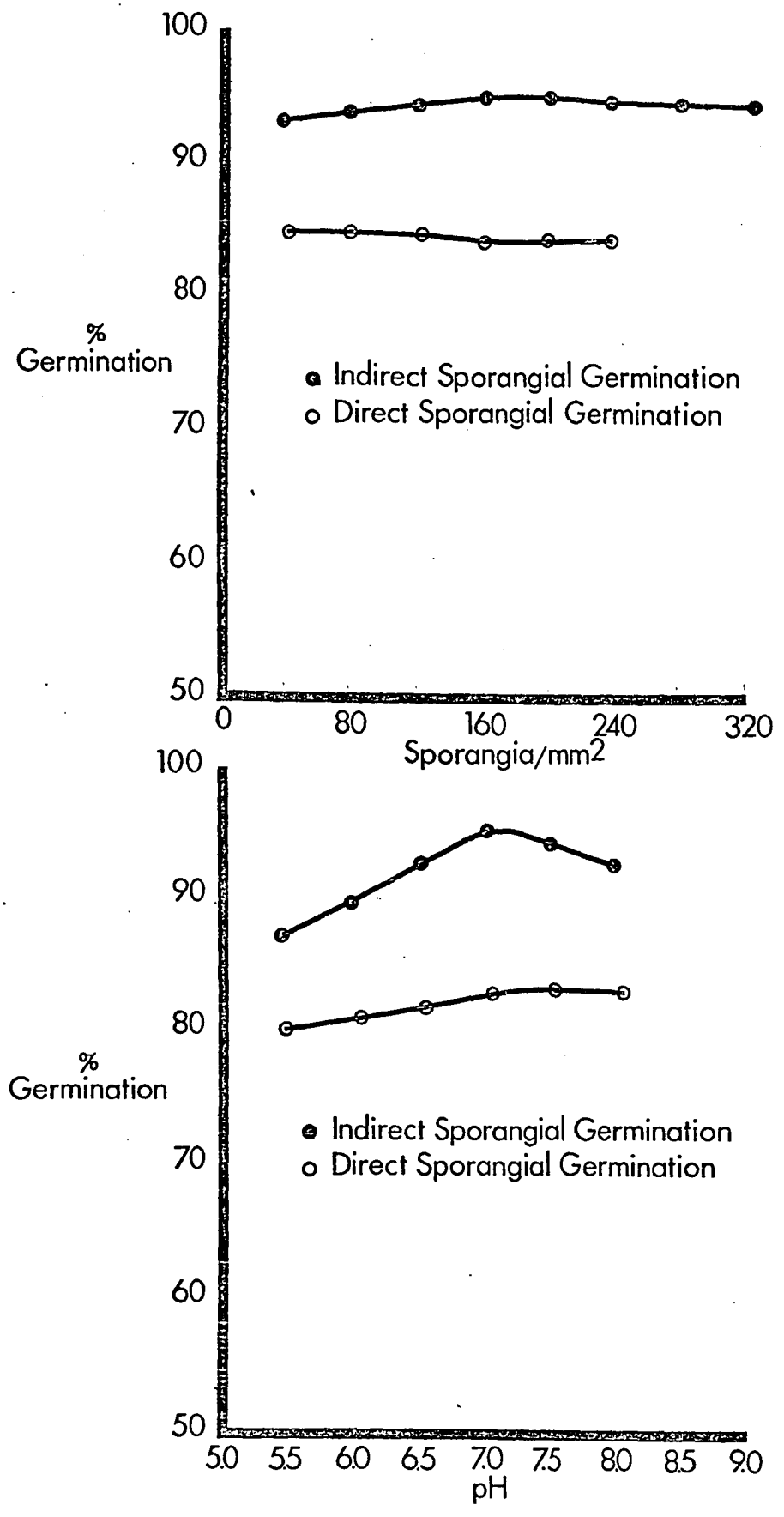

100
90
80
70
60
50
% Germination
● Indirect Sporangial Germination
o Direct Sporangial Germination
0
80
160
240
320
Sporangia/mm2
100
90
80
70
60
50
% Germination
● Indirect Sporangial Germination
o Direct Sporangial Germination
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
pH

Fig. 1-25 Effect of the age of sporangia on the culture dish on their ability to germinate by direct or indirect sporangial germination. The incubation medium for direct germination was a 50% dialyzed, autoclaved papaya extract adjusted to pH 7.0 at 28 C. Indirect germination was in 20 mM Sörenson's phosphate buffer at pH 7.0 at 28 C. Direct germination was read after 5 hrs of incubation and indirect germination after 30 min. Each point represents an average of nine separate plate counts of 200 sporangia each with an accuracy of + or - 5%.

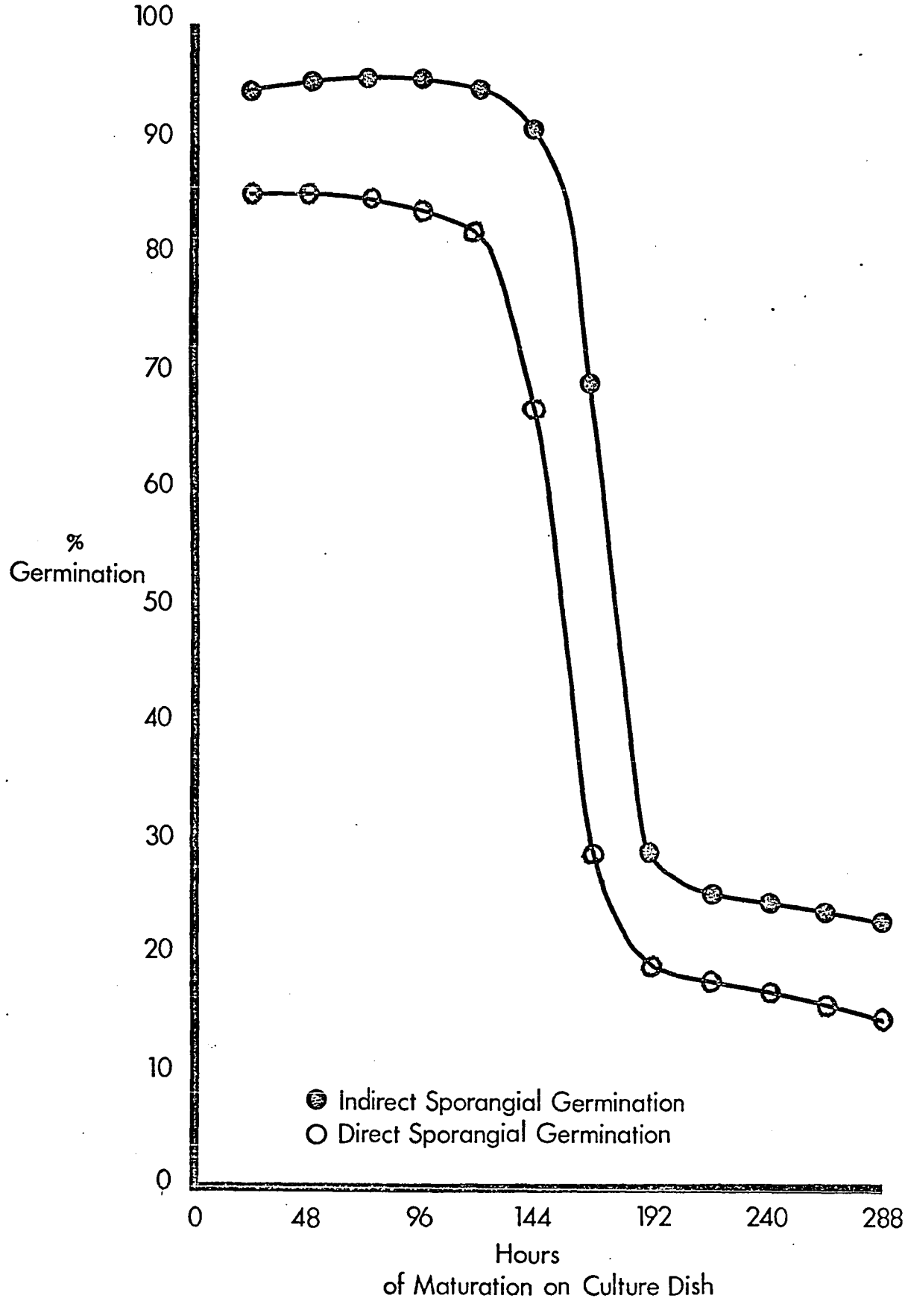

100
90
80
70
60
%
Germination
50
40
30
20
10
0
0
48
96
144
192
240
288
Hours
of Maturation on Culture Dish
Indirect Sporangial Germination
Direct Sporangial Germination

Fig. 1-26 Effects of modifications of 50% papaya extract on direct sporangial germination. Each point represents an average of nine separate plate counts of 200 sporangia each with an accuracy of + or - 5%.

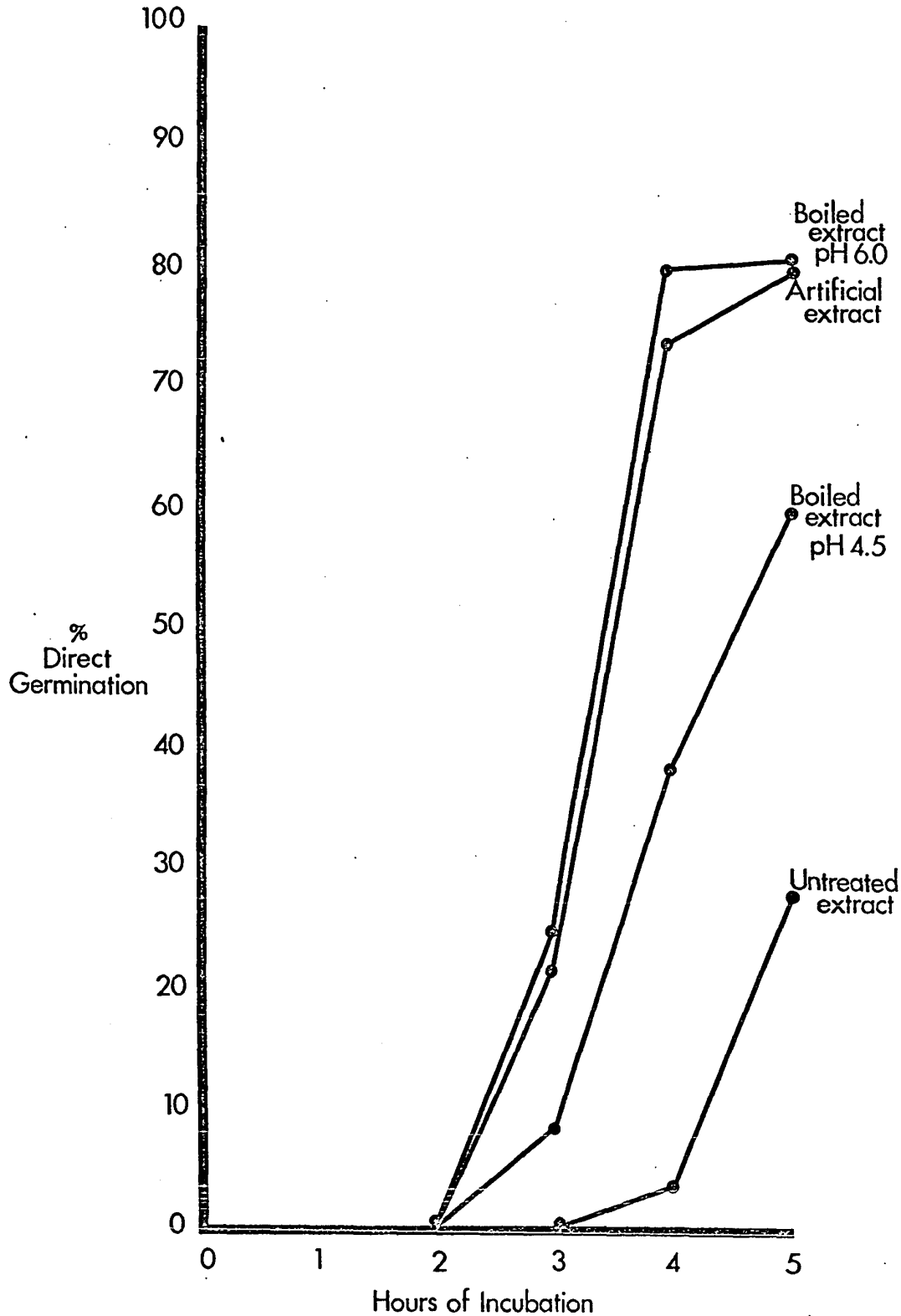

100
90
80
70
60
50
40
30
20
10
0
% Direct Germination
0
1
2
3
4
5
Hours of Incubation
Boiled extract pH 6.0
Artificial extract
Boiled extract pH 4.5
Untreated extract

Fig. 1-27 Effects of components of artificial extract on direct sporangial germination. Each point represents an average of nine separate plate counts of 200 sporangia each with an accuracy of + or - 5%.

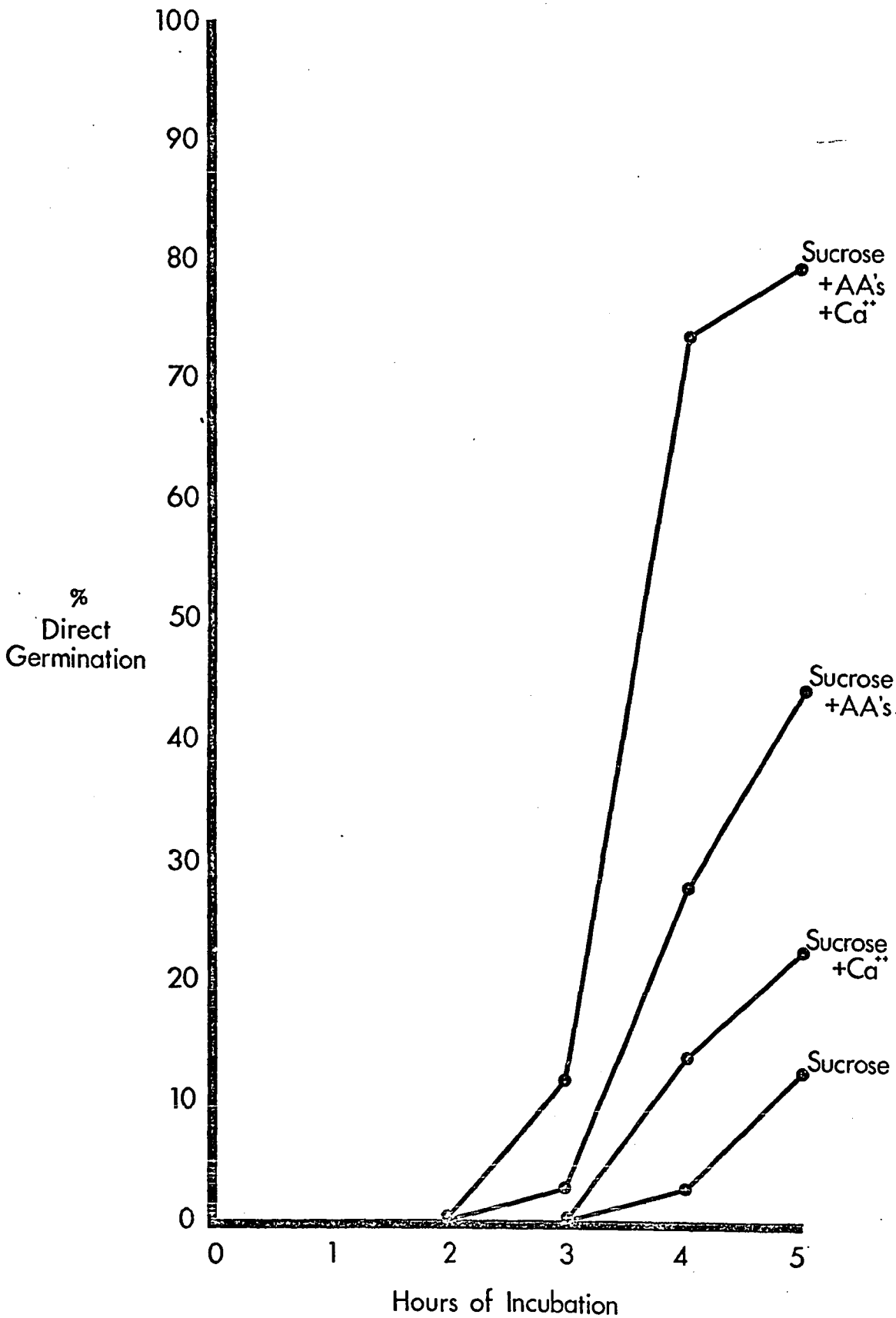

100
90
80
70
60
50
40
30
20
10
0
% Direct Germination
0
1
2
3
4
5
Hours of Incubation
Sucrose +AA's +Ca++
Sucrose +AA's
Sucrose +Ca++
Sucrose

Fig. 1-28 Specificity of carbohydrate component of artificial extract. Incubation medium contained caseamino acids and calcium ions and was adjusted to pH 7.0. Each point represents an average of nine separate plate counts of 200 sporangia each with an accuracy of + or - 5%.

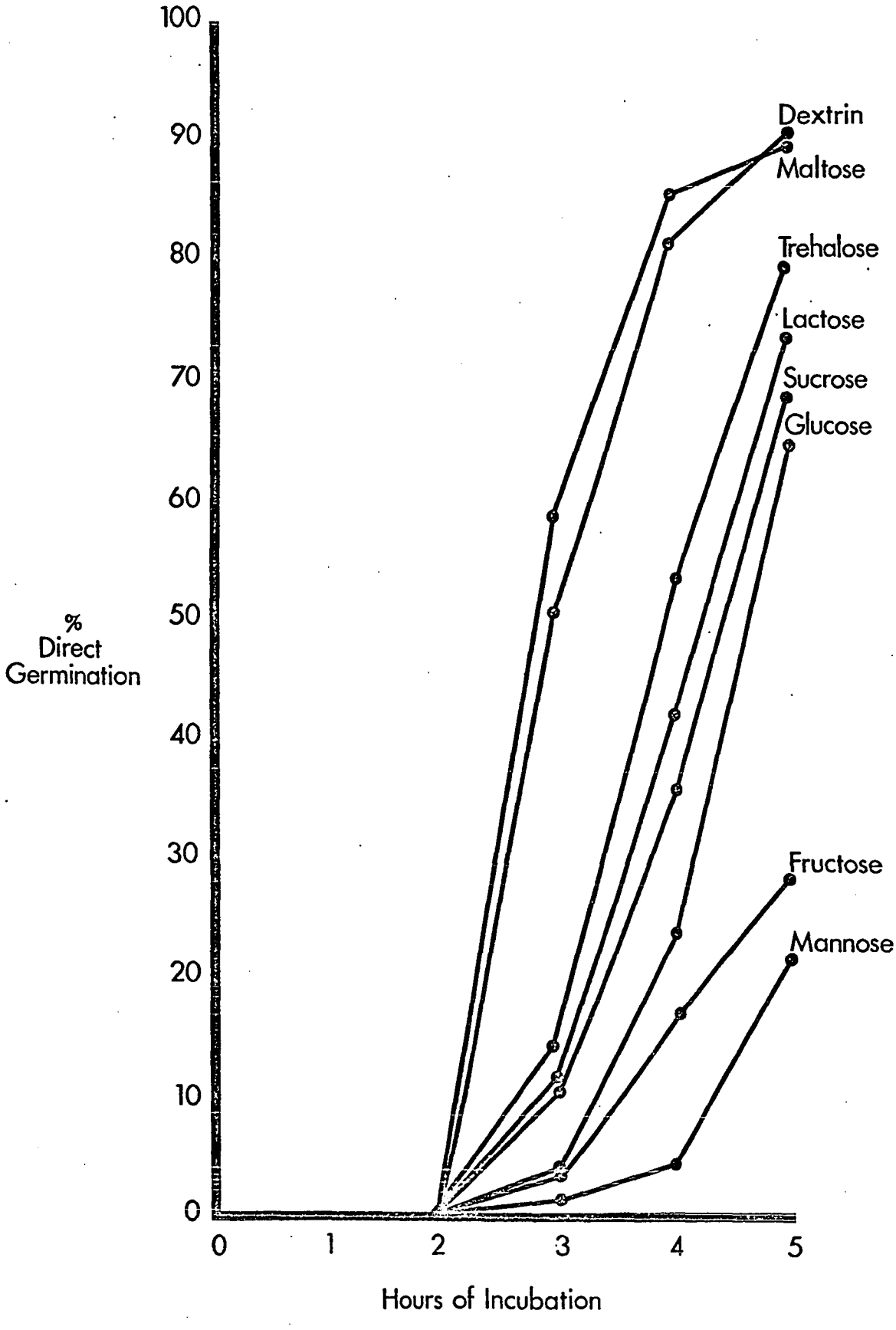

100
90
80
70
60
50
40
30
20
10
0
% Direct Germination
0
1
2
3
4
5
Hours of Incubation
Dextrin
Maltose
Trehalose
Lactose
Sucrose
Glucose
Fructose
Mannose

Fig. 1-29 Specificity of amino acid component of artificial extract. Incubation medium contained sucrose and calcium ions and was adjusted to pH 7.0. Each point represents an average of nine separate plate counts of 200 sporangia each with an accuracy of + or - 5%.

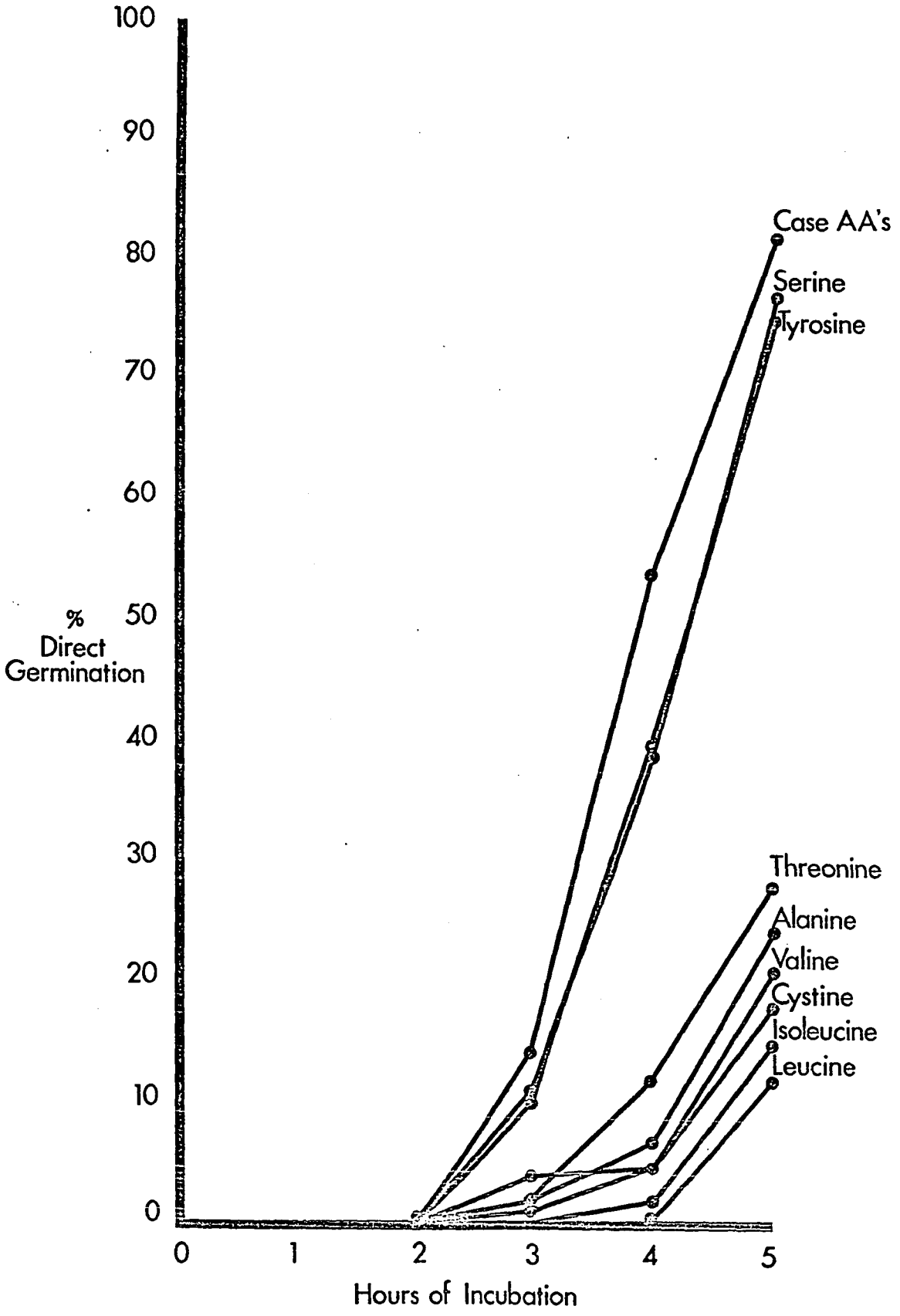
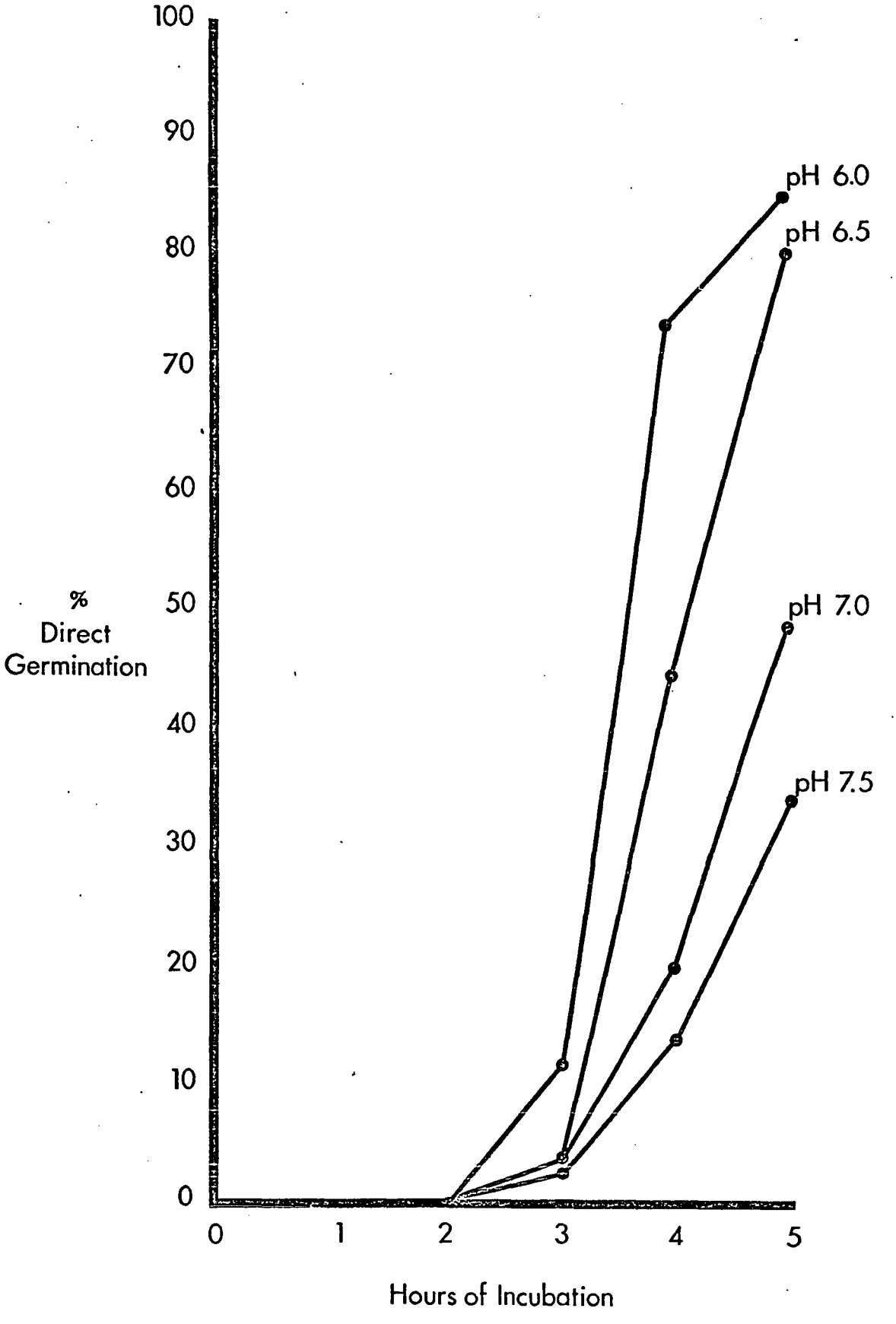

100
90
80
70
60
50
40
30
20
10
0
% Direct Germination
0
1
2
3
4
5
Hours of Incubation
Case AA's
Serine
Tyrosine
Threonine
Alanine
Valine
Cystine
Isoleucine
Leucine

Fig. 1-30 Effect of pH of artificial extract on direct sporangial germination. Each point represents an average of nine separate plate counts of 200 sporangia each with an accuracy of + or - 5%.


100
90
80
70
60
50
40
30
20
10
0
% Direct Germination
pH 6.0
pH 6.5
pH 7.0
pH 7.5
0
1
2
3
4
5
Hours of Incubation

CHAPTER IV

RESULTS

Electron Microscopy of Sporangial Germination: The ultrastructure of the fully expanded, resting sporangium has been described previously (Hohl and Hamamoto, 1967). Under the conditions employed in this study the sporangia begin to germinate by germ tubes after approximately 3 hrs. Although the sporangia are of relatively uniform age the germination process is not strictly synchronous. It is, therefore, difficult to provide an accurate temporal sequence of events occurring during germination. However, it is possible to differentiate clearly three distinct phases: the first lasting from time 0 hr up to penetration of the apical plug, the second encompassing the events of penetration of the plug by the germ tube, and the third covering the onset of hyphal tip growth. Accordingly, the description of direct germination is divided into three sections: (1) initial, intrasporangial changes; (2) perforation of apical plug and sporangial wall by germ tubes; and (3) hyphal tips.

(1) Initial, intrasporangial changes. The initial events in direct germination are indistinguishable from those of indirect germination. Flagella are formed (figs. 2-3, 2-4) and some of the large numbers of cleavage vesicles present may fuse. This development towards forming zoospores stops, however, and in samples taken after 3.5 hrs the flagella are surrounded by vesicles (figs. 2-18, 2-19, 2-20) and eventually disappear from germinating sporangia.

Occasionally, cleavage goes to completion but the zoospores are not released. Instead, they encyst within the sporangium and may later germinate, in which case the germ tubes penetrate the sporangial wall as they do in direct germination (fig. 2-8).

The next step in direct germination involves the formation of a new wall apposing the sporangial wall. A sporangial wall prior to this new wall formation is shown in figs. 2-1 and 2-2. As a first sign the plasmalemma becomes ruffled (fig. 2-9). Wall-like material is seen around aggregates of vesicular components of various sizes lying close to the periphery of the cytoplasm (fig. 2-10). The aggregates also contain ribosomes and other small cytoplasmic elements (fig. 2-16). They are, therefore, cytoplasmic in nature and distinctly different from the lomasomes that are also present. These aggregates surrounded by the wall-like material are then seen encased in the wall (figs. 2-15, 2-16). The wall is unevenly thick depending on the number of vesicular aggregates it contains (fig. 2-12). Sometimes large, localized accumulations of wall material are observed (figs. 2-13, 2-14). It may also be noted, however, that the wall exists in areas devoid of vesicular accumulations (fig. 2-11). In the end the sporangial protoplast is completely surrounded by a new wall layer, the germination wall (fig. 2-17).

The vesicular inclusions occur far less frequently in the original sporangial wall (figs. 2-12, 2-14) and have never been observed in the wall of the hyphal tip. They do occur in large numbers in the basal plug of the sporangium (figs. 2-6, 2-7; see

Chapman and Vujičić, 1965; Hohl and Hamamoto, 1967). The basal plug is made up of pockets of vesicles interlaced by fibrous wall material enclosed on each side by a continuation of the outer sporangial wall. Occasionally, large pockets of cytoplasm are found in the central portion of the basal plug.

In an attempt to analyze the cytoplasmic components that might be involved in the formation of this new wall, the following organelles were studied: plasmalemma, lomasomes, dictyosomes, polyvesicular bodies, endoplasmic reticulum, microbodies, large vacuoles, and mitochondria.

The plasmalemma of the wall-forming sporangium is highly sculptured with many anastomosing folds, forming a complex, mosaic-type surface pattern that is particularly striking in grazing sections (fig. 2-26). The same surface patterns can be seen in hyphae (fig. 2-52). The invaginations of the plasmalemma form either simple folds (fig. 2-52), such as have been observed in Penicillium by Remsen, Hess, and Sassen (1967), or they are more elaborate and form narrow channels (figs. 2-27, 2-28) or lomasomes (fig. 2-52).

The lomasomes occur in two morphologically distinct forms. The first type consists of an aggregate of small vesicles and/or tubuli of uniform diameter (approximately 300 Å) packaged into a pocket of the plasmalemma (fig. 2-25). It is found frequently at the base of the apical plug where the germ tube will penetrate (figs. 2-25, 2-39, 2-41) and also in the growing hyphae (fig. 2-48). It is most conspicuous in grazing sections through the plasmalemma (fig. 2-21).

The second type of lomasome is made up of tubular and vesicular elements in a pocket of the plasmalemma (figs. 2-22, 2-23, 2-24). The elements are usually larger and of less orderly appearance than those of type one and they have been observed only in material fixed in glutaraldehyde-osmium tetroxide (fig. 2-42).

The dictyosomes are found throughout the sporangial cytoplasm, often in a perinuclear position. They exhibit a polarity similar to that described for dictyosomes of Pythium (Bracker, 1967). One surface is often associated with a cisterna from the endoplasmic reticulum. Both the cisterna of the dictyosome and that of the endoplasmic reticulum have bulbous protuberances, and the region between the cisternae contains many small vesicles with homogeneous contents (figs. 2-31, 2-36). The configuration suggests a transport of vesicles between the cisternae. Similar configurations have been observed involving the nuclear envelope and the dictyosome (fig. 2-29). From the opposite surface of the dictyosome, vesicles appear to be pinched off which are filled with a substance of mottled texture (fig. 2-30). The vesicles are of varying size, the small ones often measuring 0.05 μ, the larger ones 0.2 μ in diam. Similar vesicles accumulate at the periphery of the sporangium (fig. 2-33) and are sometimes continuous with invaginations of the plasmalemma (fig. 2-35). Similar vesicles are also found in large numbers at the tip of the growing hyphae (figs. 2-40, 2-43, 2-49).

Polyvesicular bodies have been observed consistently but are not numerous. They measure approximately 0.3 μ in diameter and contain small vesicles in an electron-dense matrix. They are

sometimes found associated with dictyosomes (figs. 2-32, 2-36), sometimes near the plasmalemma (fig. 2-34), and often randomly dispersed in the cytoplasm (fig. 2-37). Aggregates of vesicles lacking a limiting membrane have also been observed (fig. 2-51). Although they somewhat resemble type one lomasomes, the polyvesicular bodies are smaller and much less numerous than the former.

The endoplasmic reticulum is distributed throughout the cytoplasm and frequently lines the sporangial periphery (figs. 2-9, 2-11, 2-14). There, its cisternae are often dilated and filled with electron-dense material (fig. 2-14). The microbodies (Bracker, 1967; Frederick et al., 1968) are roughly spherical to ellipsoidal in shape (fig. 2-10). They measure approximately 0.5 μ in diameter, are surrounded by a single unit membrane, and have homogeneously granular contents. Sometimes they contain small sets of concentric, lamellar material (fig. 2-12, 2-25).

In addition to the organelles described, large vacuoles filled with fibrillar material are frequently found close to the plasmalemma (fig. 2-1). They are surrounded by a membrane which appears to be easily ruptured during fixation. The mitochondria also frequently accumulate at the cytoplasmic periphery, their membranes often touching the plasmalemma but never fusing with it (fig. 2-38). They occasionally contain looped tubules (figs. 2-21, 2-38), as also observed by Ehrlich and Ehrlich (1966).

(2) Perforation of apical plug and sporangial wall by germ tubes. The germ tubes penetrate either the sporangial walls or the apical

plug. The region below the apical plug contains microbodies, dictyosome vesicles, endoplasmic reticulum, and type-one lomasomes (fig. 2-40). The lomasomes penetrate deep into crevices in the apical plug (figs. 2-39, 2-40). The germ tube forces its way through the central section of the plug (fig. 2-41) while the peripheral portions of the plug remain in their original position (fig. 2-43). The newly formed germination wall on the inside of the sporangium is continuous with the wall of the germ tube (figs. 2-45, 2-47). Thus the hyphal wall represents an extension of the germination wall. Beyond the plug area, the germ tube is swollen (figs. 2-44, 2-45) and from this bulge one or more hyphal tips originate (fig. 2-46). The emerging germ tube may also branch directly without forming a bulge (fig. 2-47), or may not branch at all. At a later stage of germination, germ tubes also penetrate the walls of the sporangium by dissolving and pushing their way out (fig. 2-48) in much the same way as has been described for the apical plug area.

(3) Hyphal tips. The cytoplasm at the hyphal tip, where most of the growth takes place, has a structure that differs from the rest of the cytoplasm. It is filled with vesicles (fig. 2-49) that resemble those near the dictyosomes (fig. 2-43). In addition, the endoplasmic reticulum is prominent and forms a network of anastomosing tubules rather than flat cisternae (fig. 2-43). Aside from the vesicles having a diameter of about 0.2 μ there are smaller ones measuring about 500 Å (figs. 2-43, 2-49).

Behind the vesicular zone at the hyphal tip numerous mitochondria are found. They are elongated, their long axis oriented parallel

to the axis of the hypha, and they are located mainly at the periphery of the cytoplasm (figs. 2-50, 2-51, 2-53). In older sections of the hypha vacuoles are found that occupy the central portion of the cytoplasm (fig. 2-51). The wall of the hypha appears vaguely two-layered (fig. 2-53). A loose, fluffy outer layer covers an inner, solid and fibrillar one. The two layers are clearly recognizable in germ tubes of germinating cysts (fig. 2-54).

Fig. 2-1 A fully expanded, resting sporangium of Phytophthora parasitica Dast. Note the random distribution of sporangial organelles: a nucleus (N), dictyosomes (D), microbodies (Mb), fibrillar vacuoles (Fv), mitochondria (M), and the sporangial wall (W) are shown. Glut-$KMnO_4$. X 12,100

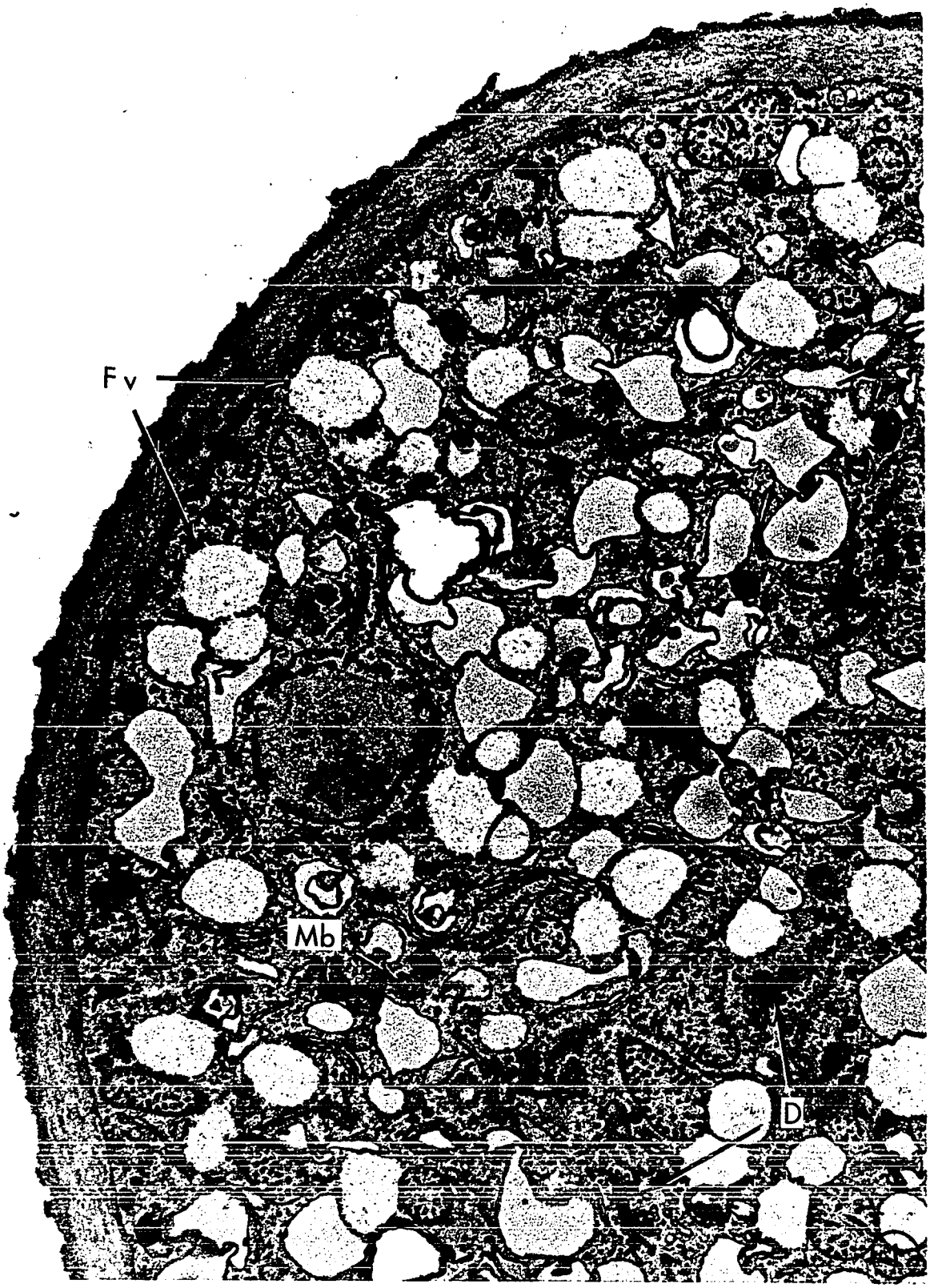
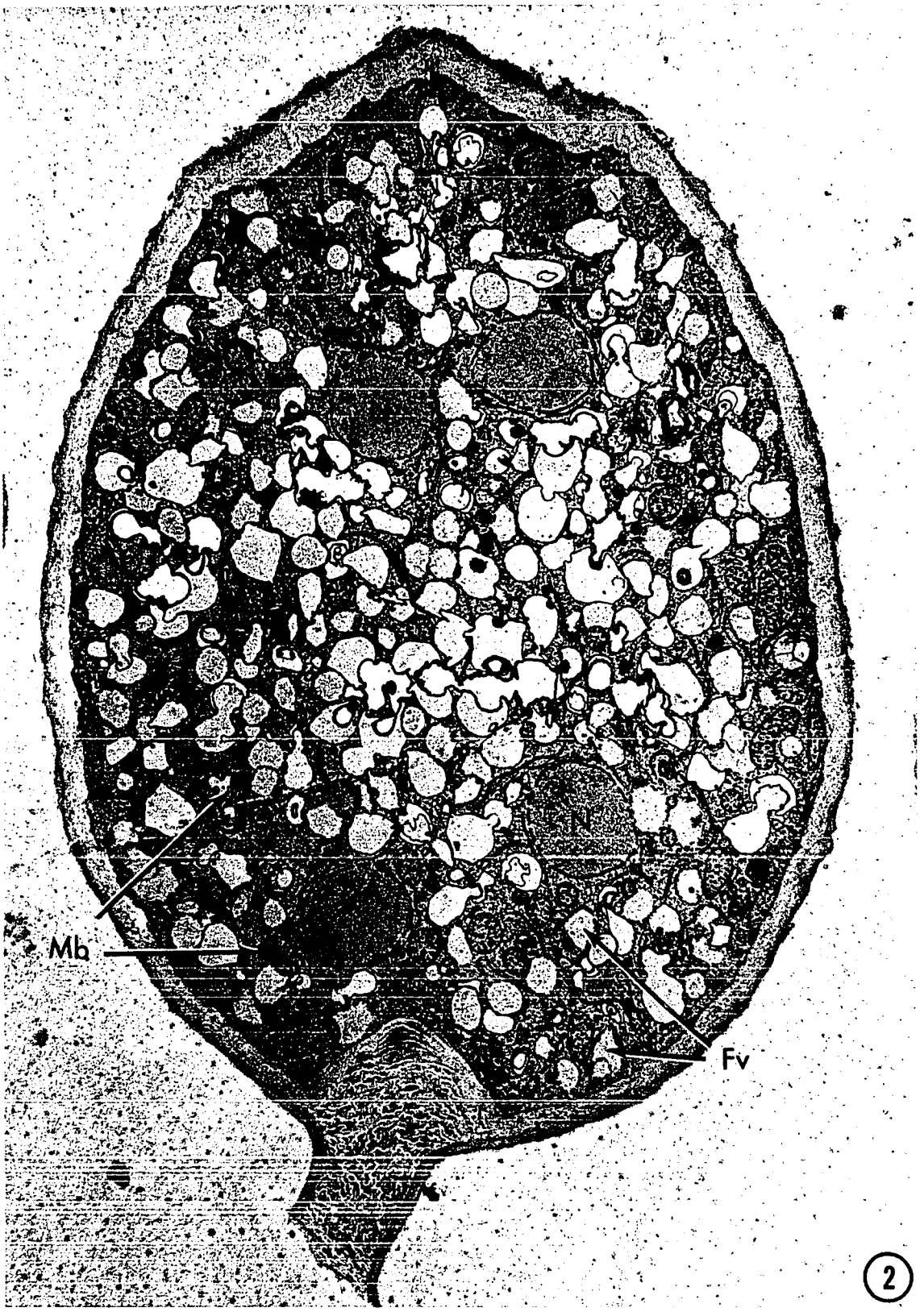
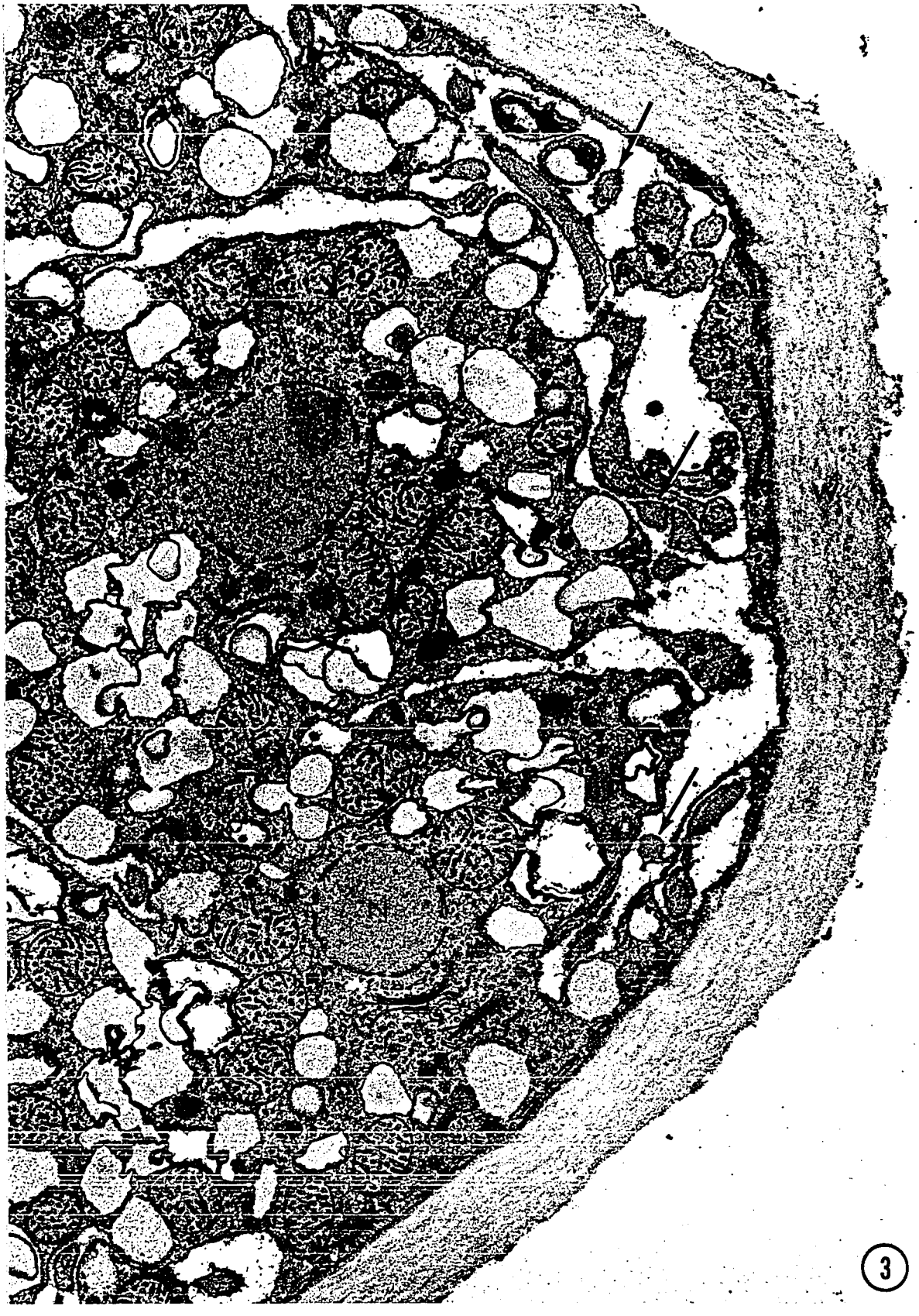

Fv
Mb
D

Fig. 2-2 Section through sporangium and basal attachment showing general distribution of organelles. Mitochondria (M) and microbodies (Mb) are slightly more concentrated near the periphery of the sporangium, whereas, fibrillar vacuoles (Fv) are located randomly. Nuclei (N). Glut-$KMnO_4$. X 7,000



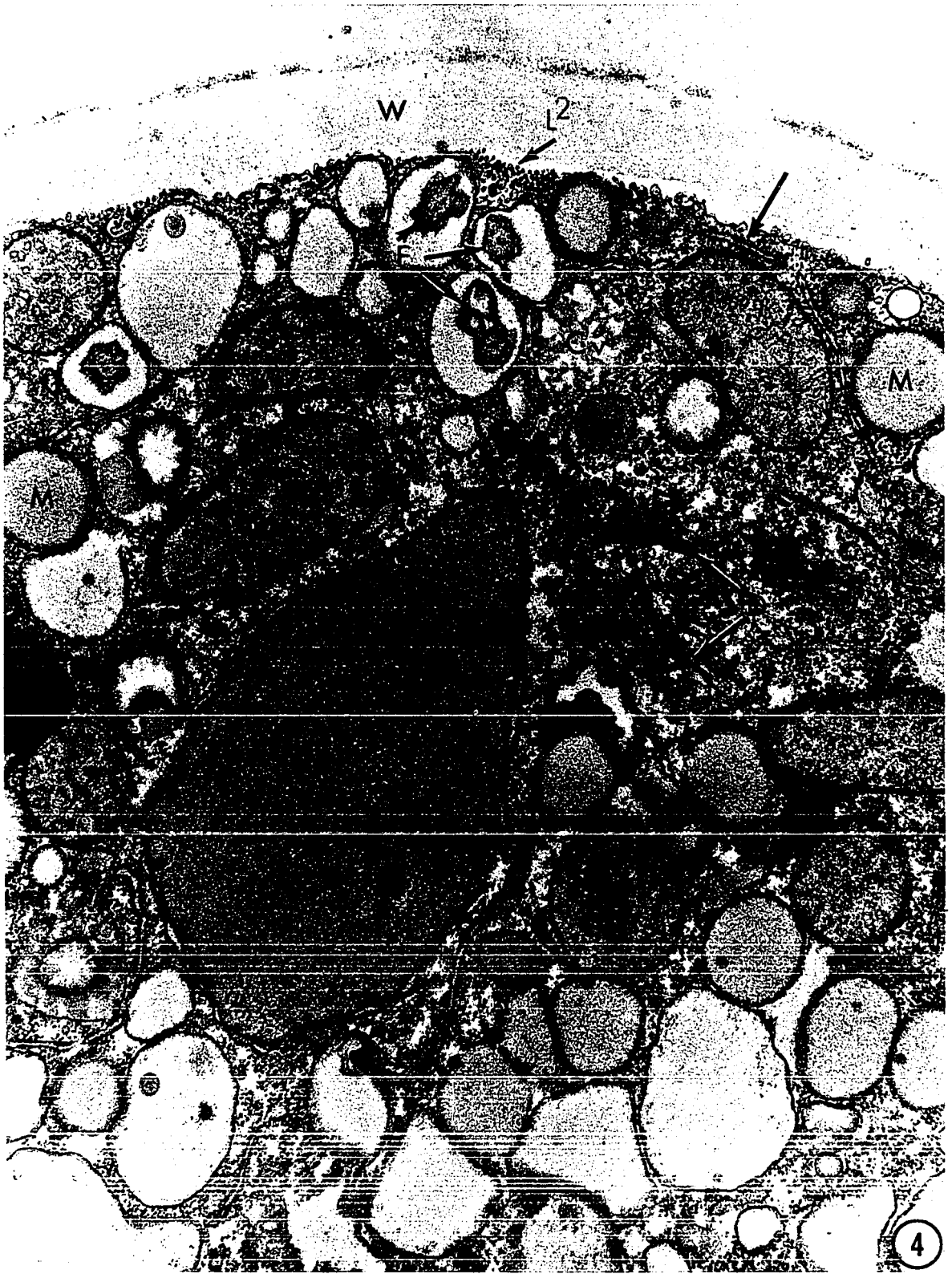
(2)

Fig. 2-3 Sporangium before formation of germination wall showing the partial cleavage of the cytoplasm equidistant from the nuclei (N) to form zoospores. Flagella (arrows), sporangial wall (W). Glut-$KMnO_4$. X 12,100



3

Fig. 2-4 Sporangium fixed with glutaraldehyde followed by osmium tetroxide before germination wall formation. Note the type II lomasome (L^2), endoplasmic reticulum (arrow), and microbodies (M) near the sporangial wall (W). Nucleus (N), dictyosomes (D), flagella (F), and crystalline vacuoles (Cv). Glut-OsO_4. X 19,100

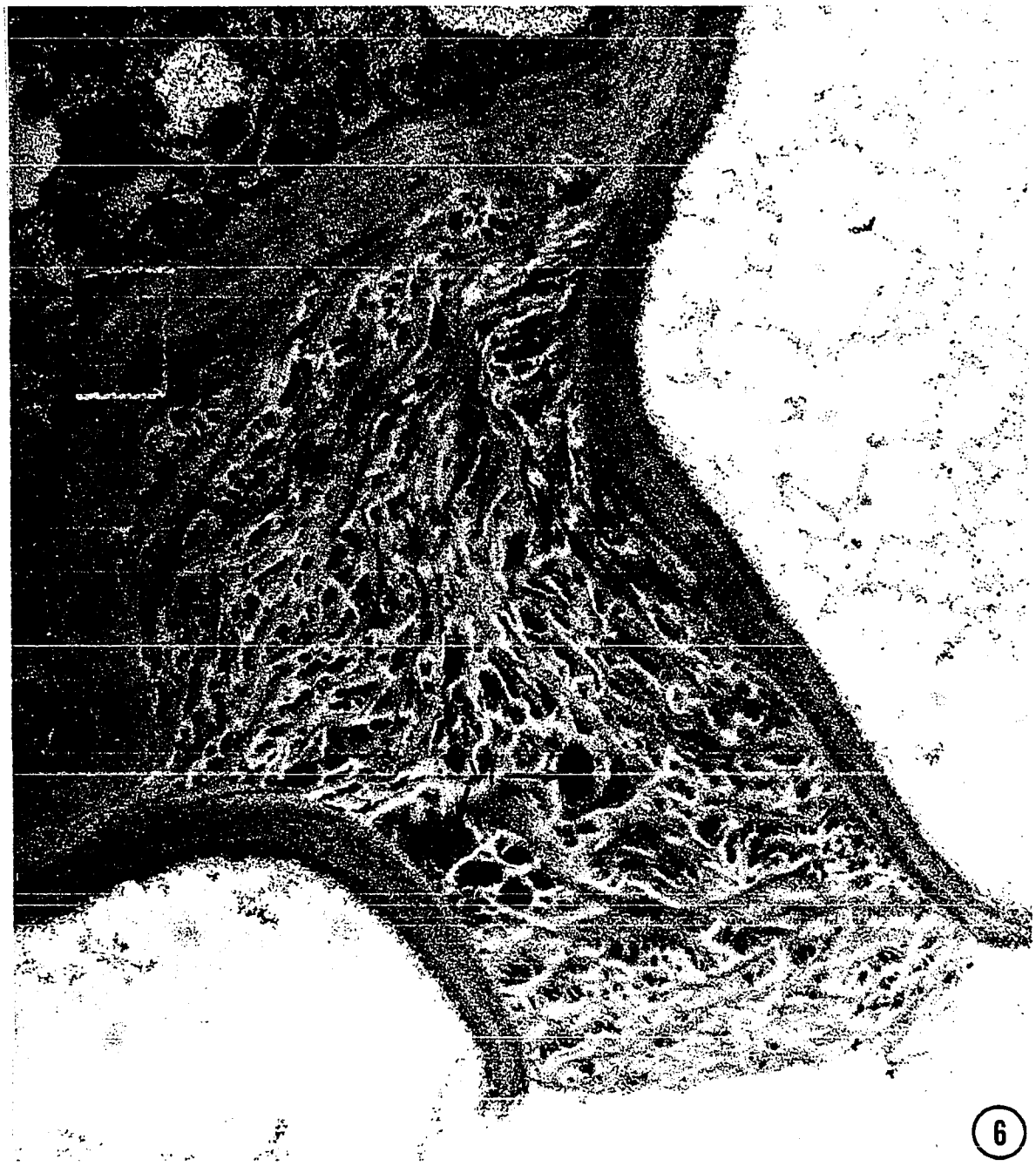


4

Fig. 2-5 Sporangial plug (P) of fully-expanded sporangium. Note difference in electron density from sporangial wall (W) and groove containing type I lomasome (arrow). Glut-$KMnO_4$. X 16,100

5

Fig. 2-6 Basal sporangial plug containing many vesicular elements (Vi) and surrounded by continuations of the sporangial wall (W). Glut-$KMnO_4$. X 28,200



6

Fig. 2-7 Basal sporangial plug fixed with glutaraldehyde-osmium demonstrating enclosed vesicular elements. GOP. X 37,000

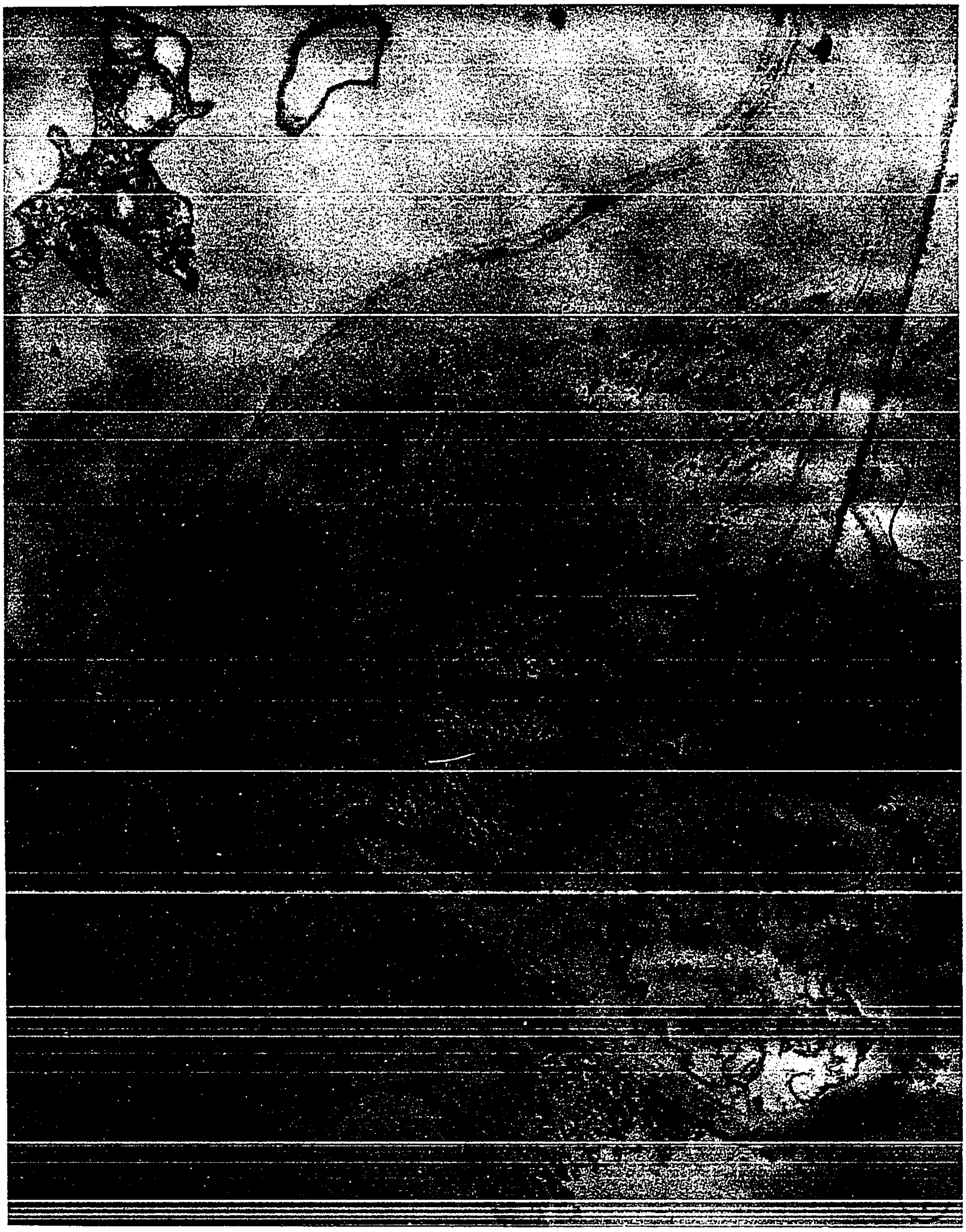
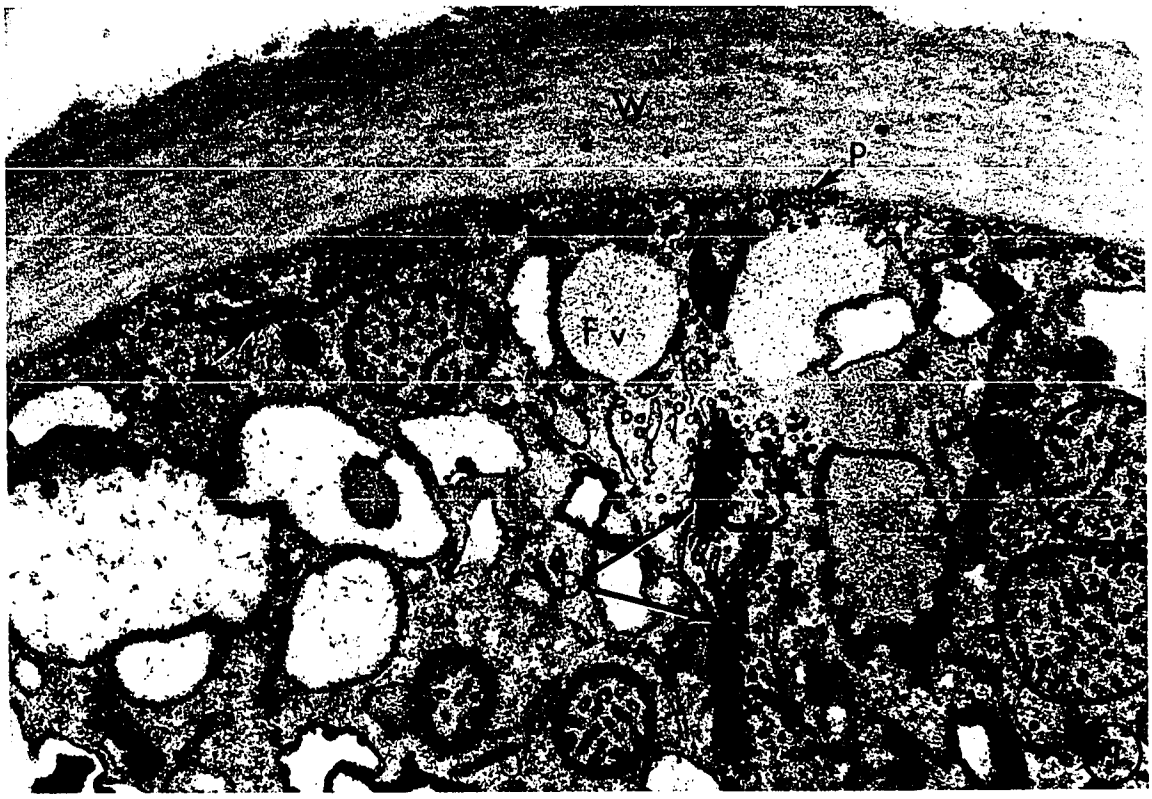


Fig. 2-8 Sporangium in which cleavage has gone to completion. Zoospores have encysted and begun to germinate through the sporangial wall (arrows). GOP. X 9,500

Fig. 2-9 Sporangial periphery prior to formation of germination wall showing the sporangial wall (W), fibrillar vacuole (Fv), endoplasmic reticulum (Er), dictyosomes (D), and ruffled plasmalemma (P). Glut-$KMnO_4$. X 20,000

Fig. 2-10 A thin layer of wall material is seen around an aggregation of vesicular elements (arrow). Original sporangial wall (W), microbodies (M), and dictyosome (D) are also shown. Glut-$KMnO_4$. X 23,900


W
P
Fv

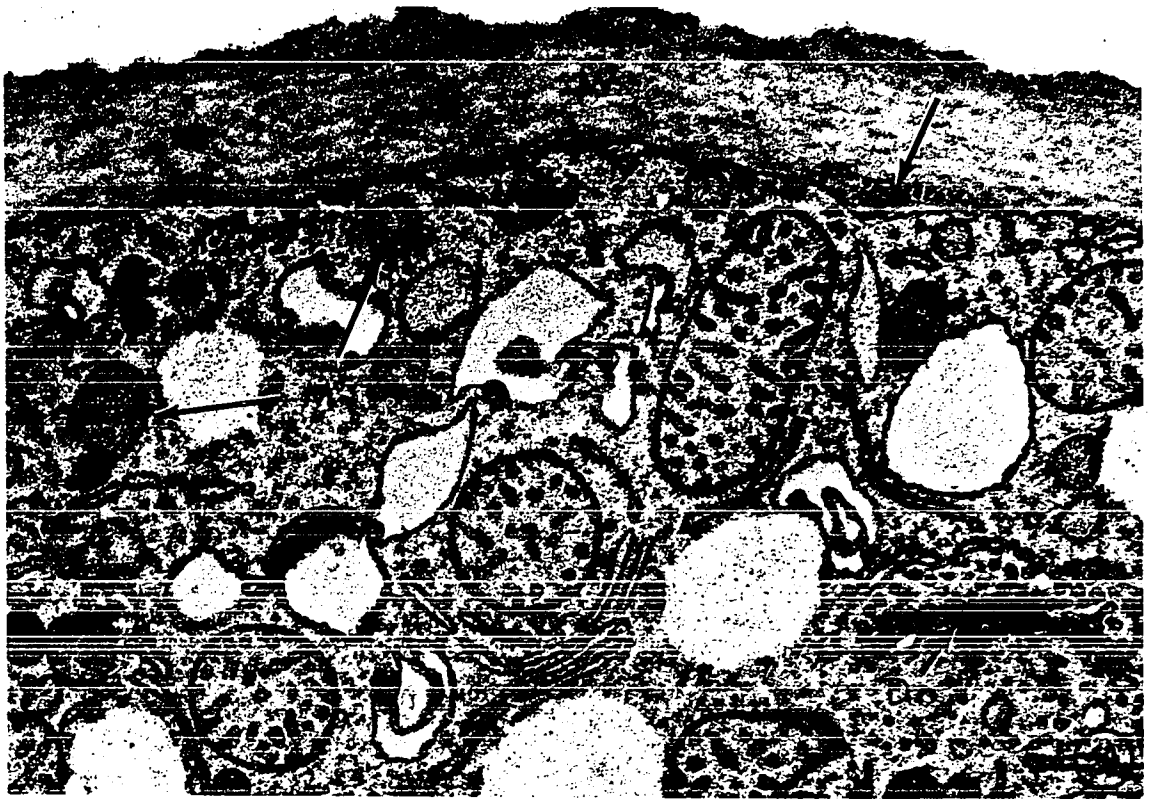


Fig. 2-11 Encased vesicular elements (Vi) within the inner sporangial wall. Segments of endoplasmic reticulum (arrow) and fibrillar vacuoles (Fv) are near the sporangial periphery. Original sporangial wall (W), germination wall (G), flagella (F). Glut-$KMnO_4$. X 23,000

Fig. 2-12 Thicker accumulation of the germination wall containing abundant vesicular elements (Vi). Note the vesicular deposit in the original sporangial wall. Fibrillar vacuoles (Fv), flagella (F), germination wall (G), microbody (Mb), original sporangial wall (W). Glut-$KMnO_4$. X 27,000


F
11

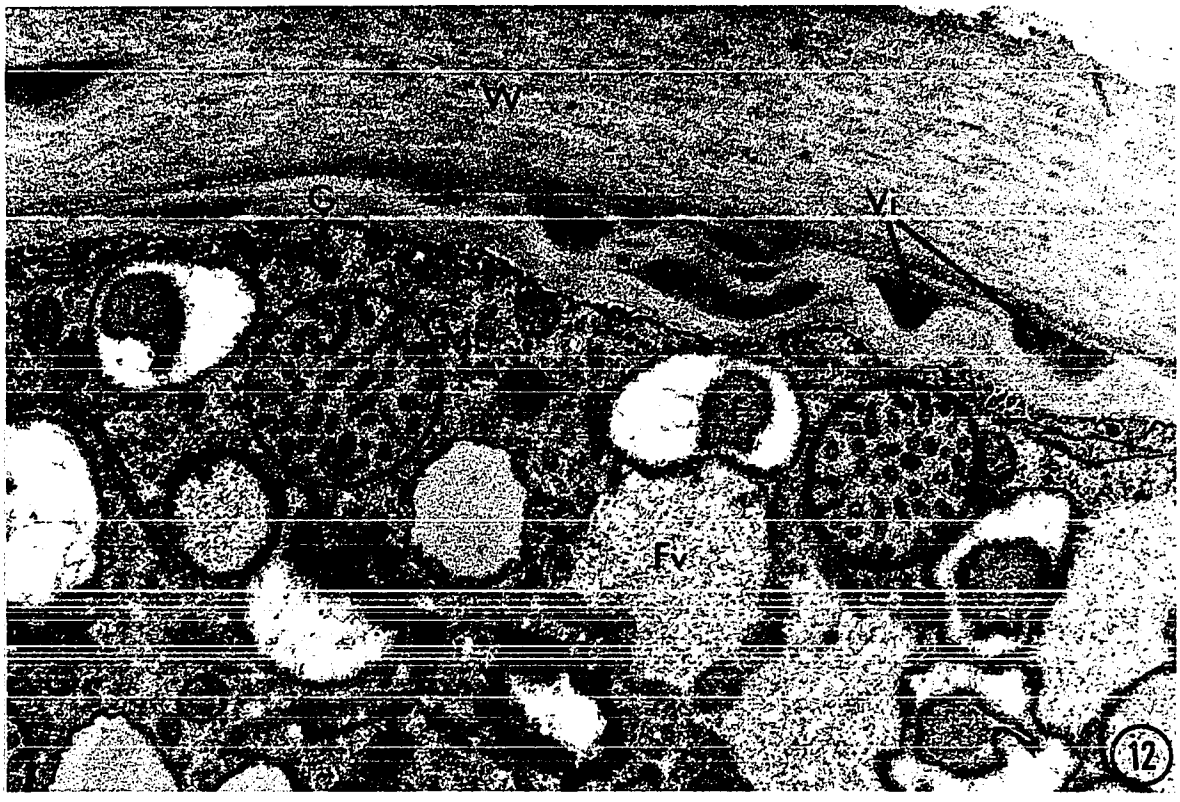
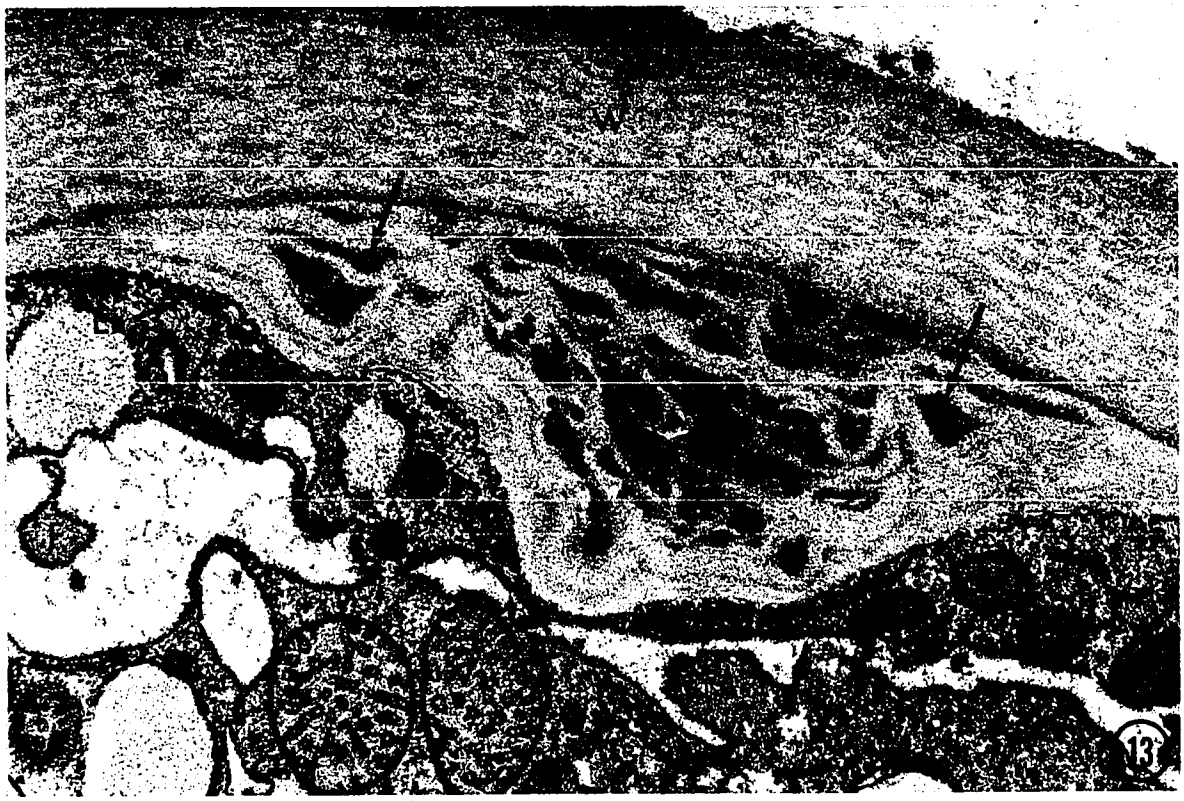

W
C
Vt
Fv
12

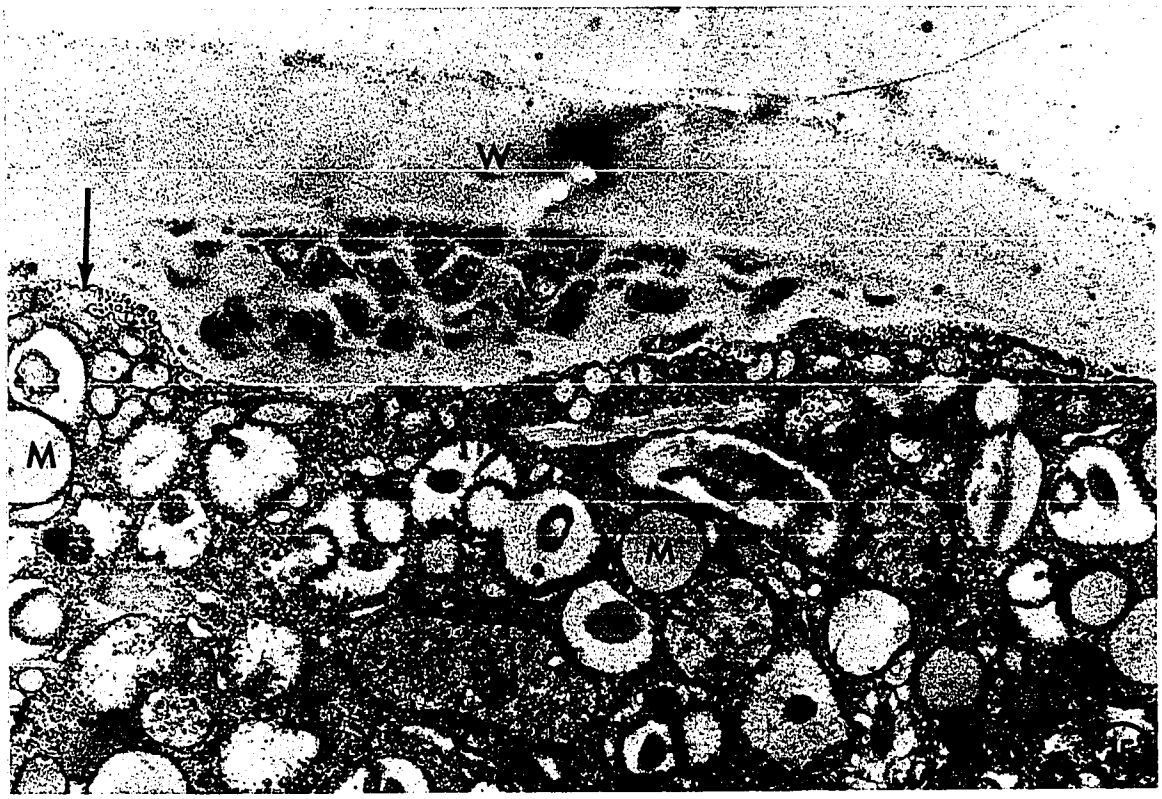
Fig. 2-13 and 2-14 Thicker accumulation of the germination wall containing abundant vesicular elements (arrows). Note the occasional vesicular deposits in the original sporangial wall (W) in fig. 2-14. Flagella (F) are still found in the cytoplasm. Microbodies (M), endoplasmic reticulum (Er). Glut-$KMnO_4$. X 23,000 and 12,000




Er

Fig. 2-15 Glutaraldehyde-osmium fixed sporangium with material (arrow) of same density as wall seen in the cytoplasm adjacent to the plasmalemma. Microbodies (M), a flagellum (F), original sporangial wall (W). Glut-OsO_4. X 20,200

Fig. 2-16 Same as fig. 2-15. In addition ribosomes can be seen in the vesicular elements (arrows) encased within the wall. Glut-OsO_4. X 17,500


W
M
M

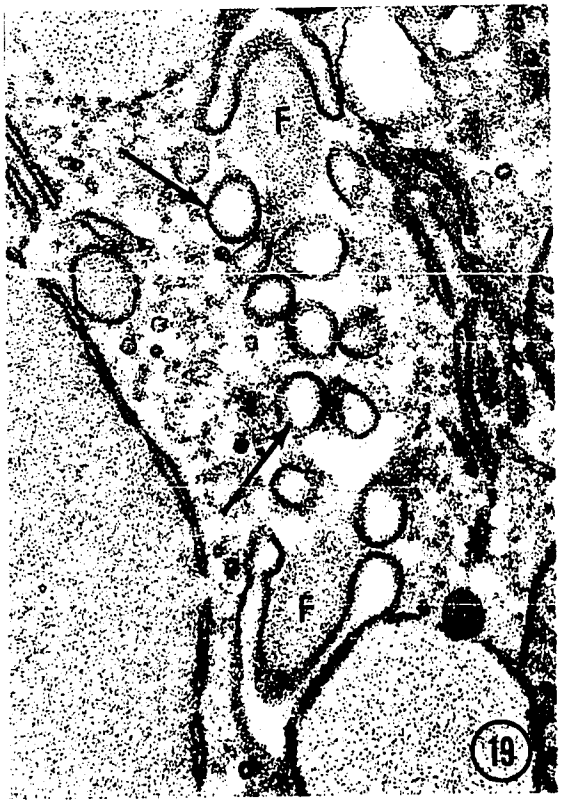

W

Fig. 2-17 Sporangium after germination wall (Gw) has formed showing that the cytoplasm is still partially cleaved, which is true of many of these sporangia. Flagella (arrows), original sporangial wall (W), nucleus (N). Glut-$KMnO_4$. X 12,200

Gw

Fig. 2-18 and 2-19 and 2-20 Accumulations of vesicles (arrows) around flagella (F) in directly germinating sporangia. Glut-$KMnO_4$. X 28,700, 29,000, and 38,500


F
18


F
F
19

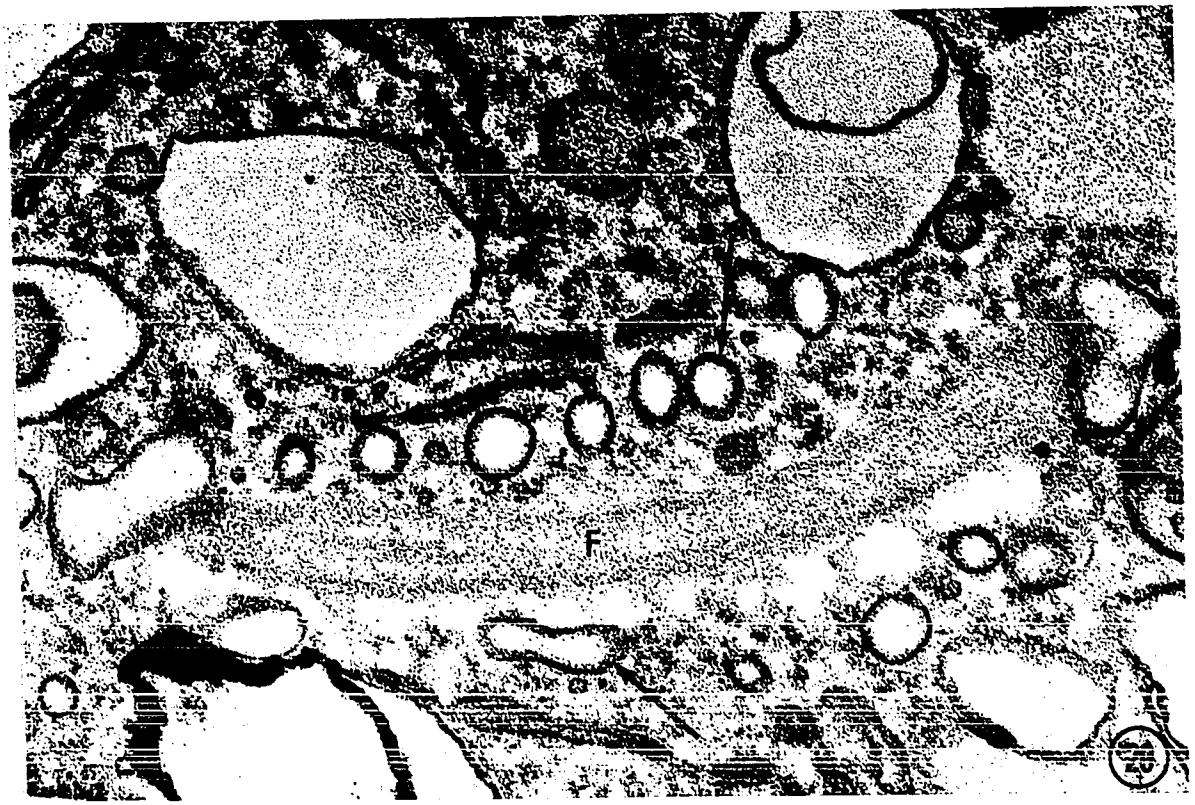

F
20

Fig. 2-21 Grazing section through sporangial periphery showing highly structured plasmalemma and several type I lomasomes (L^1). Note loops in mitochondria (M). Sporangial wall (W). Glut-$KMnO_4$. X 24,000

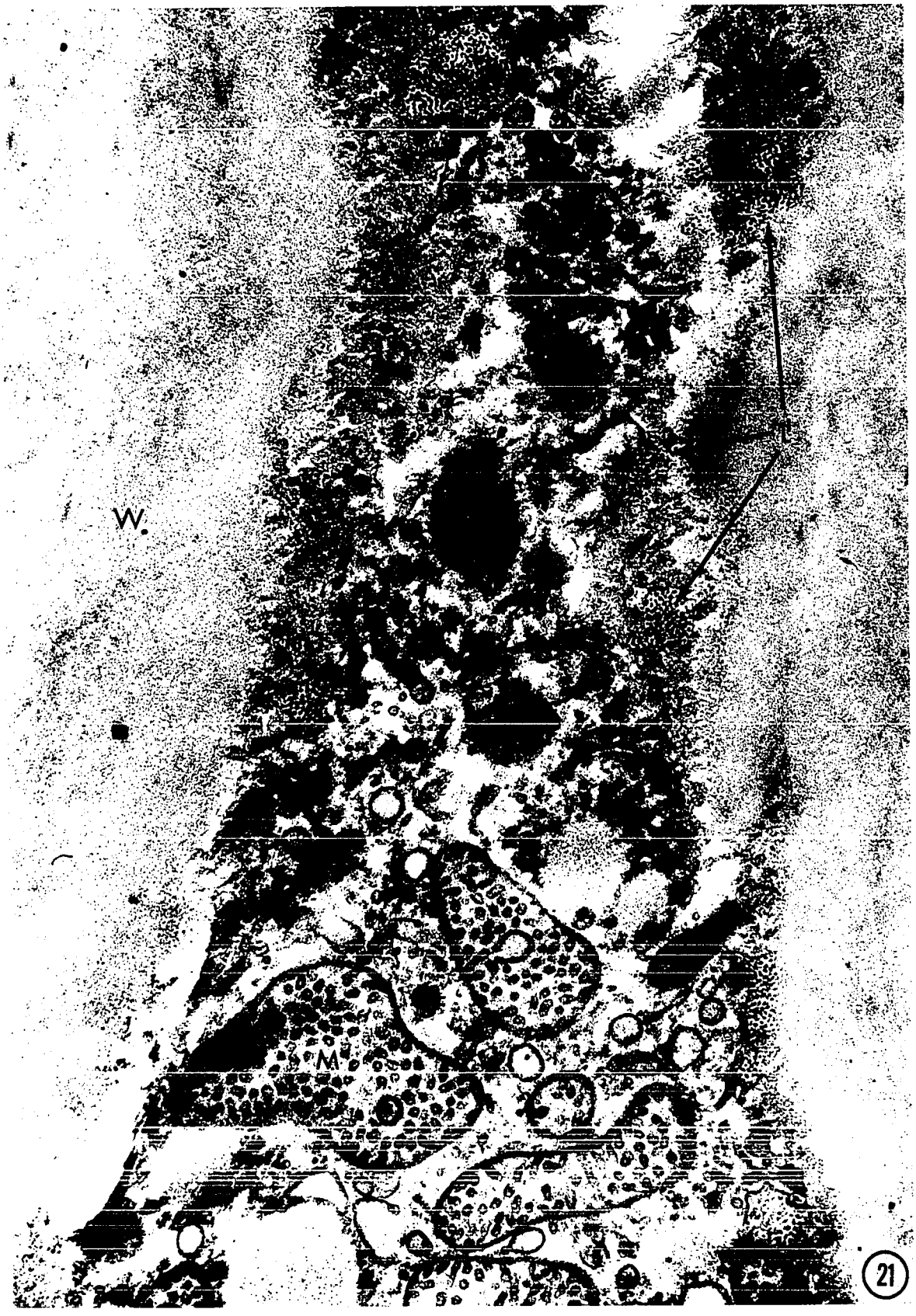

W.
M
21

Fig. 2-22 Type II lomasomes (arrows) containing spherical profiles encased by unit membranes. Sporangial wall (W). Glut-$KMnO_4$. X 53,300

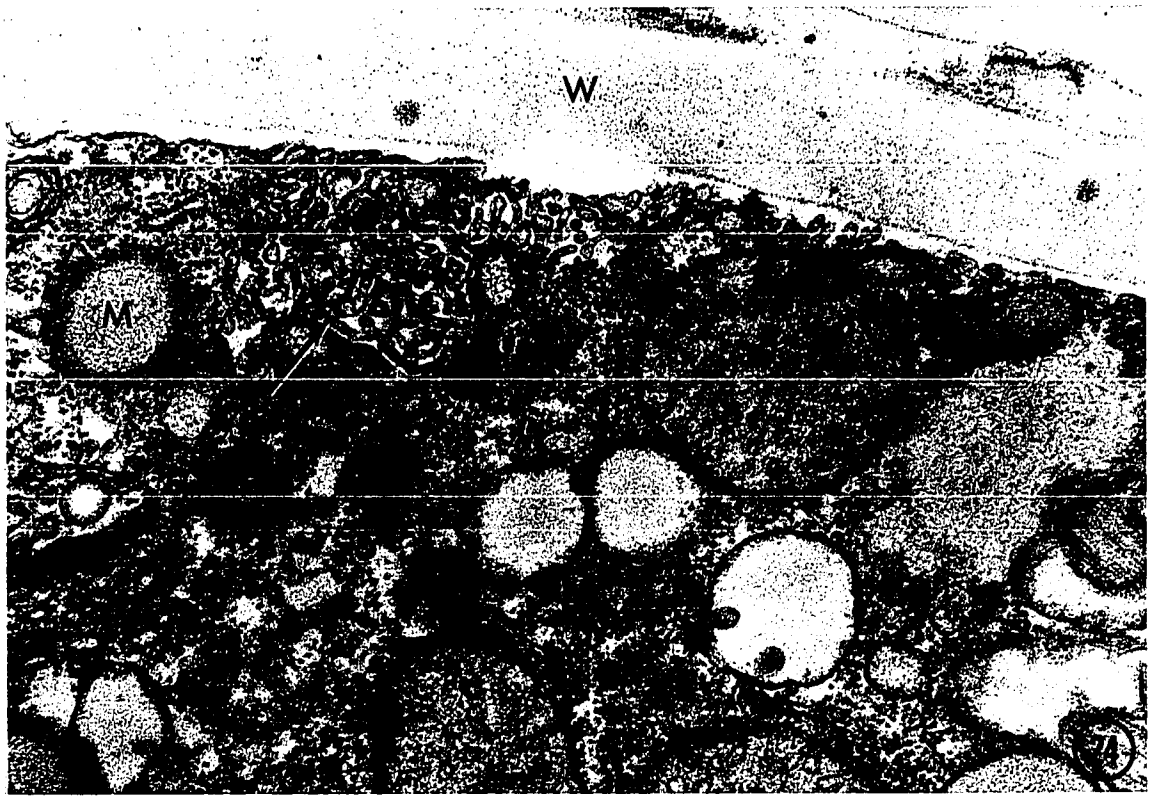
Fig. 2-23 Type II lomasomes (L^2) containing tubular profiles. Flagellum (F), microbody (M), sporangial wall (W). Glut-OsO_4. X 37,100

W

W
25

Fig. 2-24 Lomasome (L^2) containing spherical and tubular profiles. Dictyosome (D), microbody (M), sporangial wall (W). Glut-OsO_4. X 41,200

Fig. 2-25 Appearance of lomasomes (arrows) fixed with permanganate. Note the regularity of size and distribution of the spherical profiles. Microbody (M) containing typical lamellar inclusion. Sporangial plug (P), sporangial wall (W). Glut-$KMnO_4$. X 39,600


W
M
4

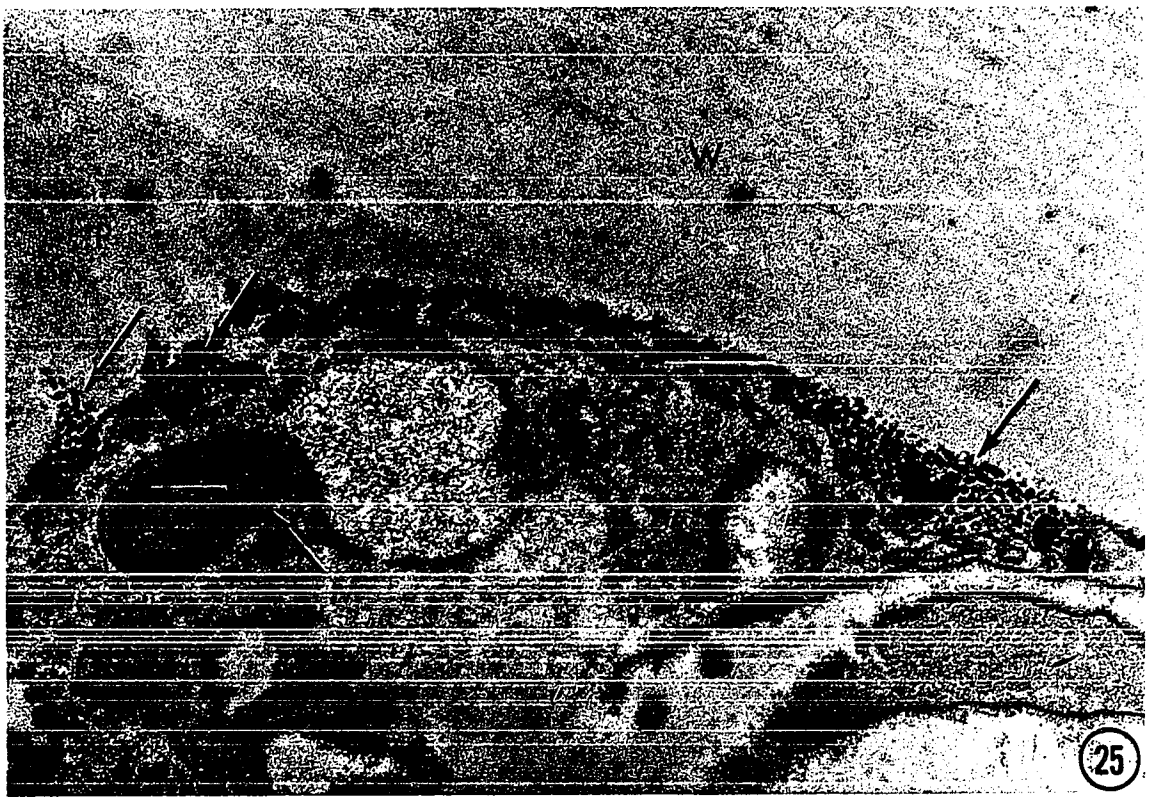
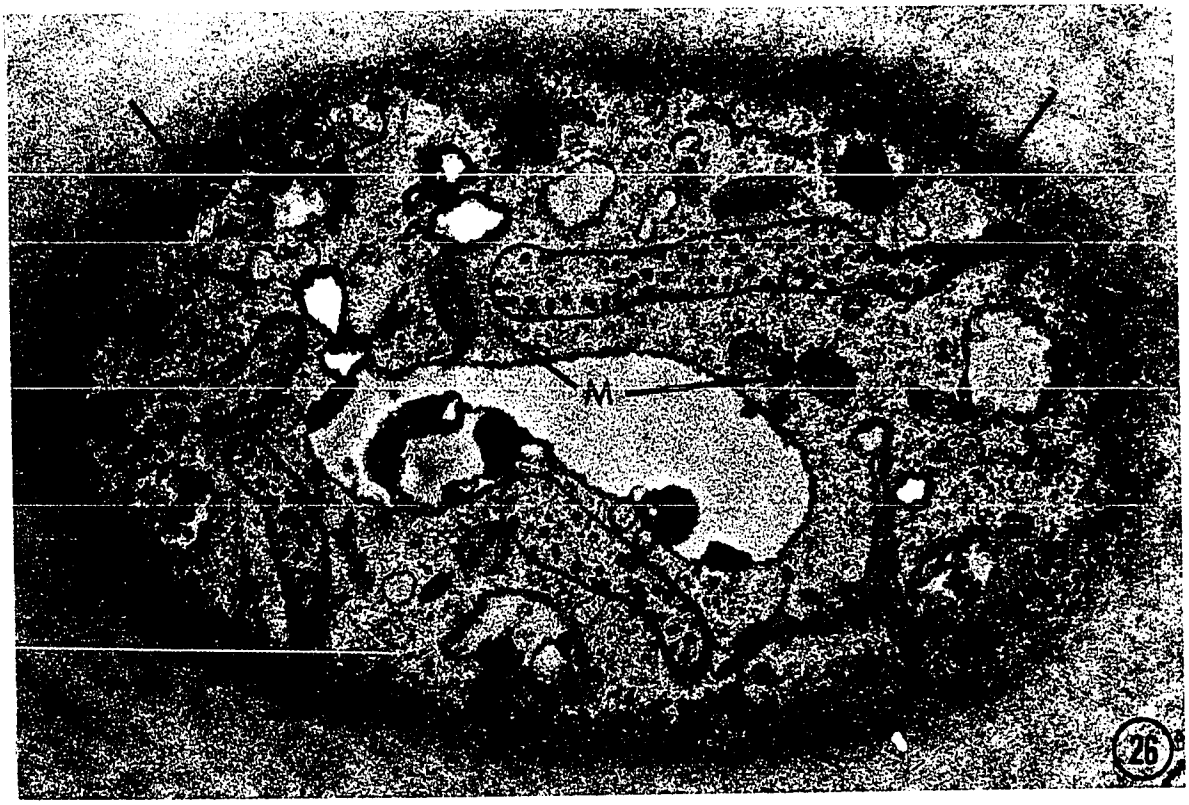
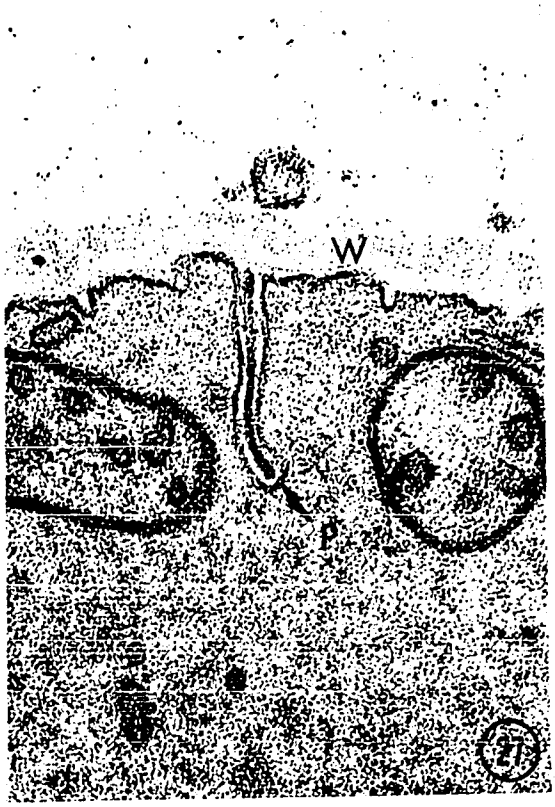

W
25

Fig. 2-26 Grazing section through sporangium demonstrating the complex folding of the plasmalemma (arrows). Microbodies (M). Glut-$KMnO_4$. X 15,600

Fig. 2-27 and 2-28 The plasmalemma (P) extends deeply into the cytoplasm. Hyphal wall (W). Glut-$KMnO_4$. X 53,300 and 81,900


M
26


W
P
27


W
28

Fig. 2-29 Dictyosome in perinuclear position. Note vesicles blebbing off nuclear envelope (arrow). Nucleus (N), Glut-$KMnO_4$. X 34,300

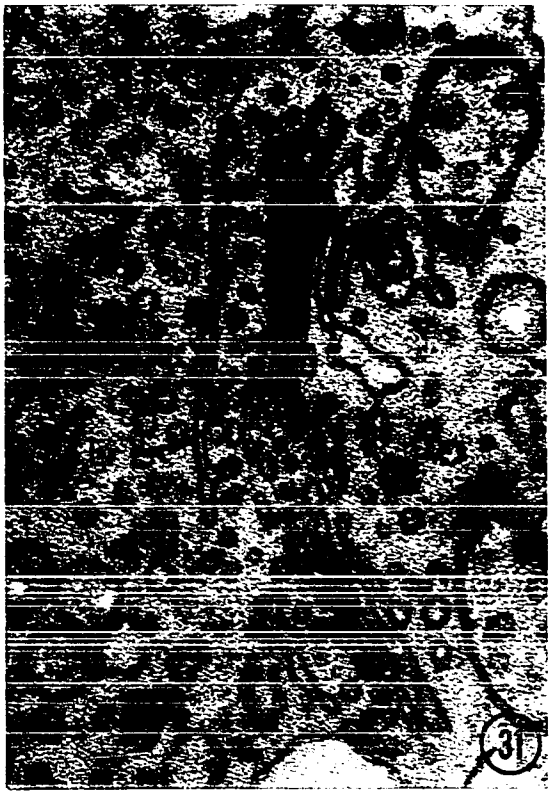
Fig. 2-30 Mottled-vesicles (arrows) being pinched from cisternae of the dictyosomes. Glut-$KMnO_4$. X 41,600

Fig. 2-31 Dictyosome in association with cisternae of endoplasmic reticulum (Er). Note the vesicles blebbing from the endoplasmic reticulum. Glut-$KMnO_4$. X 79,200

Fig. 2-32 Whorl of vesicles near dictyosome which may fuse to form polyvesicular bodies. Glut-$KMnO_4$. X 30,500


29


30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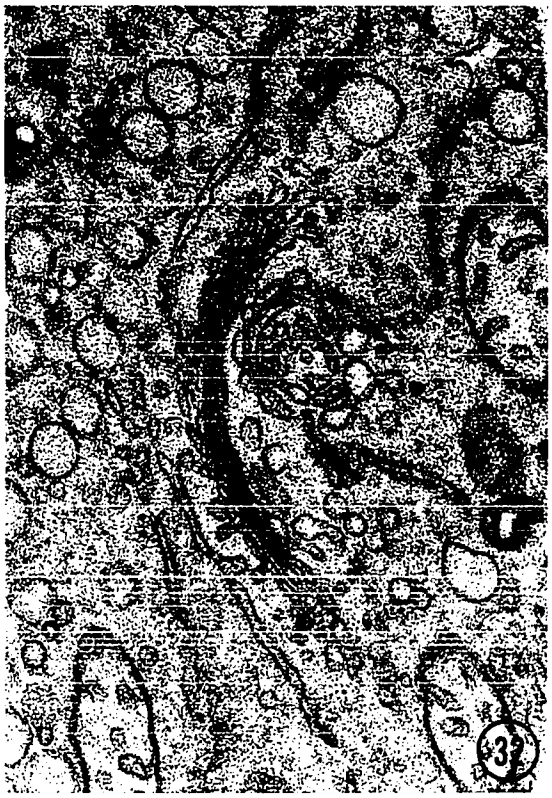
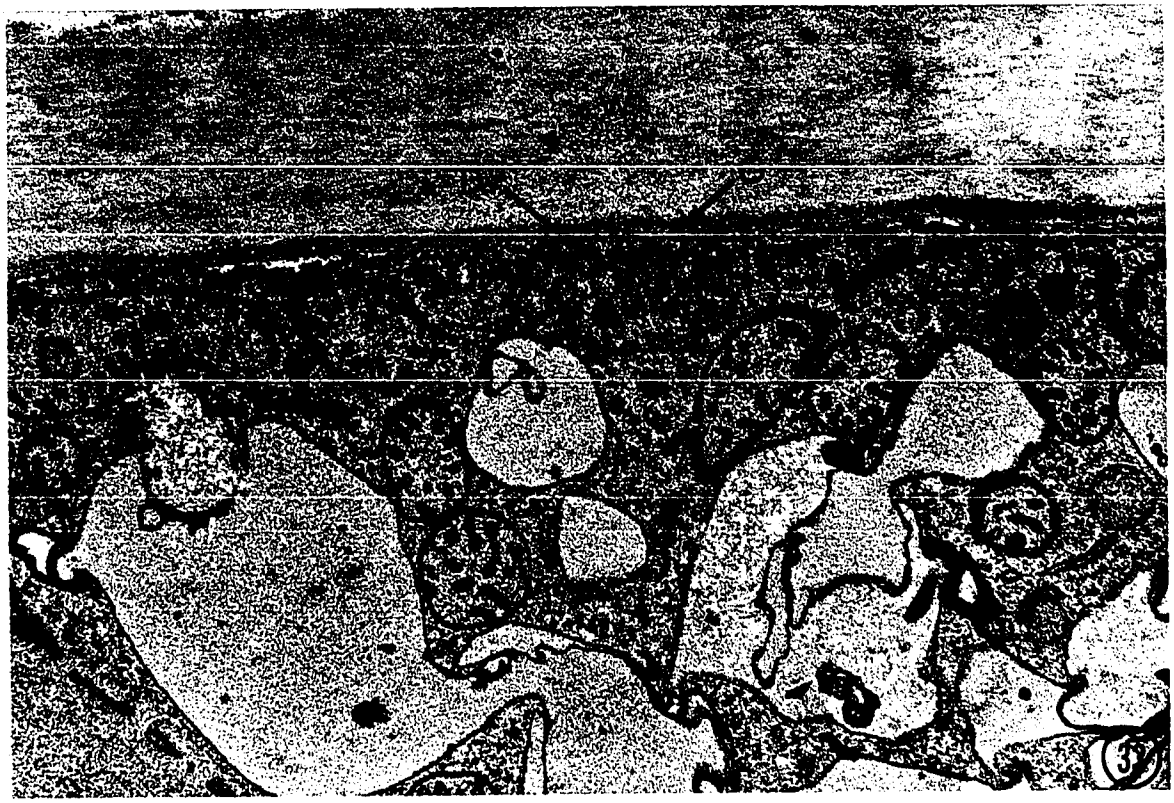

32

Fig. 2-33 Aggregate of vesicles (Dv) near sporangial wall (W). Germination wall (G). Glut-$KMnO_4$. X 21,000

Fig. 2-34 Polyvesicular body (Pvb) near the plasmalemma (P) of a hypha. Dictyosome (D). Glut-$KMnO_4$. X 41,000

Fig. 2-35 Continuity between plasmalemma invagination and a vesicle (Dv). Vesicular elements (Vi), sporangial wall (W), germination wall (G). Glut-$KMnO_4$. X 61,500

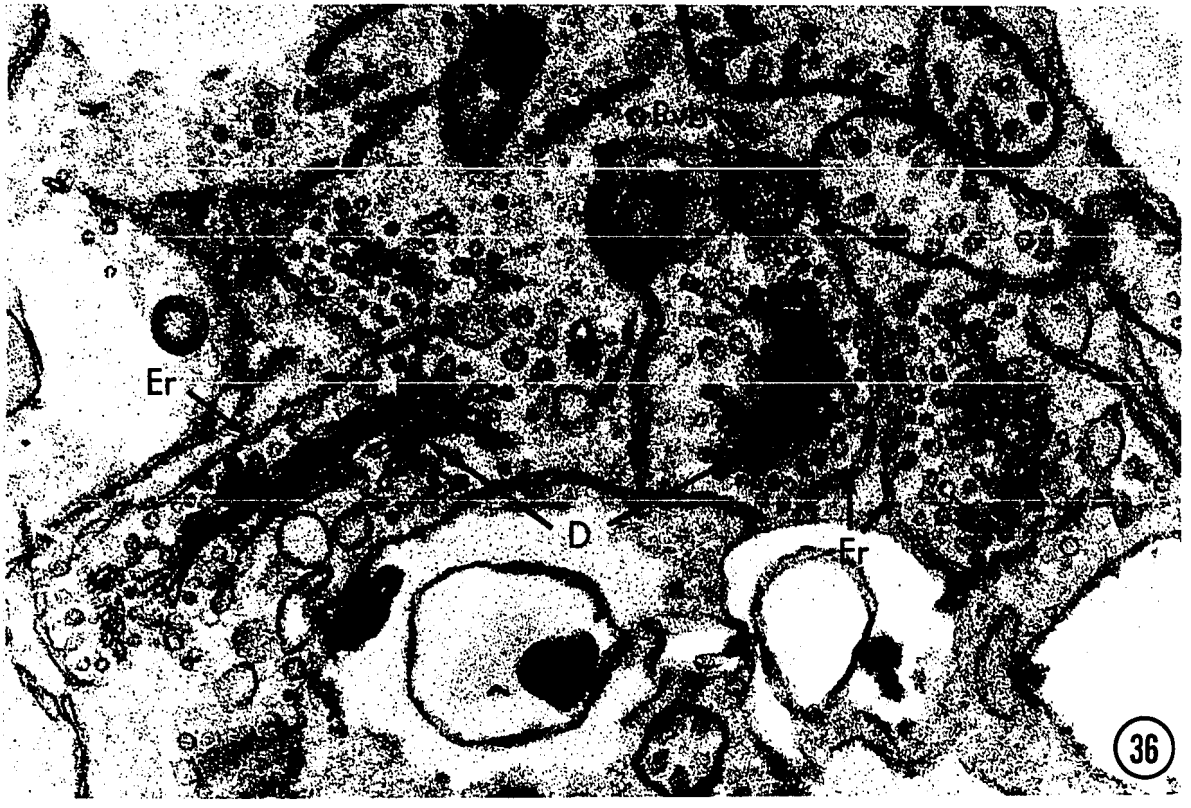

33


34


Vi
W
35

Fig. 2-36 Dictyosomes (D) associated with cisternae of endoplasmic reticulum (Er). Note the association of the polyvesicular body (Pvb) with the dictyosome. Glut-$KMnO_4$. X 37,000

Fig. 2-37 Polyvesicular bodies (Pvb) in the cytoplasm of a hypha. Glut-$KMnO_4$. X 30,000


Er
D
Er
36

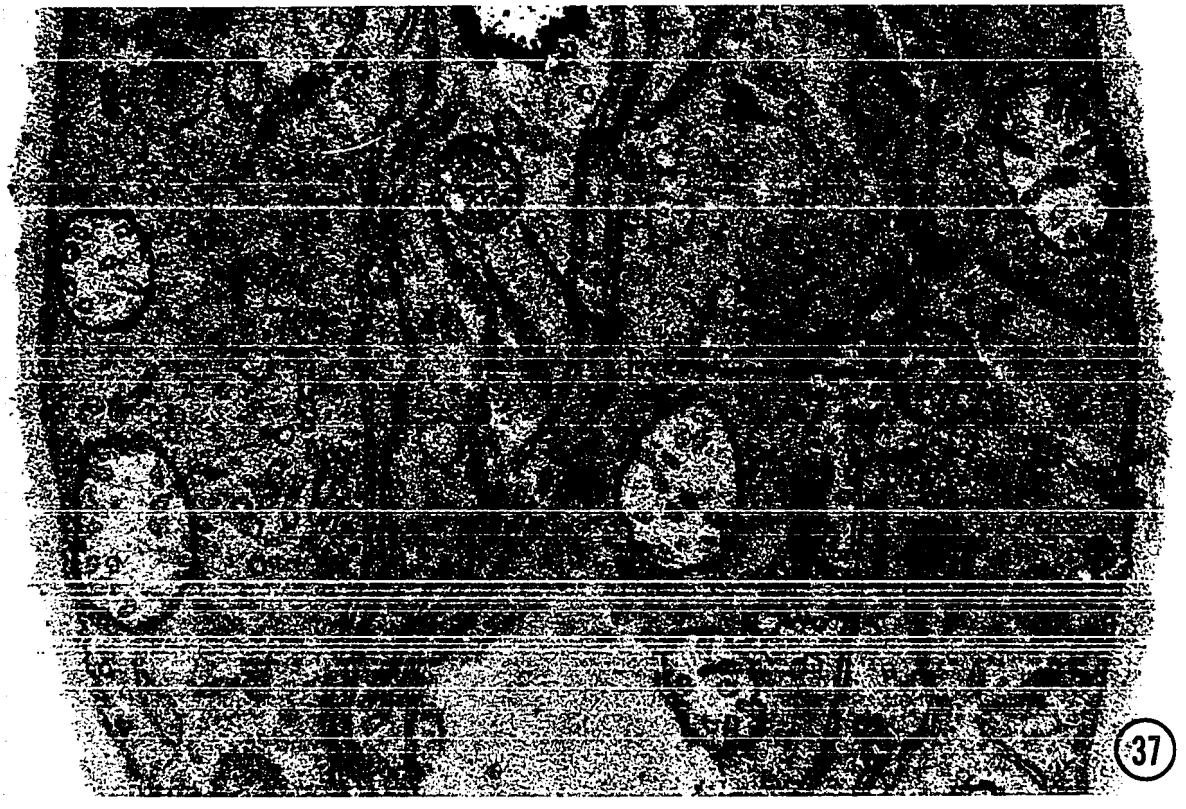

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Fig. 2-38 Grazing section of sporangium near "neck" of sporangium showing large accumulation of mitochondria (M) containing loops (arrows). Type I lomasome (L^1), sporangial wall (W). Glut-$KMnO_4$. X 25,300

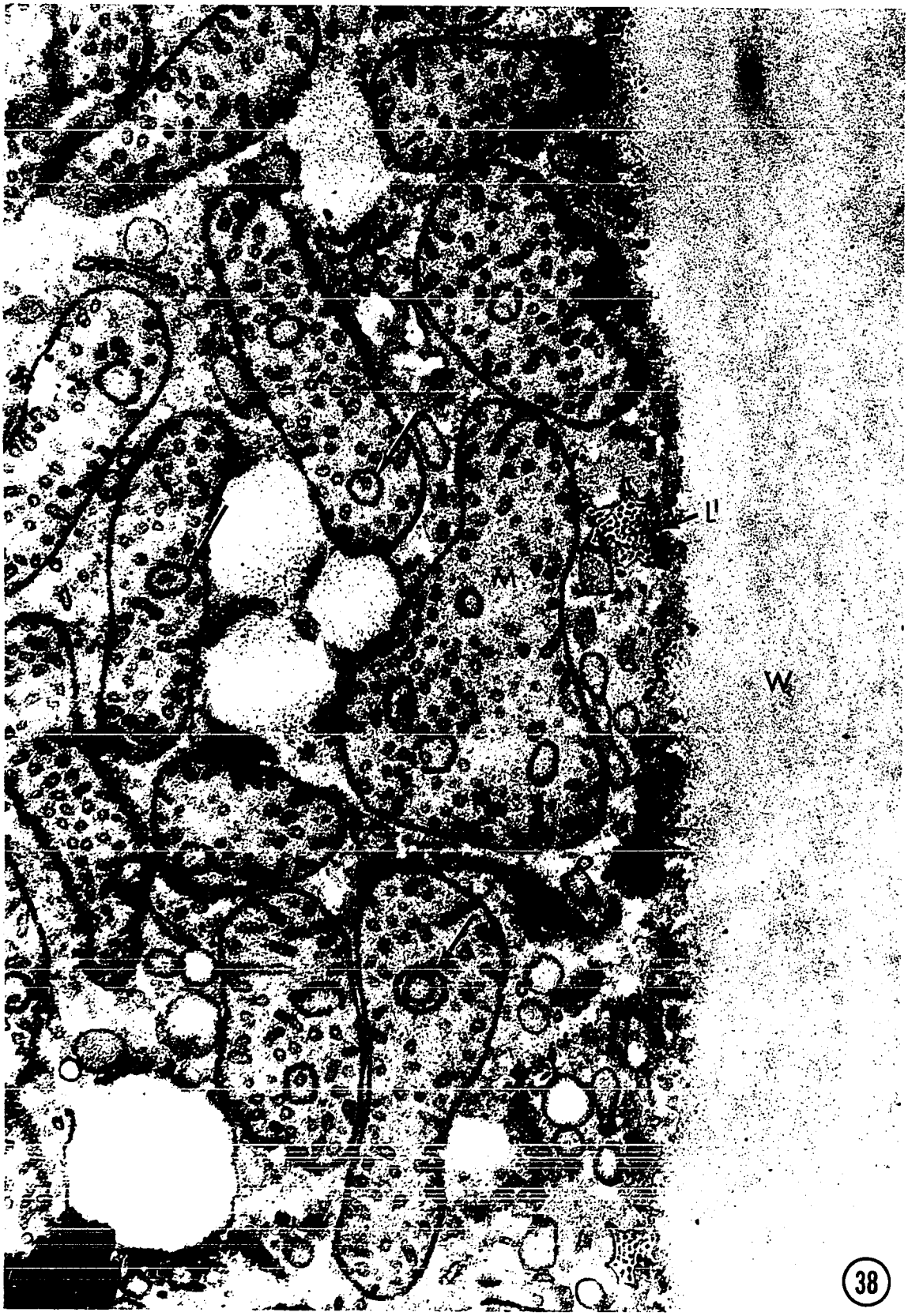
L'
M
W

Fig. 2-39 Apical plug (P) located in the neck formed by the sporangial wall (W), with type I lomasomes (L^1) at the base of crevices (arrows). Glut-$KMnO_4$. X 39,600

W

Fig. 2-40 Accumulation of vesicles (Dv) near the apical plug (P). Type I lomasomes (L^1) are located at the base and in crevices of the apical plug. Mitochondria (M). Glut-$KMnO_4$. X 29,400

L
P
Q

Fig. 2-41 Cytoplasmic channel through the apical plug area (P). Lomasomes (L^1) are again prominent. Glut-$KMnO_4$. X 45,000

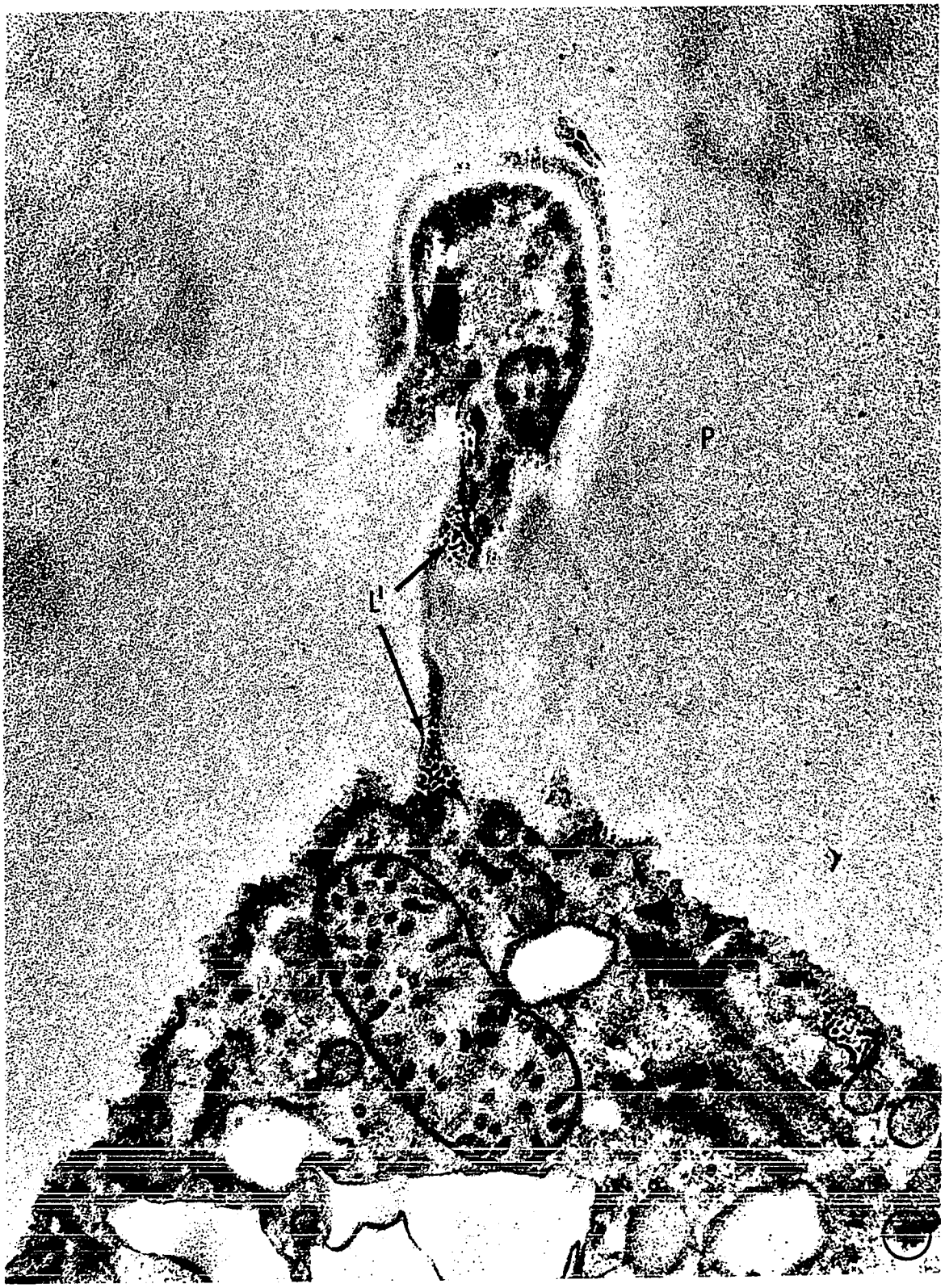
P
L'

Fig. 2-42 Grazing section of sporangial "neck" fixed with glutaraldehyde-osmium to demonstrate the vesicular composition of the lomasomes (arrows) in comparison with permanganate fixed material in figure 2-41. Sporangial papilla (P), germination wall (Gw). GOP. X 40,200

Fig. 2-43 Top of apical plug (P) as hypha emerges. Note vesicles of different sizes (Dv), tubular endoplasmic reticulum (Er), and a fibrillar vacuole (Fv). Glut-$KMnO_4$. X 26,100

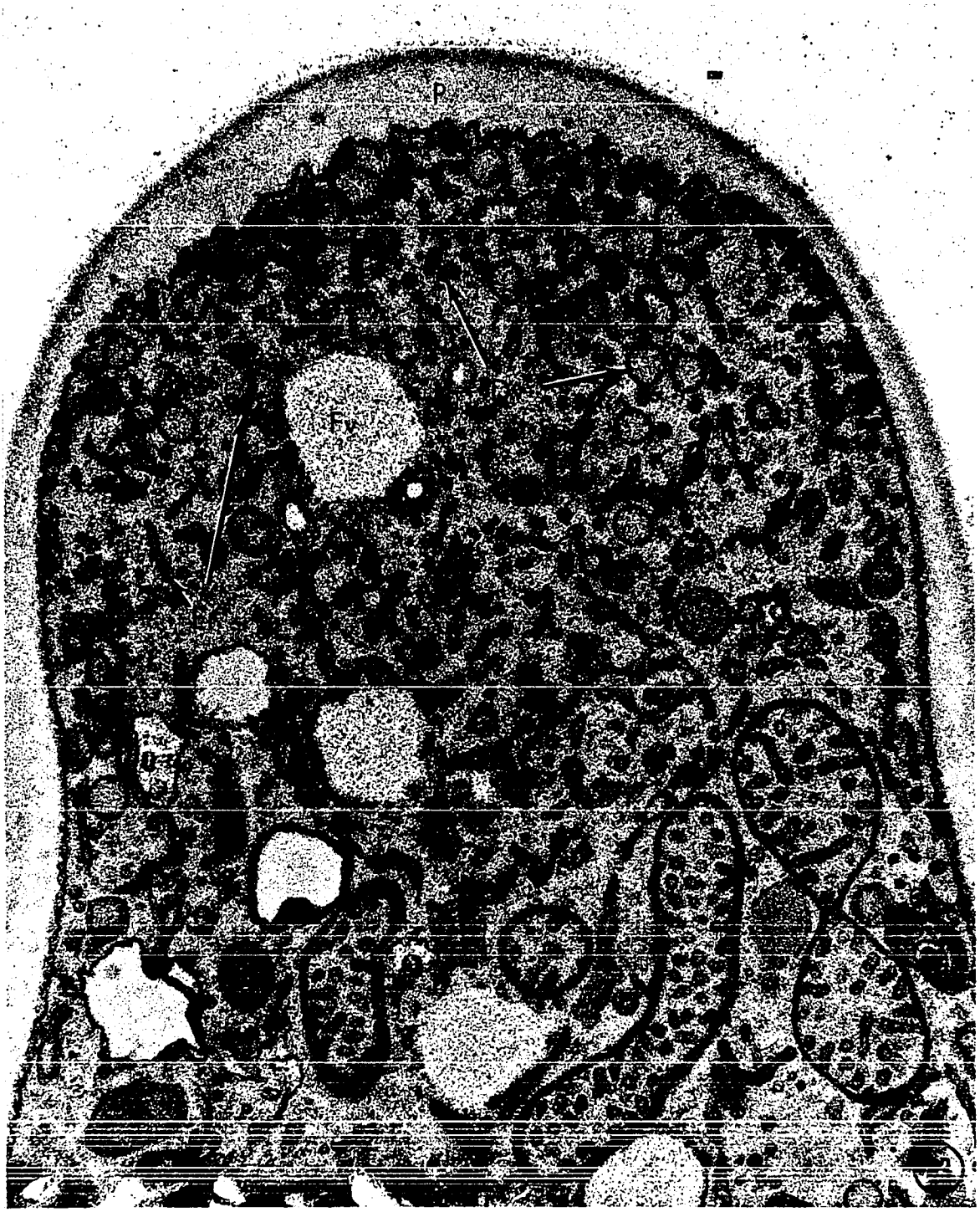
P
Fv

Fig. 2-44 Similar to fig. 2-43, but the germ tube has not penetrated the plug (P) completely. The plug material at the tip of the germ tube has been stretched out of its original position in the neck formed by the sporangial wall (W). Nucleus (N). Glut-$KMnO_4$. X 17,000


P
W
44

Fig. 2-45 Apical bulge of cytoplasm demonstrating the continuity of the hyphal wall (W) with the germination wall (G). Also note the small vesicles and tubular endoplasmic reticulum (arrows) at the periphery of the apical bulge. Type I lomasomes (L^1), sporangial plug (P). Glut-$KMnO_4$. X 16,800

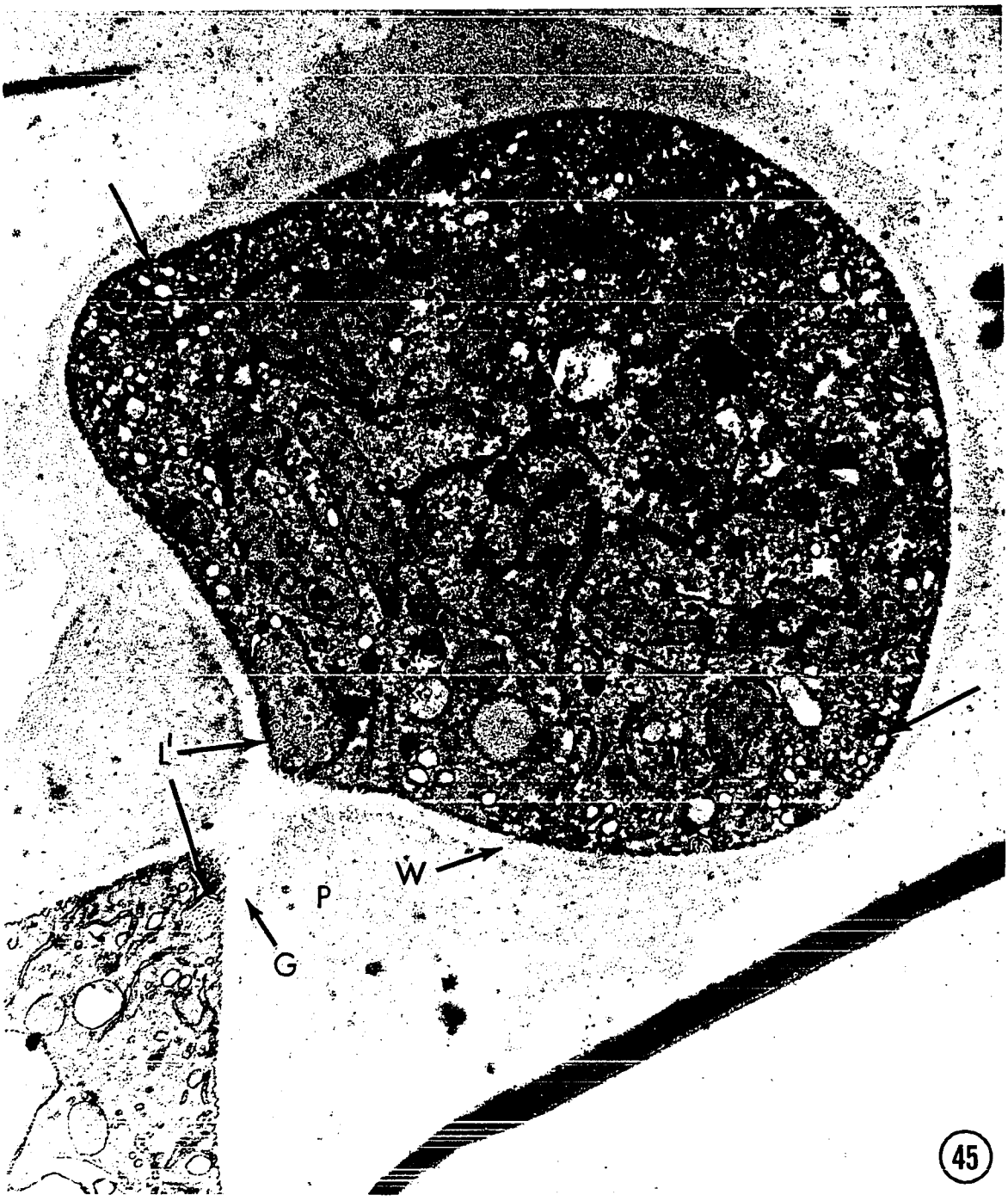
L
W
P
G

Fig. 2-46 Bulged hypha after complete penetration of the plug (P). Note elongated nucleus (N) in the germ tube emerging from the sporangium. Glut-$KMnO_4$. X 13,500

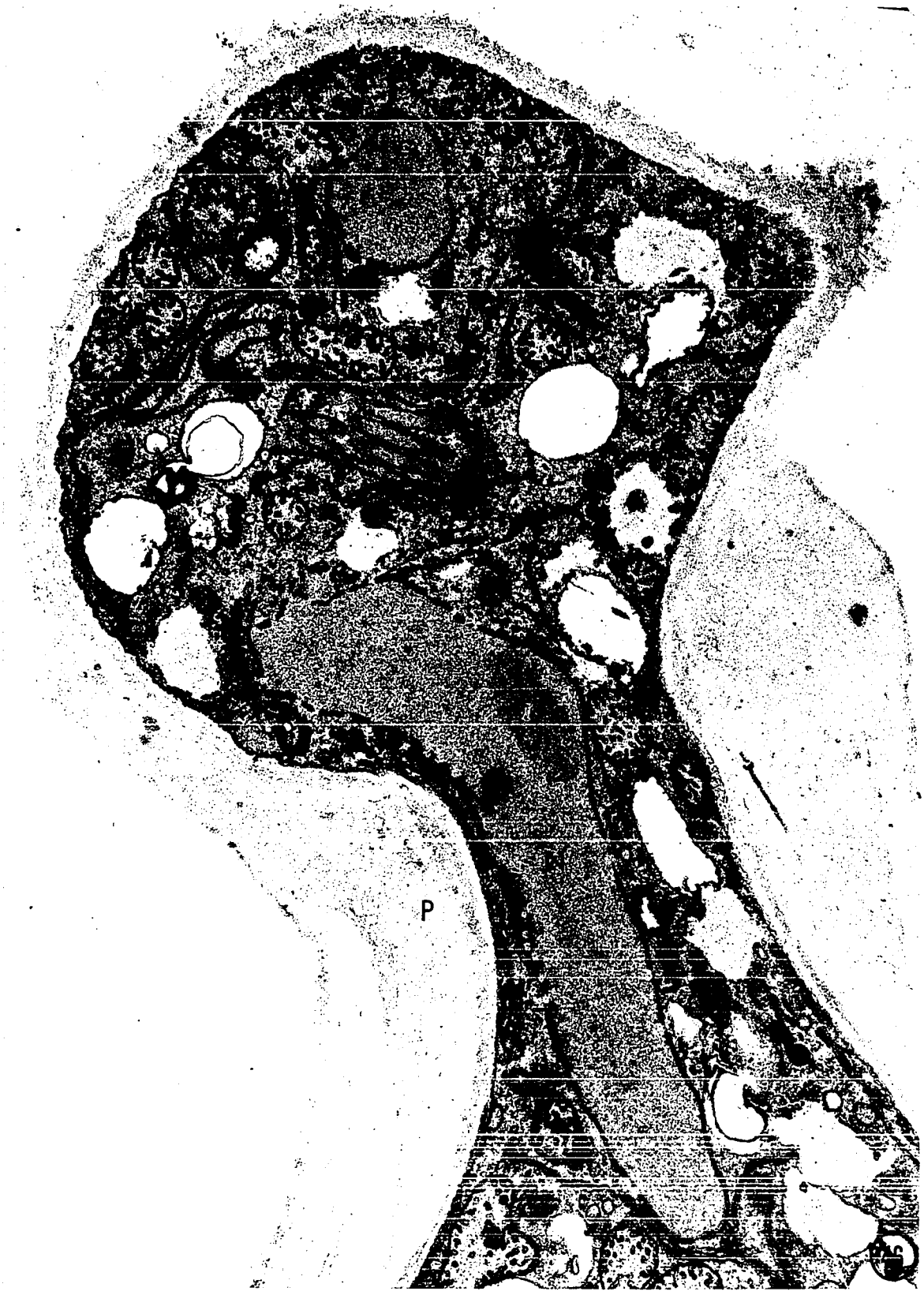
P

Fig. 2-47 Branching of hyphae (H) without formation of an apical bulge. Hyphal wall (Hw) is a continuation of the germination wall (G). Glut-$KMnO_4$. X 10,800

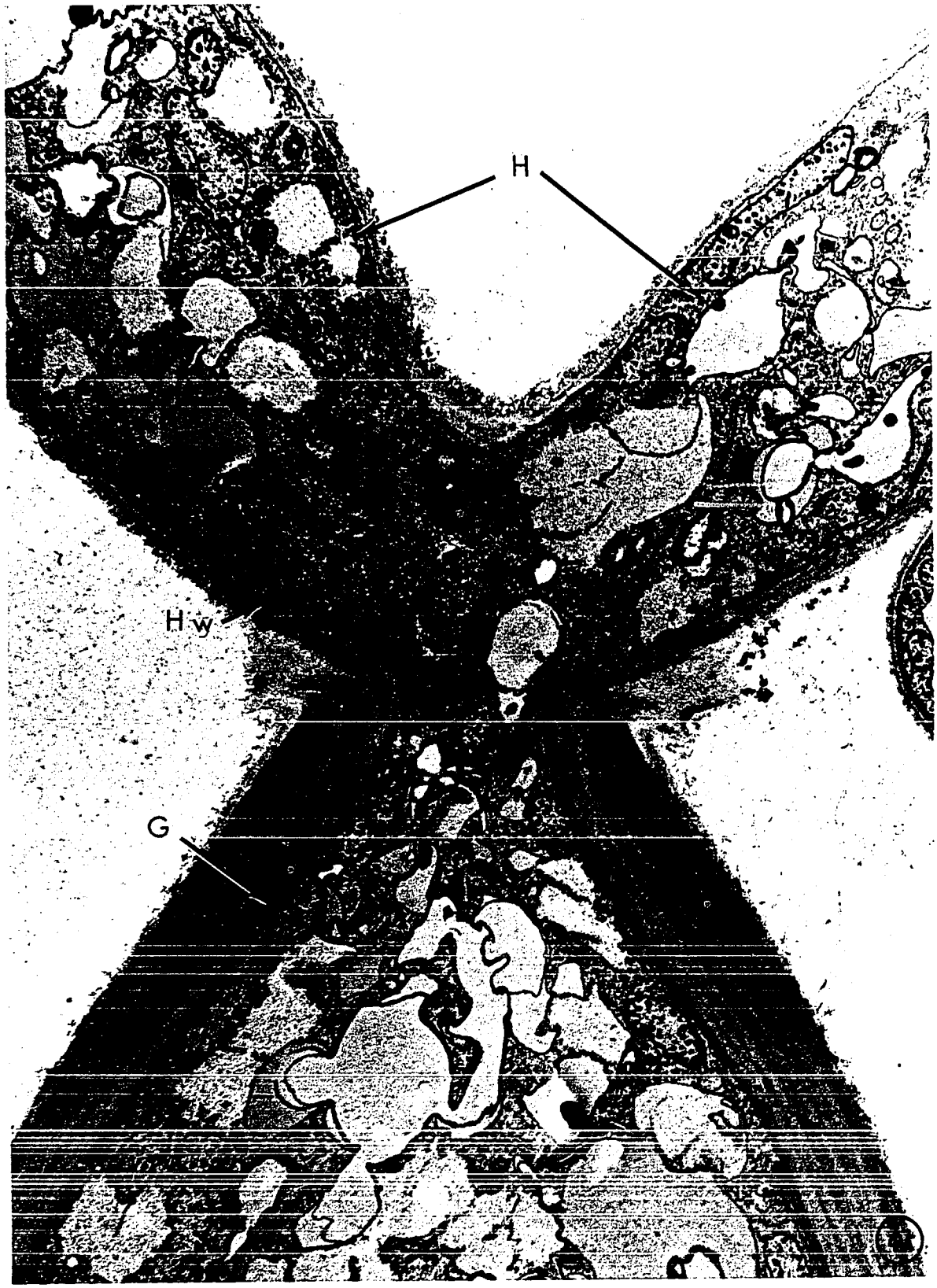
H
Hw
G

Fig. 2-48 Germ tube after penetration of sporangial wall (W) showing hyphal wall (Hw) and type I lomasome (L^1). This section is cut tangentially through the basal attachment of the sporangium (Ba). Glut-$KMnO_4$. X 35,200


L'
Hw
W
Bc

Fig. 2-49 Accumulation of vesicles (Dv) at the hyphal tip. Similar vesicles are located near the dictyosomes (D). Glut-$KMnO_4$. X 28,000

Fig. 2-50 Cross-section of hypha demonstrating the peripheral orientation of the mitochondria (M) parallel to the hyphal tube. Lomasome (L^1). Glut-$KMnO_4$. X 46,000

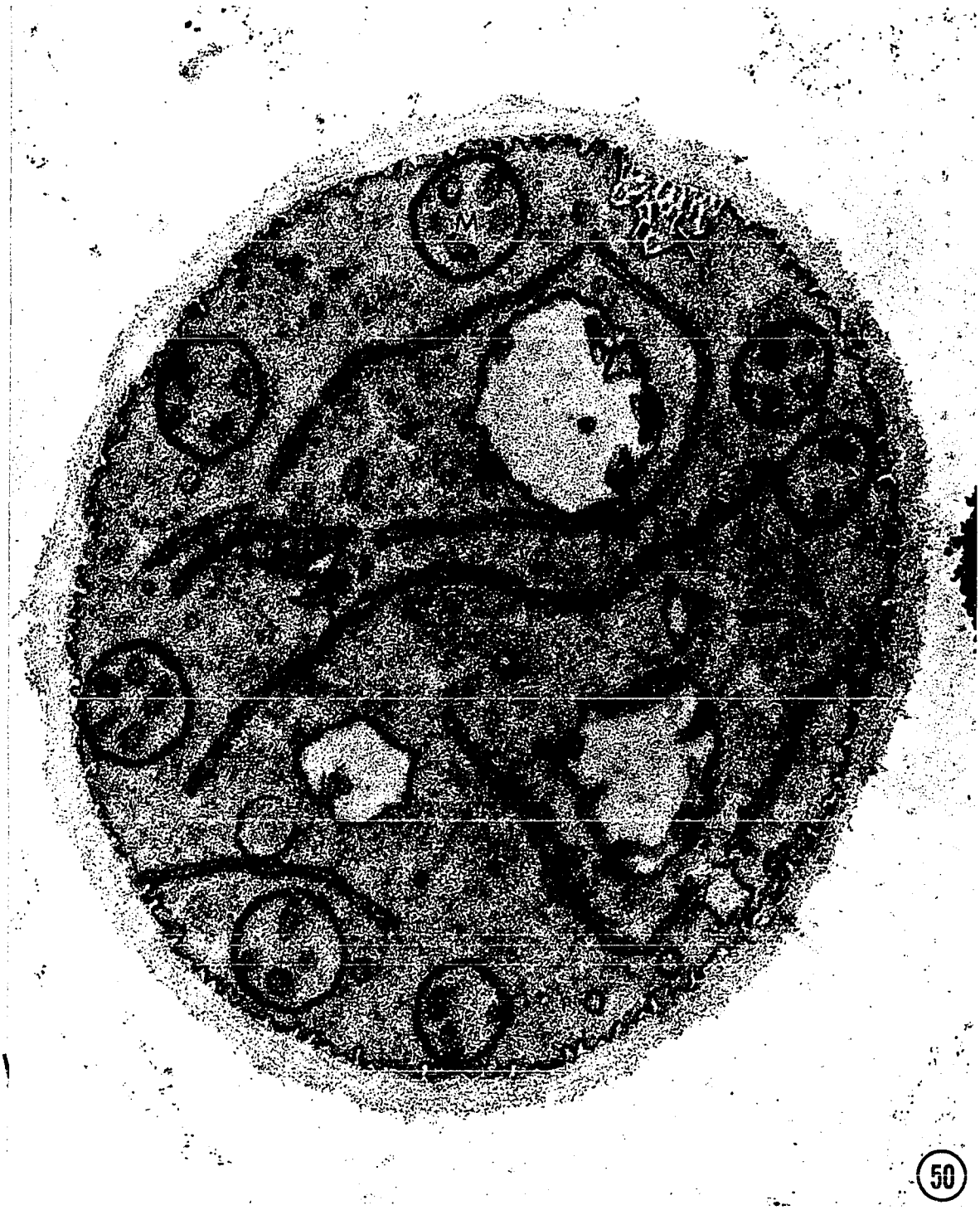

Fig. 2-51 Oblique section of a hypha with peripheral arrangement of mitochondria (M) and central vacuoles (V). Note the vesicular aggregates (arrows) and fibrillar vacuoles (Fv). Glut-$KMnO_4$. X 24,800


Ev
M
V

Fig. 2-52 Tangential section through hypha showing folding pattern of plasmalemma (P). Type I lomasome (L^1). Glut-$KMnO_4$. X 25,200

Fig. 2-53 Longitudinal section of hypha demonstrating the orientation of mitochondria (M) parallel to the germ tube. Glut-$KMnO_4$. X 26,500

52

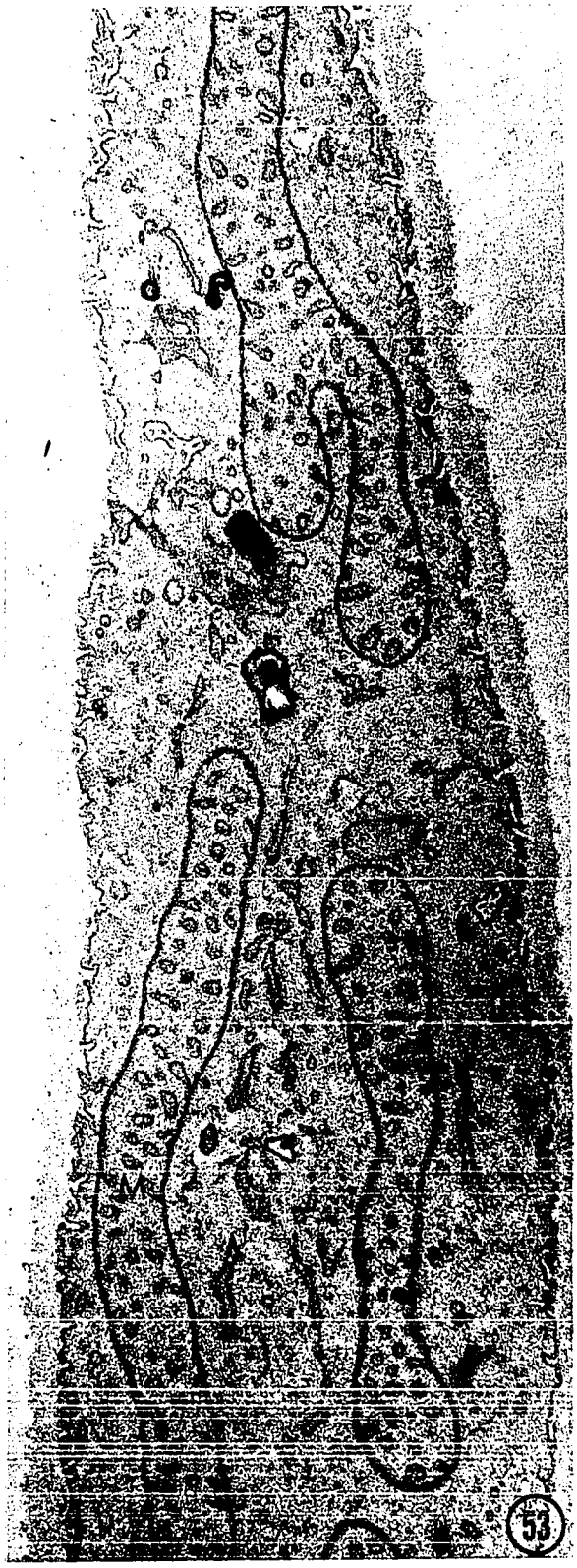

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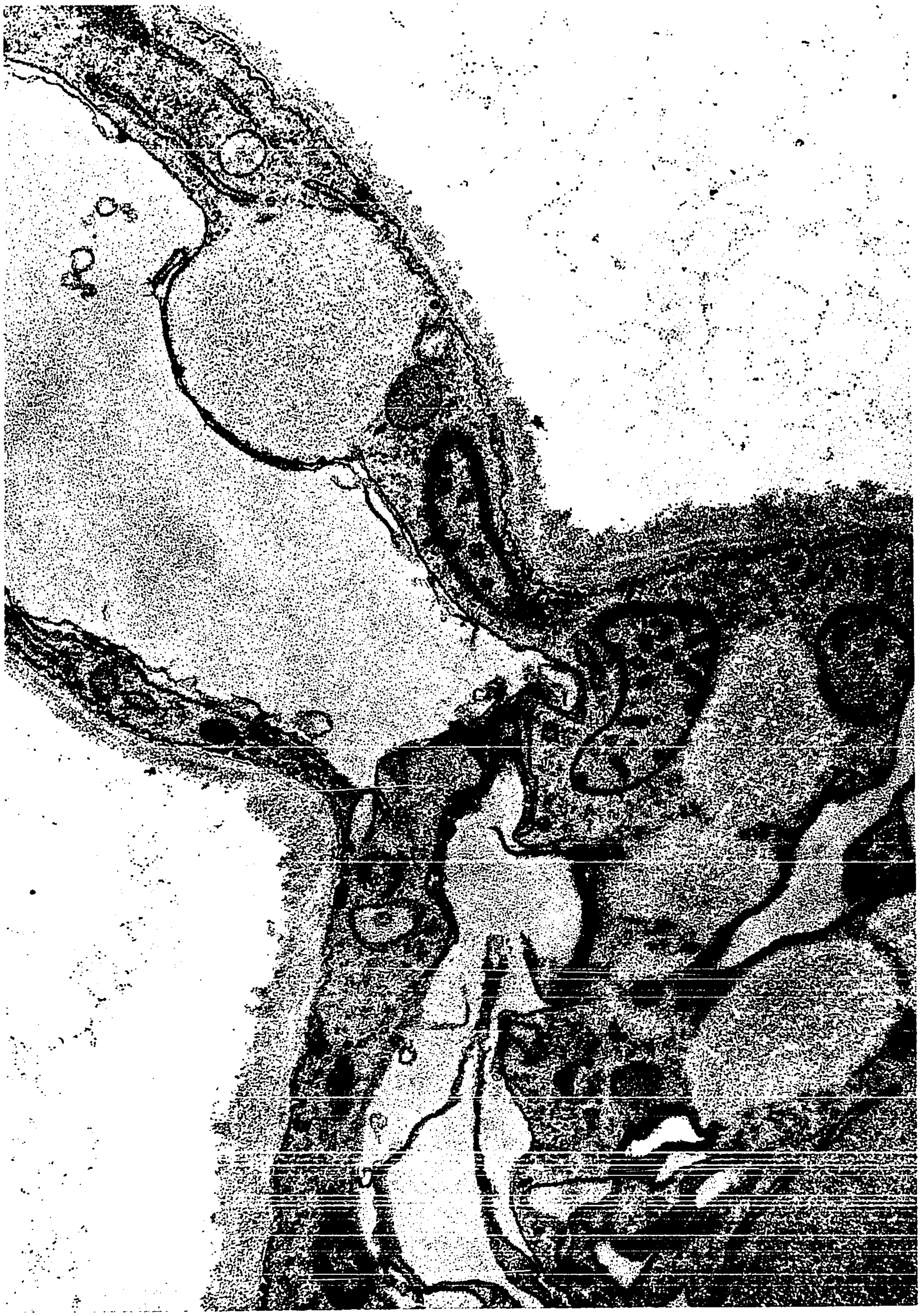
Fig. 2-54 Germ-tube wall fixed with permanganate demonstrating the two layered nature of the hyphal wall. Note the inner fibrillar layer covered by a "fluffy" outer wall. Glut-$KMnO_4$. X 30,500



CHAPTER V

RESULTS

Electron Microscopy of Encystment and Cyst Germination: Cysts of Phytophthora parasitica may germinate in different ways depending on the composition of the incubation medium (fig. 1-1). The two types of cyst germination which appear to be analagous to direct and indirect sporangial germination (figs. 1-6, 1-9) were chosen for comparative study. The results are divided into three parts: (1) encystation, (2) production of germ tubes (Direct Cyst Germination), and (3) production of secondary zoospores (Indirect Cyst Germination).

One of the basic differences in the ultrastructure of the zoospore of Phytophthora parasitica as described by Reichle (1969) and the cyst is the redistribution of the cellular organelles. Encystation. Once the zoospore begins to round up, the flagella are lost leaving the basal bodies, microtubules, and other flagellar structures intact near the nucleus (fig. 3-5). No evidence of retraction of flagella into or around the cyst is observed. The large vacuole found at the side of the nucleus in the zoospore disappears as the nucleus assumes a central position within the cyst. The dictyosomes increase in number and are no longer associated with the small 75 mμ vesicles seen in the zoospore (fig. 3-9), but are surrounded with large numbers of irregular shaped vesicles 15-350 mμ in size (fig. 3-10). Mitochondria are not concentrated at the cell periphery as in the zoospore, but are scattered throughout the

cytoplasm. Other organelles such as "crystalline vacuoles", lipid granules, endoplasmic reticulum, and bundles of microtubules continue to be randomly distributed throughout encystation and both types of cyst germination. The lamellation of the "crystalline vacuoles" (Reichle, 1969) has been observed to appear under the electron beam and must be classified as an artifact. However, by this reaction this vacuole can be distinguished from fibrillar and other vacuoles of the cytoplasm (fig. 3-6).

There are two distinct types of microbody-like structures within the cyst. One type has an ultrastructure consisting of a central core of fibers surrounded by a peripheral plate (fig. 3-7). It is found in large numbers at the periphery of the zoospore (fig. 3-1), but randomly spread throughout the cyst. This type of microbody is also found near the flagella in cysts producing secondary zoospores (figs. 3-23, 3-25). The second type of microbody-like structure is distinguished by the presence of concentric lamellae within a homogeneous matrix (fig. 3-8). This organelle, rarely found in the zoospore, is prominant in the cyst.

The second noticable change occurring upon encystation is the loss of the flattened vesicles and fibrillar vacuoles lining the plasmalemma of the zoospore (figs. 3-2, 3-3). These vesicles decrease in number as the cyst rounds up leaving in their place fibrillar areas of cytoplasm devoid of ribosomes (figs. 3-3, 3-4). Once the cyst wall has formed, occasional fibrillar areas may still be seen, and flattened vesicles are but rarely found (figs. 3-5, 3-11).

The first indication of germ tube production is an evagination of the cyst wall and plasmalemma proximal to a large accumulation of vesicles morphologically identical to those closely associated with the dictyosomes near the nucleus (fig. 3-12). These vesicles appear to contain a small amount of fibrillar material. As the germ tube extends, the vesicles continue to accumulate at the tip and occupy approximately the first 2 μ of the germ tube cytoplasm (fig. 3-13). Numerous pockets of membranous material are found outside the plasmalemma near the vesicles along the germ tube and germ tube tip (fig. 3-13). The membranous material occurs in form of short segments of membrane or small vesicles, 300 mμ in diameter (figs. 3-14, 3-15). These membranous aggregates, or lomasomes, are also found at the periphery of the cyst once germ tube extension has begun (fig. 3-16).

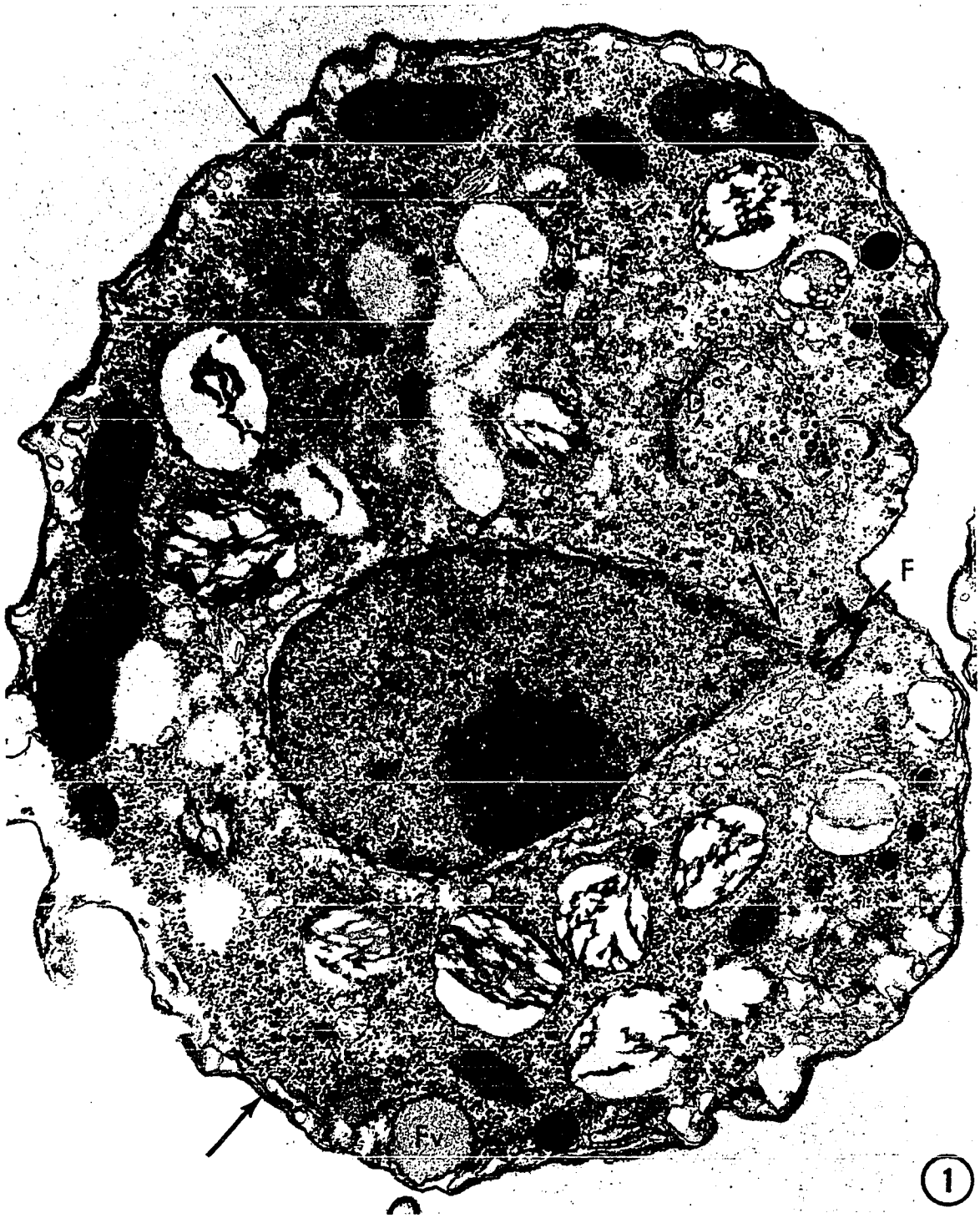
In cysts incubated in distilled water, the first indication of secondary zoospore production is the formation of a cap at the tip of the germ tube. The caps form after the hyphae have attained an average length of 6 μ (figs. 3-17, 3-18, 3-19). The vesicles at the germ tube tip decrease in number as the cap becomes larger and are absent in older cysts where the caps average 2 μ in thickness (fig. 3-18).

Coincident with cap formation, large numbers of vesicles accumulate around dictyosomes randomly spaced within the cell. These vesicles appear to coalesce along with fibrillar vacuoles to form an extensive central vacuole near the cyst nucleus (fig. 3-22). Initially, the secondary flagella with their attached basal bodies

are seen enclosed within this vacuole (figs. 3-20, 3-21). At the time of secondary flagellar development the cyst cytoplasm becomes very dense (figs. 3-24, 3-25).

The flagella are eventually located outside the cell between the plasmalemma and cyst wall (fig. 3-23). The remainder of the fibrillar vacuoles which do not coalesce with the flagellar vacuole appear to be released at the plasmalemma creating a gap between the plasmalemma and cyst wall (fig. 3-25). The "crystalline vacuole" is the only type of vacuole left in the cell immediately before release of the zoospore. There is no ultrastructural indication as to the fate of the cap, but it appears to lose electron density as the zoospore matures (fig. 3-25). After release, the secondary zoospore once again assumes the ultrastructural organization and morphology described by Reichle (1969) for Phytophthora parasitica zoospores.

Fig. 3-1 Zoospore with pyriform nucleus (N) joined by microtubules (Mt) to the flagellar apparatus (F). Note the system of flattened vesicles (arrows), fibrillar vacuoles (Fv), and microbodies (Mb) at the cell periphery. Dictyosome (D), "crystalline vacuoles" (Cv), and mitochondria (M). GOP. X 27,500



1

Fig. 3-2 Higher magnification of zoospore periphery illustrating the flattened vesicles (arrows) and fibrillar vacuoles (Fv). "Crystalline vacuoles" (Cv). GOP. X 45,700

Fig. 3-3 Rounded zoospore. Note the reduction in numbers of flattened vesicles (V) and appearance of fibrillar areas (arrows) at the cell periphery. Fibrillar vacuoles (Fv), nucleus (N), dictyosome (D). GOP. X 21,000


V
fv
3

Fig. 3-4 High magnification of rounded zoospore periphery demonstrating reduction of flattened vesicles (V) and appearance of fibrillar areas (arrows). Dictyosome (D), fibrillar vacuoles (Fv), nucleus (N), microbody (M). GOP. X 33,000

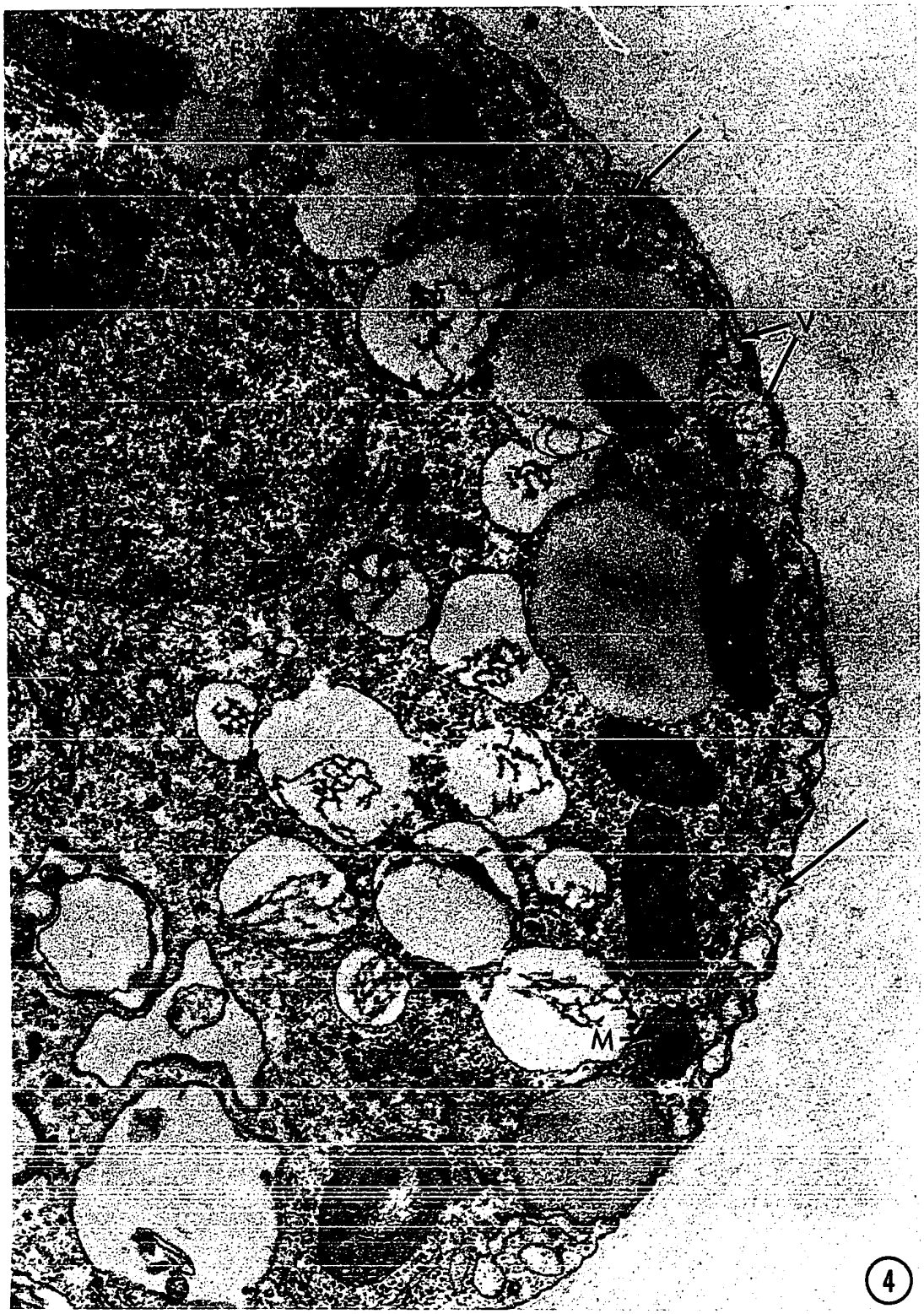


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Fig. 3-5 Immature cyst with faintly staining wall (W). Note remnants of flagellar apparatus (F) near nucleus (N) of the cell. Note the absence of fibrillar vacuoles and flattened vesicles at the periphery (arrows). GOP. X 23,600

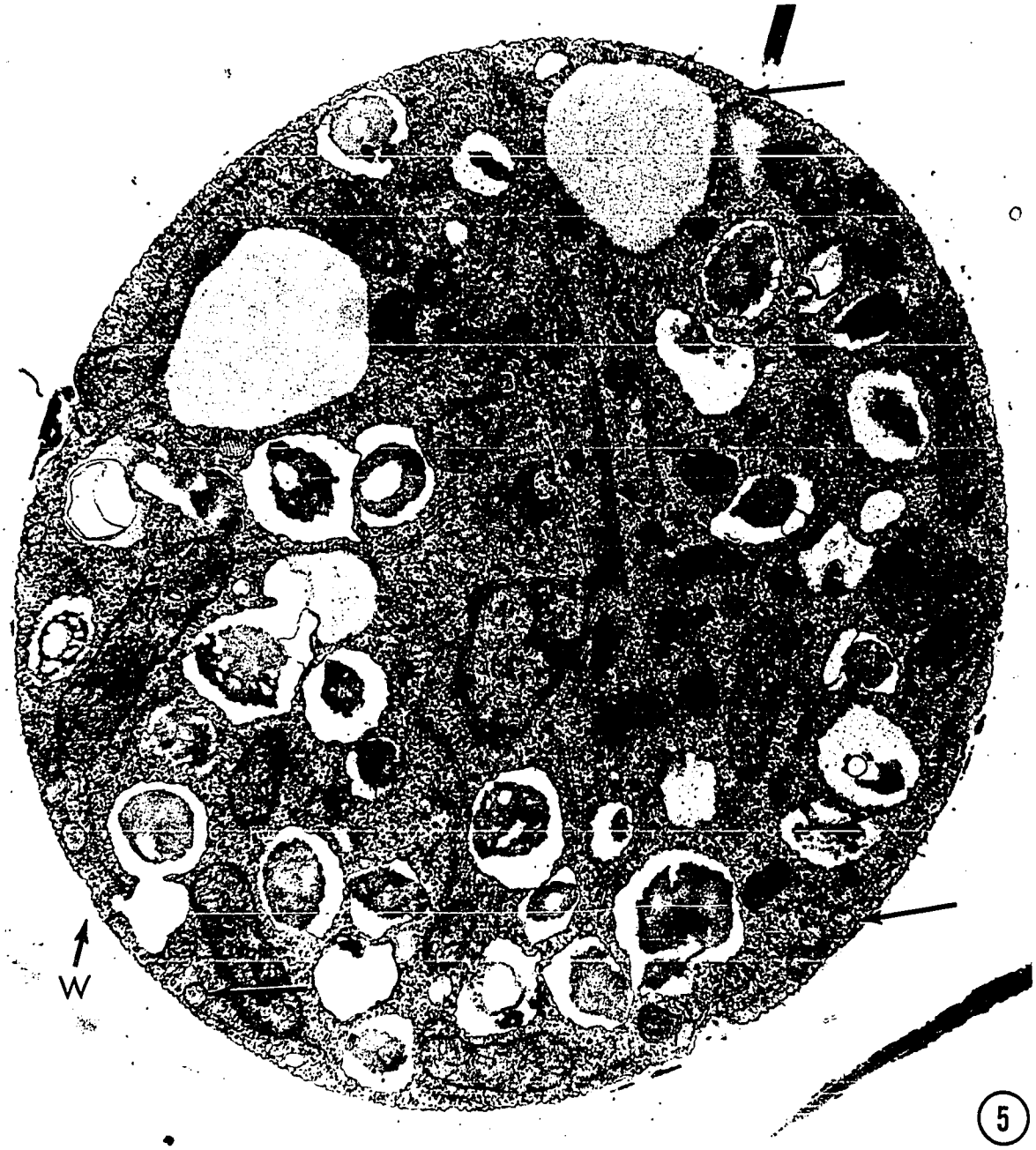

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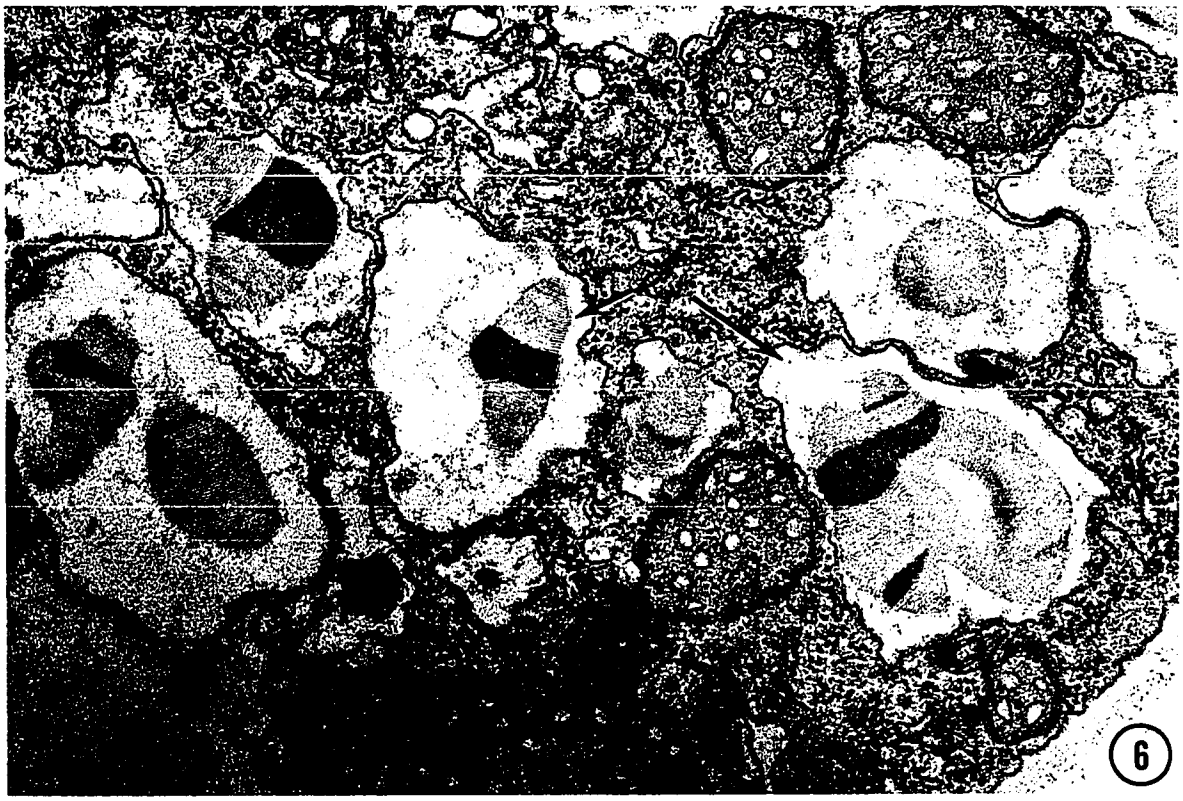
Fig. 3-6 "Crystalline vacuoles" (Cv) which remain through all the life cycle of Phytophthora parasitica. GOP. X 37,000

Fig. 3-7 Type of microbody (M) found concentrated at the zoospore periphery and near flagella within the cyst. GOP. X 72,000

Fig. 3-8 Type of microbody (M) found in the cyst. GOP. X 55,500



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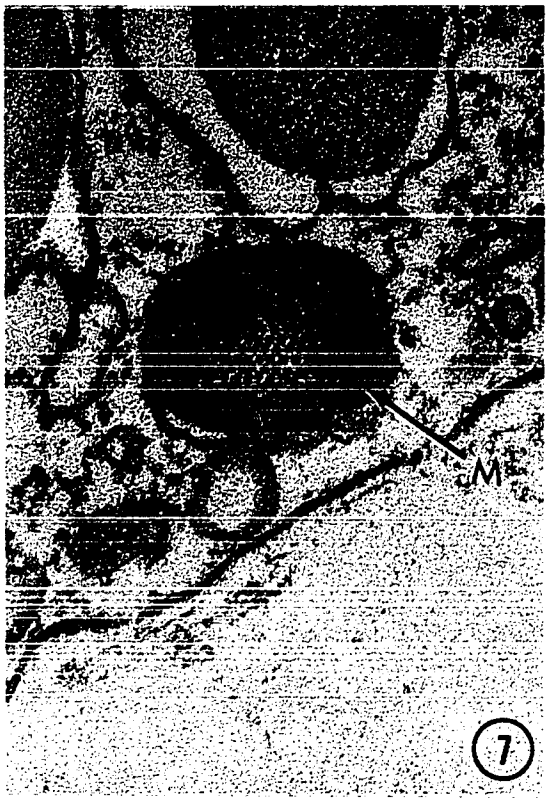

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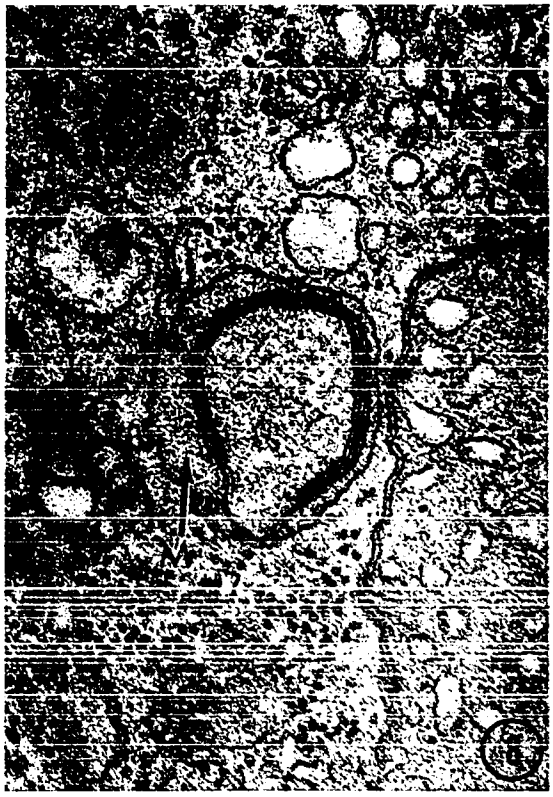

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Fig. 3-9 Dictyosome (D) with small vesicles, 75 mμ in size, seen in zoospores and immature cysts. GOP. X 55,000

Fig. 3-10 Dictyosome (D) with large vesicles, 150-350 mμ in size, seen in cysts with readily stainable walls. GOP. X 64,800

Fig. 3-11 Mature cyst prior to germination. Note the presence of dictyosomes (D) surrounded by vesicles and two types of microbody-like structures (M). Lipid body (L), nucleus (N), and crystalline vacuoles (Cv). GOP. X 21,300

11

Fig. 3-12 Initiation of cyst germination. Note the extension of the cyst near the accumulation of vesicles (arrows). Lomasomes (L), nucleus (N). GOP. X 21,000

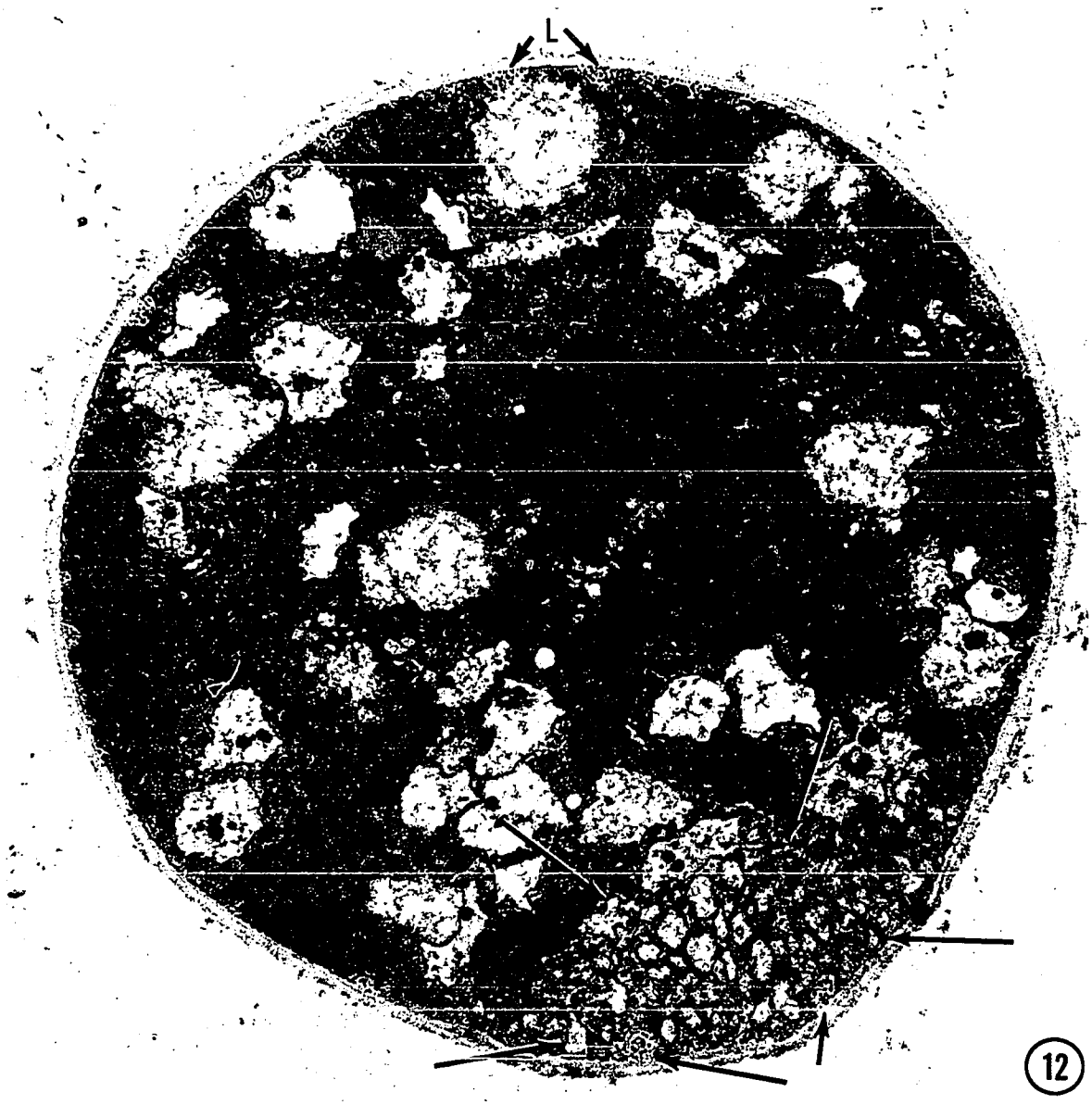

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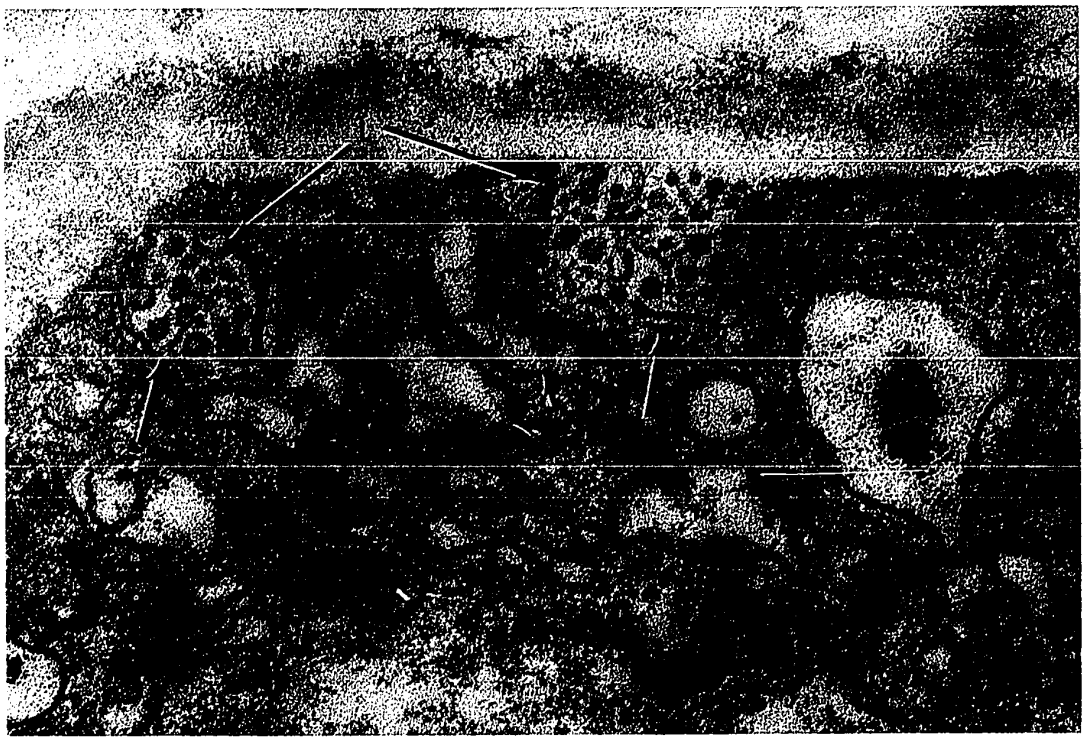
Fig. 3-13 Germ tube tip containing the characteristic accumulation of vesicles (V) and lomasomes (L). GOP. X 55,800


V
13

Fig. 3-14 Lomasomes (L) at the germ tube periphery containing vesicles and membrane fragments (arrows). Note abundance of vesicles (V) proximal to the lomasomes. Germ tube wall (W). GOP. X 86,200

Fig. 3-15 Tangential section of germ tube illustrating the abundance of vesicular material (arrows) at the periphery of the germ tube. Germ tube wall (W). GOP. X 54,800



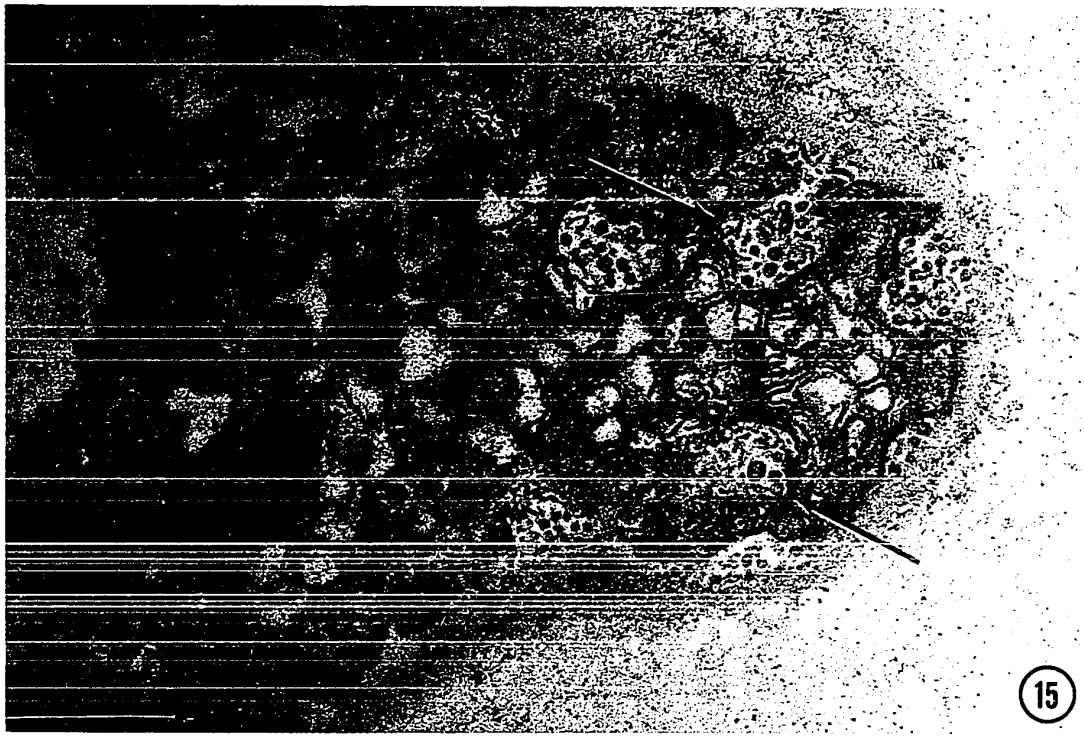


Fig. 3-16 Accumulation of vesicles (V) at the cyst periphery. Note the apparent continuity of a vesicle and lomasome (arrows). Lomasome (L), microbody (M), and hyphal wall (W). GOP. X 82,000

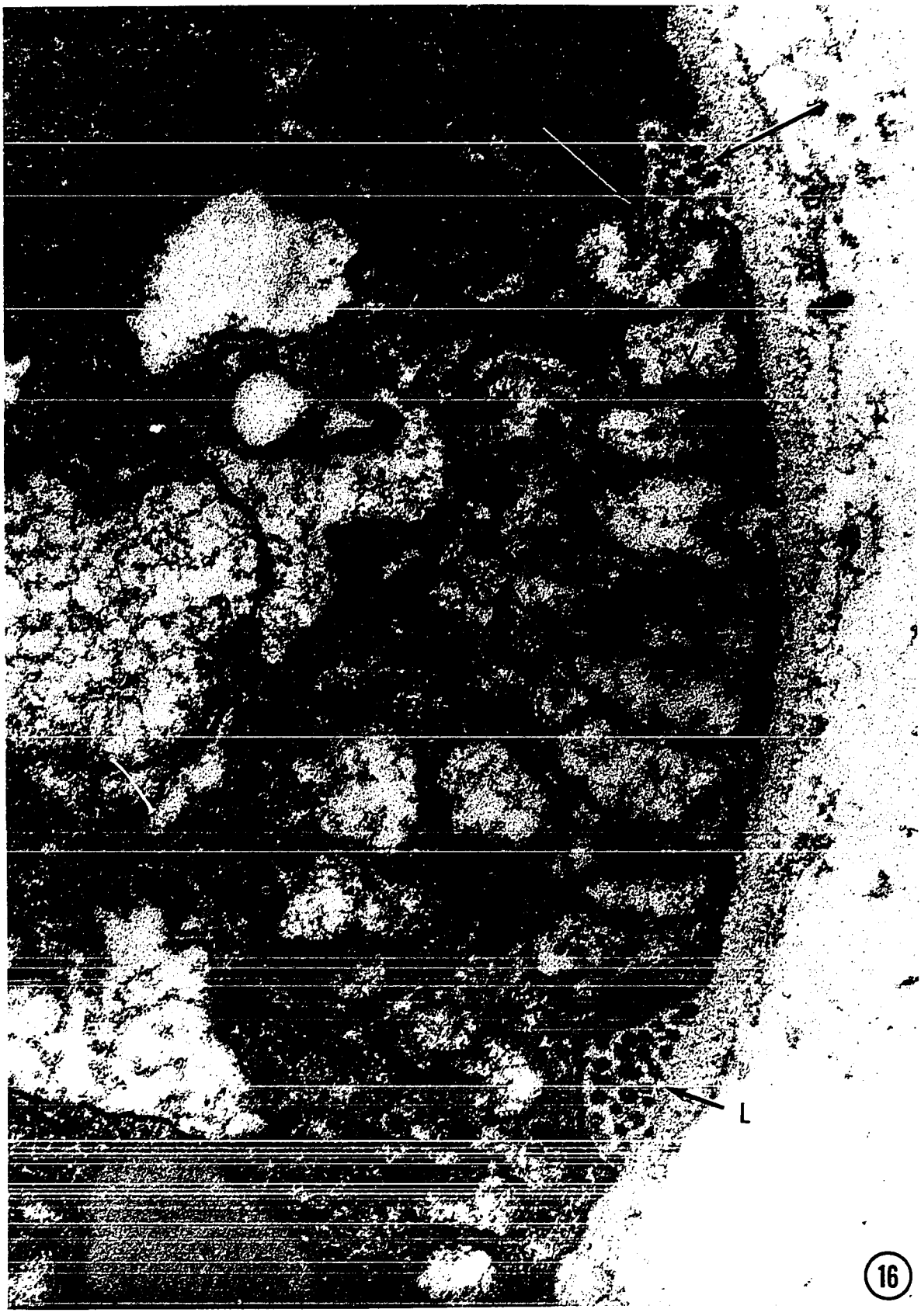


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Fig. 3-17 Cap (P) formation at the germ tube tip. Note the vesicles (V) with fibrillar material near the papillar cap. Crystalline vacuoles (Cv), microbodies (M). GOP. X 37,500

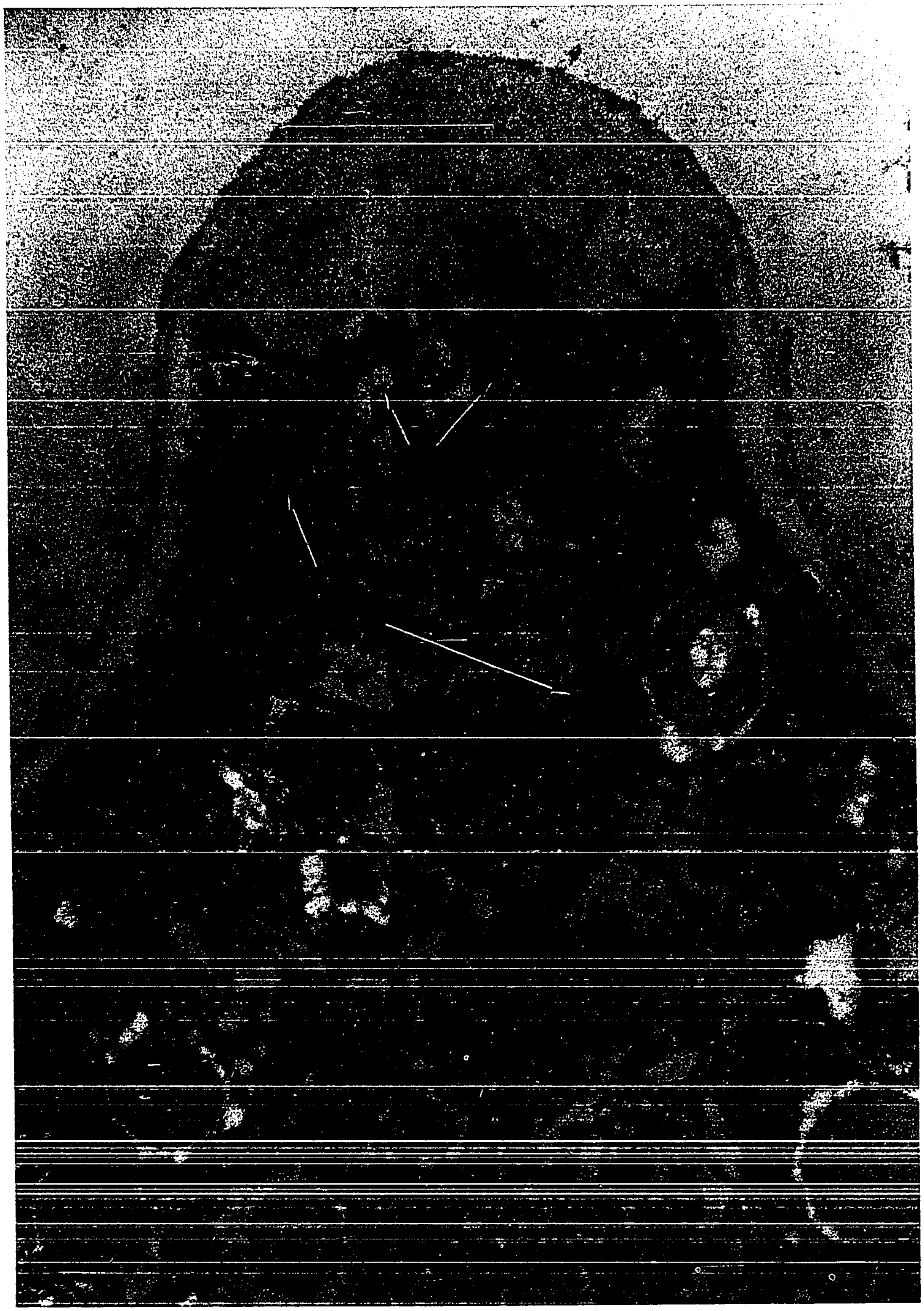


Fig. 3-18 Nearly mature cap (P) at the germ tube tip. Notice the reduction of vesicles (V) in comparison with fig. 3-17. Crystalline vacuoles (Cv), microbodies (M). GOP. X 42,300

Fig. 3-19 Cyst with cap (P) prior to flagella formation. Dictyosome (D), microbodies (M), lipid bodies (L), nucleus (N). GOP. X 19,500

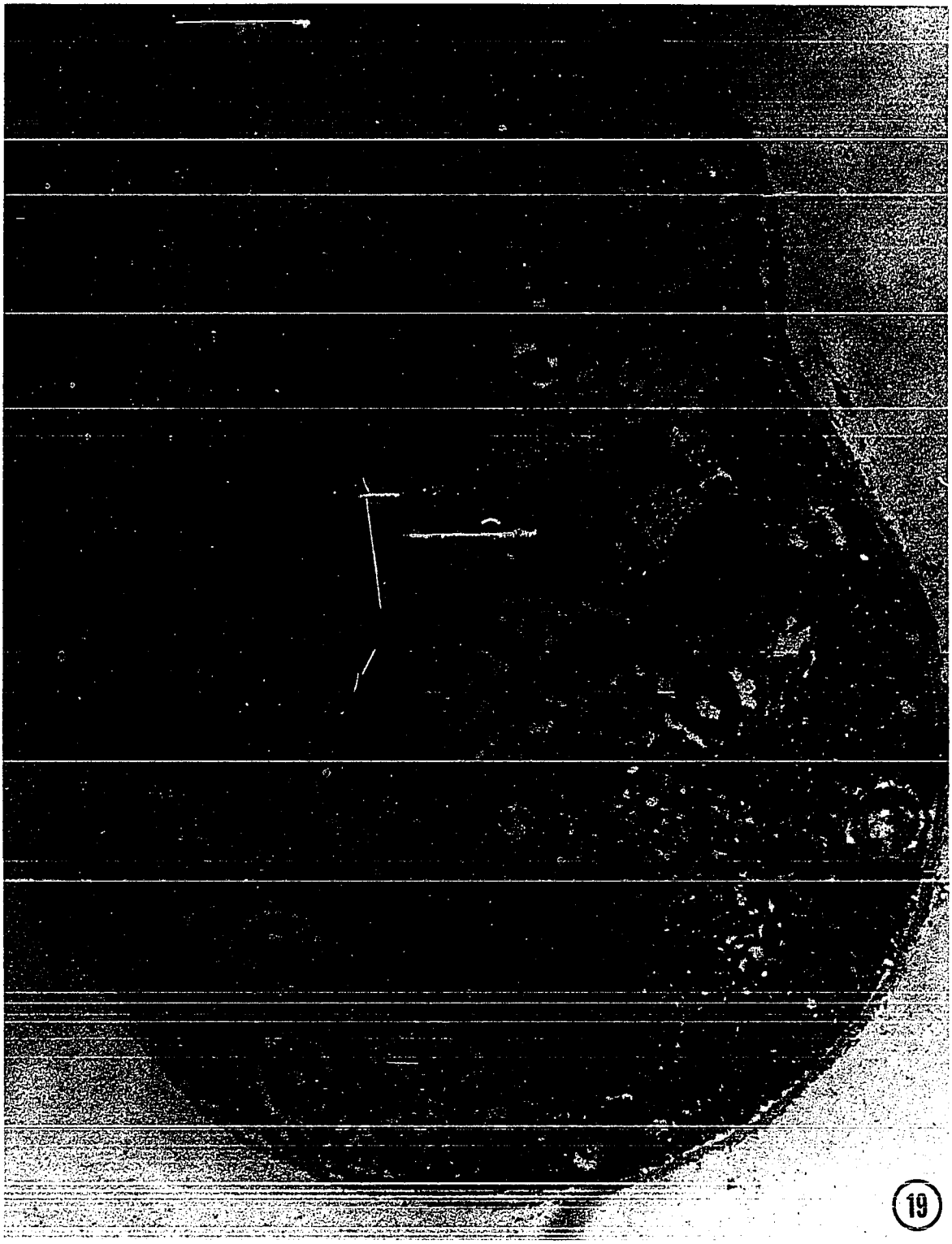
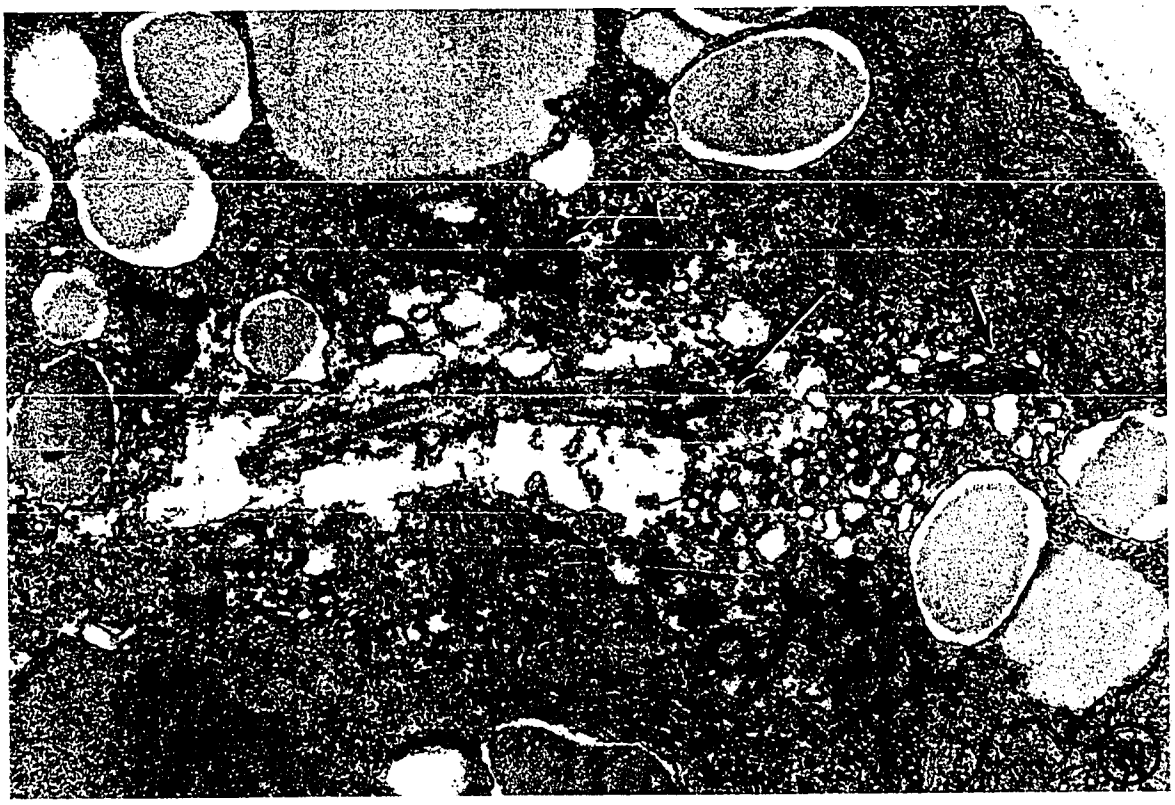


Fig. 3-20 Flagellar formation within vacuole in the cyst. Note the proximity of a dictyosome (D) and microbodies (M). Basal body or kinetosome (arrow), nucleus (N). GOP. X 31,700

Fig. 3-21 Flagella (F) within cyst near papillar cap (P). GOP. X 51,600



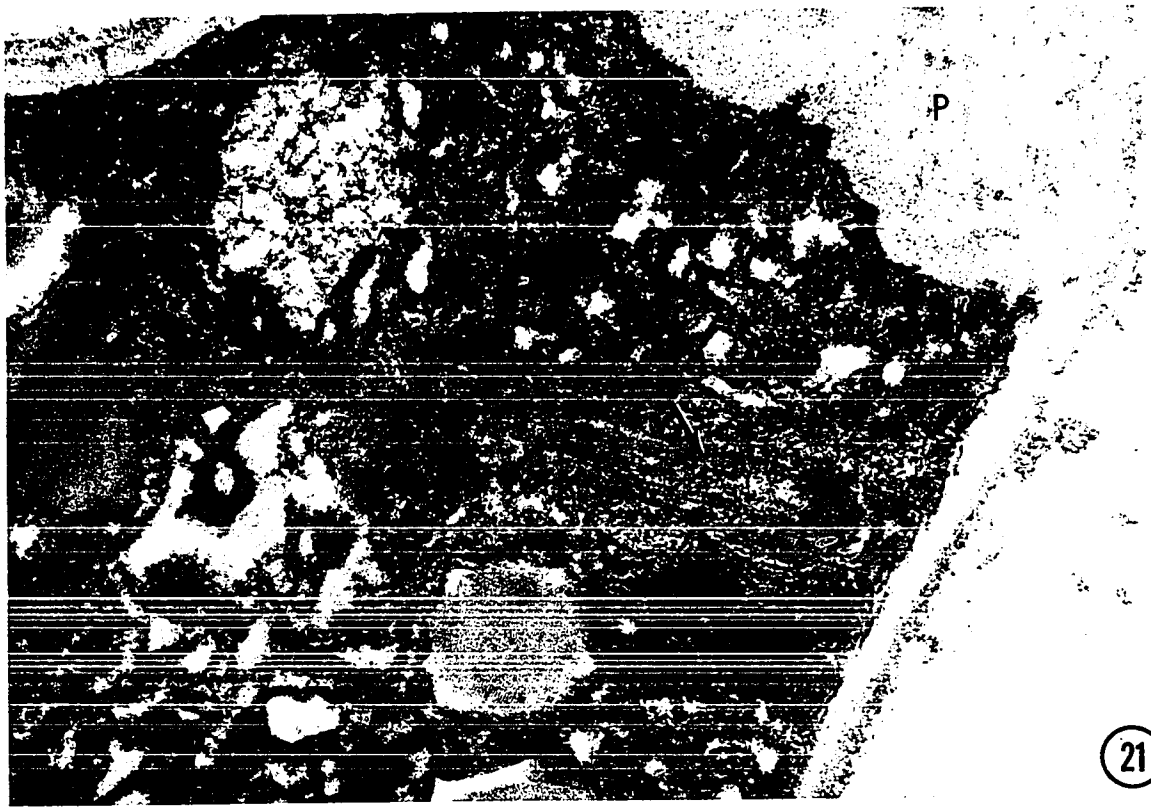

P

Fig. 3-22 Area of flagella (F) formation within a cyst. Note proximity of dictyosome (D) and fibrillar vacuoles (Fv). "Crystalline vacuoles" (Cv), nucleus (N). GOP. X 41,300

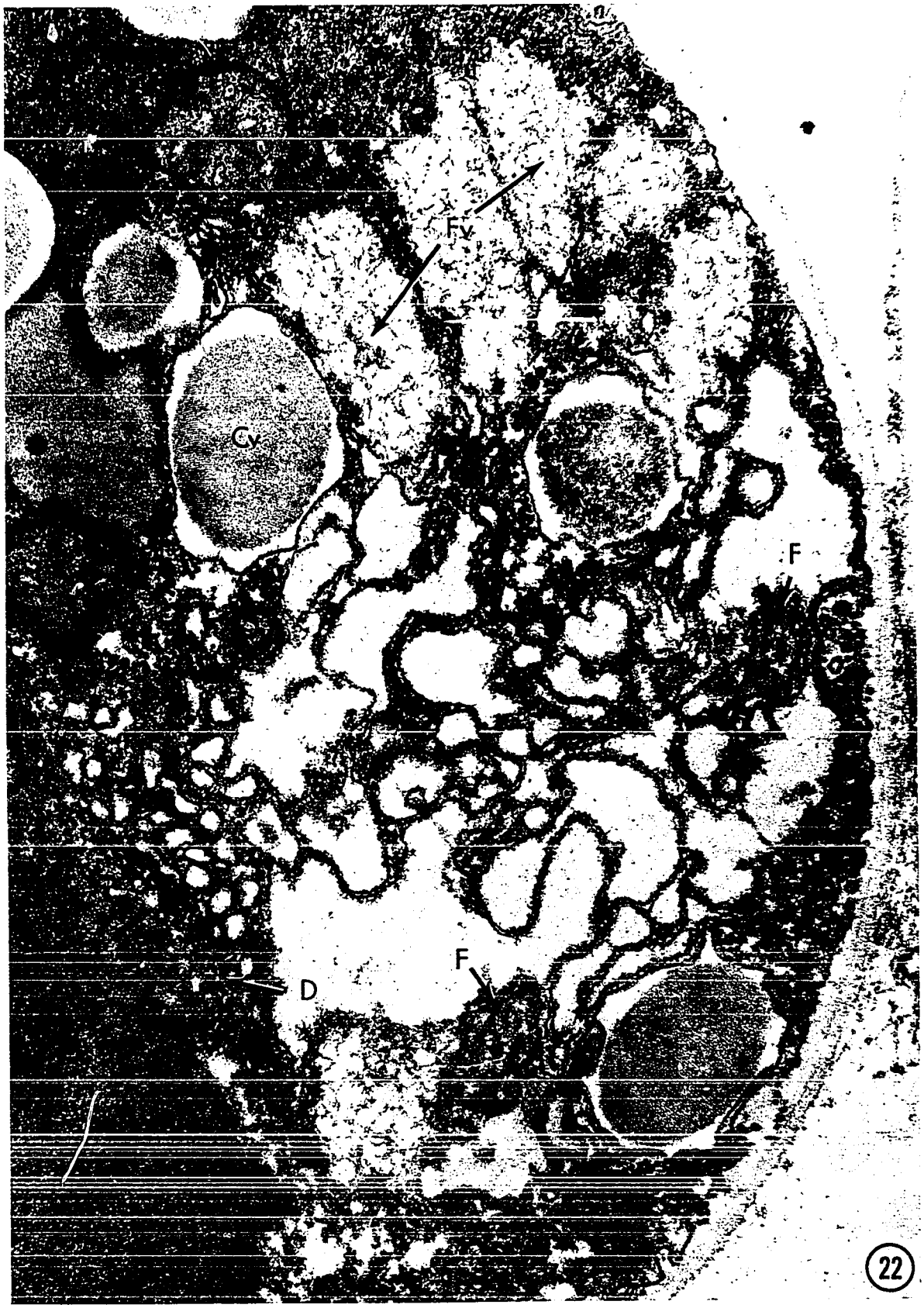

Fv
Cv
F
F
D

Fig. 3-23 Flagellum (arrows) outside of the plasmalemma bordering the cyst wall (W). "Crystalline vacuoles" (Cv), microbody (Mb), mitochondria (M), and lipid body (L). GOP. X 40,400

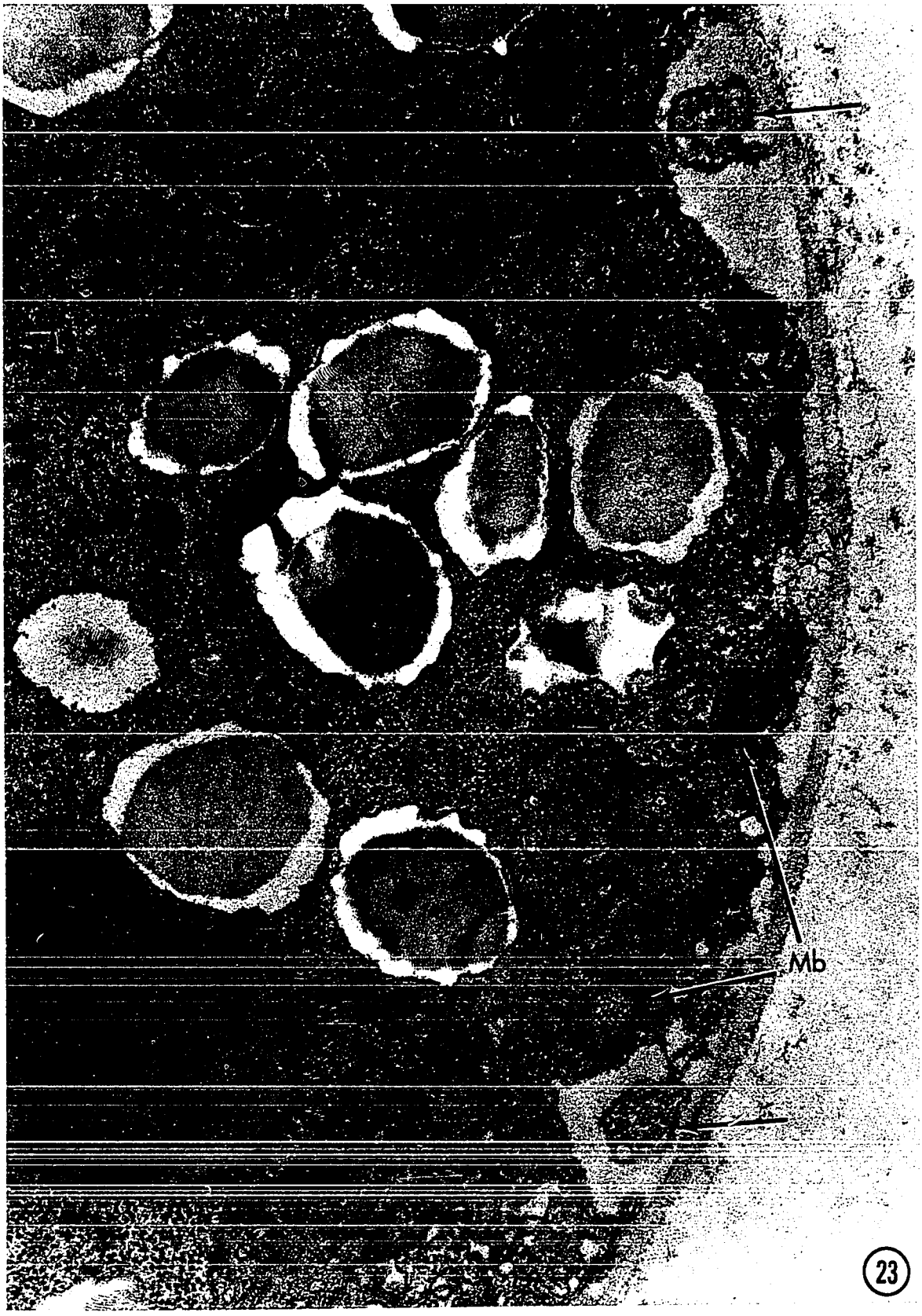

Mb

Fig. 3-24 Zoospore prior to release from cyst case (W). Note sections through flagella (arrows) and absence of fibrillar vacuoles. Also note the increased density of the cyst cytoplasm. Crystalline vacuoles (Cv), nucleus (N), papillar cap (P). GOP. X 19,400

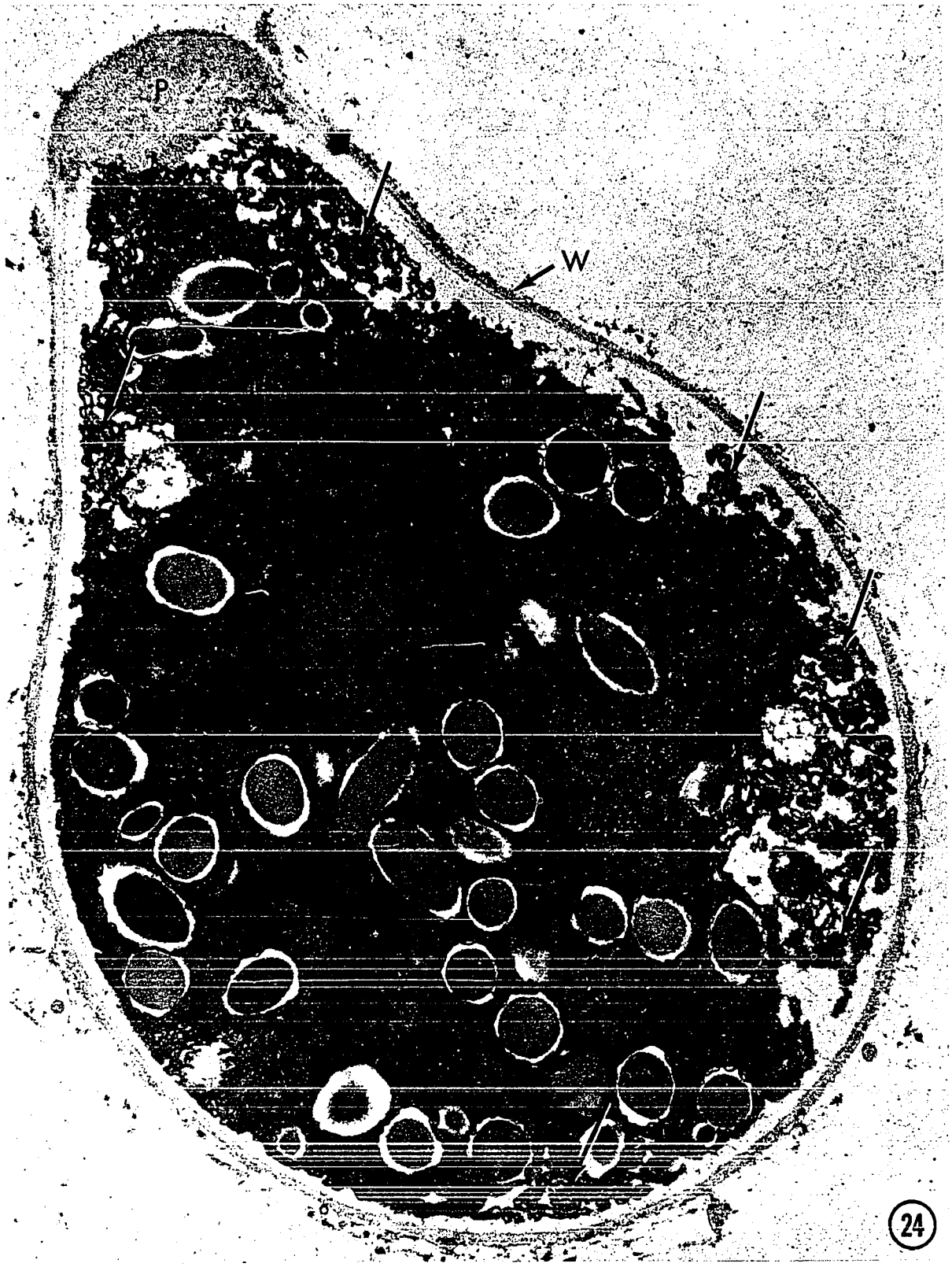
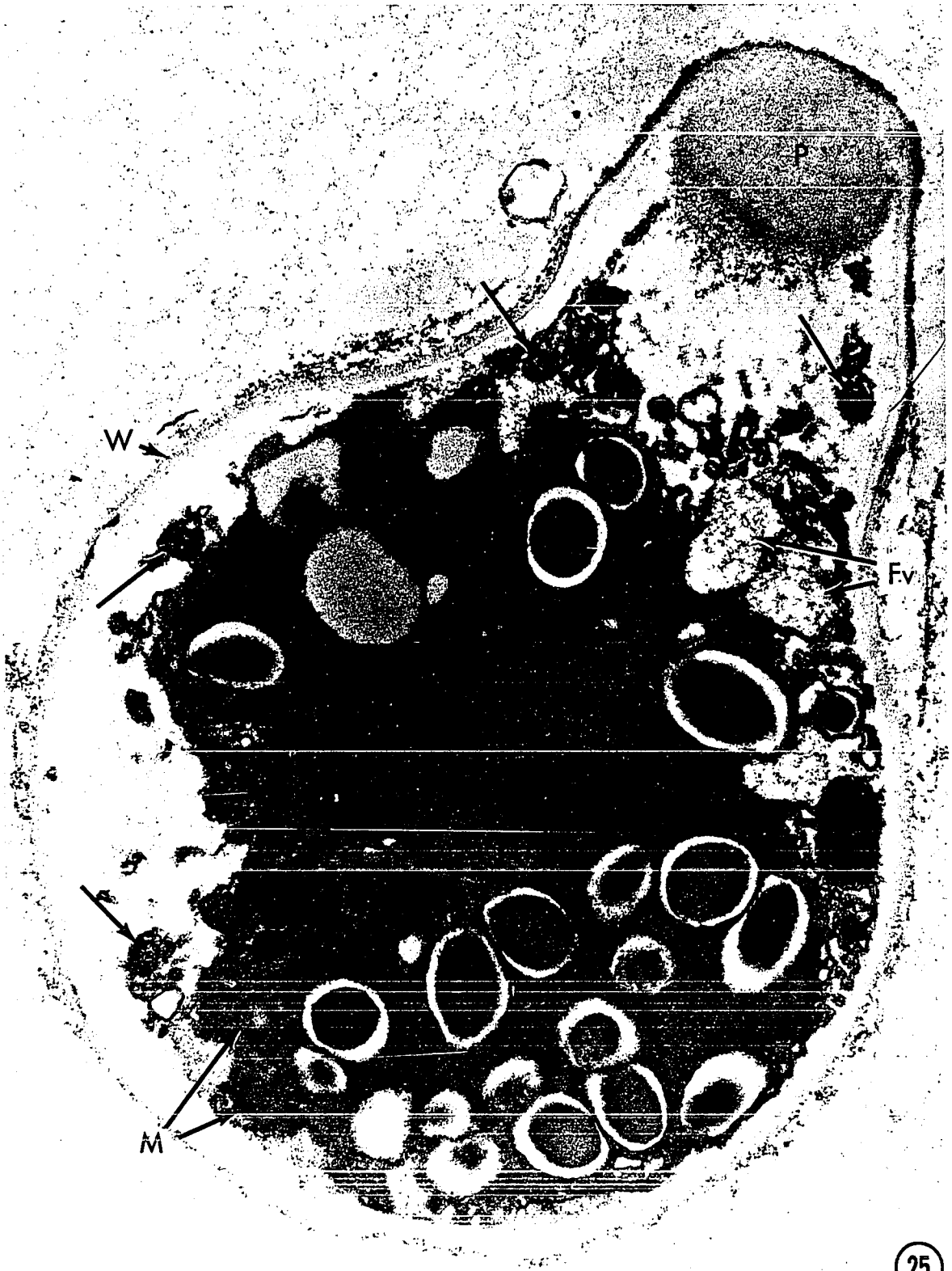

P
W

Fig. 3-25 Zoospore prior to release from cyst case (W). Note fibrillar vacuoles (Fv) and microbodies (M) at zoospore periphery. "Crystalline vacuoles" (Cv), flagella (arrows), nucleus (N), and cap (P). GOP. X 20,700


P
W
Fv
M

CHAPTER VI

RESULTS

Electron Microscopy of Inhibition of Germination: Emergence of germ tubes in direct sporangial germination is completely inhibited when sporangia are incubated in 50% papaya extract solutions containing from 10^{-3} to 10^{-6} g/ml cycloheximide. However, indirect sporangial germination is not inhibited. Zoospores are released from sporangia in aqueous solutions of cycloheximide and encyst at approximately the same rate as control samples without cycloheximide. Cyst germination does not begin as germ tube extension is again inhibited. Attempts to reverse the inhibition were unsuccessful.

Samples of inhibited cysts in 50% papaya extract and distilled water were fixed 1, 3, and 5 hrs after zoospore release, when control samples without cycloheximide had produced extensive hyphae.

Ultrastructurally, large accumulations of membranous elements are found in inhibited cysts (figs. 4-1, 4-2, 4-3). Flattened cisternae which appear to surround and isolate small segments of cytoplasm make up the bulk of the membranous aggregation. Basal bodies, microbody-like organelles, and small dictyosome-derived vesicles, 75 mμ in diameter, comprise the remainder of the vesicular material (fig. 4-3).

A second observation is the apparent inhibition of complete wall formation (fig. 4-5). In sections stained with uranyl acetate and lead citrate, a faint outline is the only indication of the presence

of a wall in five-hour inhibited cysts. This is comparable to the initial wall formed prior to germ tube formation in normally germinating cysts (fig. 3-5). However, poststaining with $BaMnO_4$ demonstrates that a wall matrix is present around inhibited cysts (fig. 4-4).

The absence of lomasomes is conspicuous in inhibited cysts. Lomasomes may be occasionally observed when the aggregation of vesicular material occurs near the periphery of the cyst, but is uncommon. Both "crystalline vacuoles" and fibrillar vacuoles are observed.

Fig. 4-1 Five-hour cyst incubated in 50% papaya extract containing 10^{-3} g/ml cycloheximide. Note the aggregation of vesicular material (arrows), and "looped" mitochondrion (M), and faintly-staining wall. "Crystalline vacuole" (Cv), Fibrillar vacuole (Fv), and nucleus (N). GOP. X 30,900

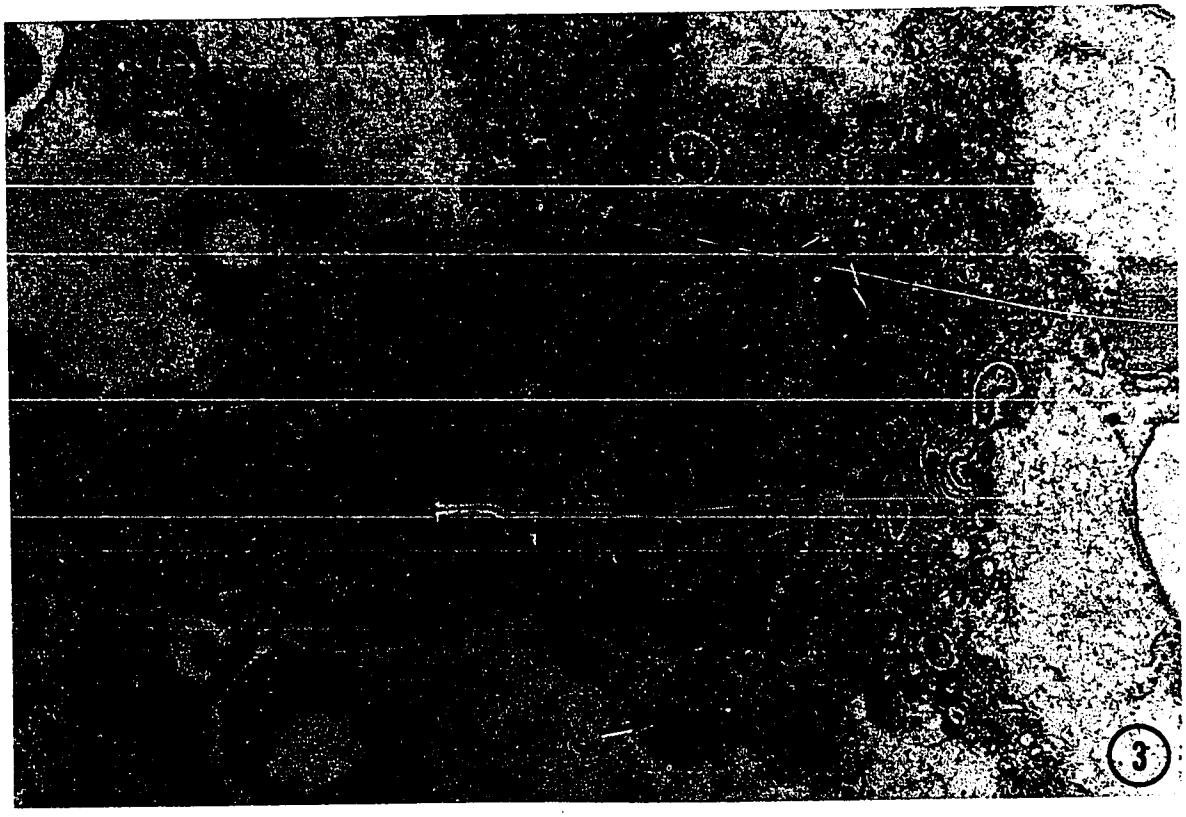

M
1

Fig. 4-2 Aggregation of vesicular material surrounding dictyosomes in directly germinating cysts inhibited by 10^{-3} g/ml cycloheximide. Note the cisternae which enclose segments of cytoplasm (arrows). Microbody-like organelles (M). GOP. X 51,000

M
P
2

Fig. 4-3 Aggregation of vesicular material surrounding dictyosomes in directly germinating cysts inhibited by 10^{-3} g/ml cycloheximide. Note the presence of basal bodies (B) and microbody-like structures (M). Also note the cytoplasmic inclusions within the vesicles. GOP. X 36,900

Fig. 4-4 Cyst wall of inhibited cyst stained with 0.5% $BaMnO_4$. GOP. X 50,600


3

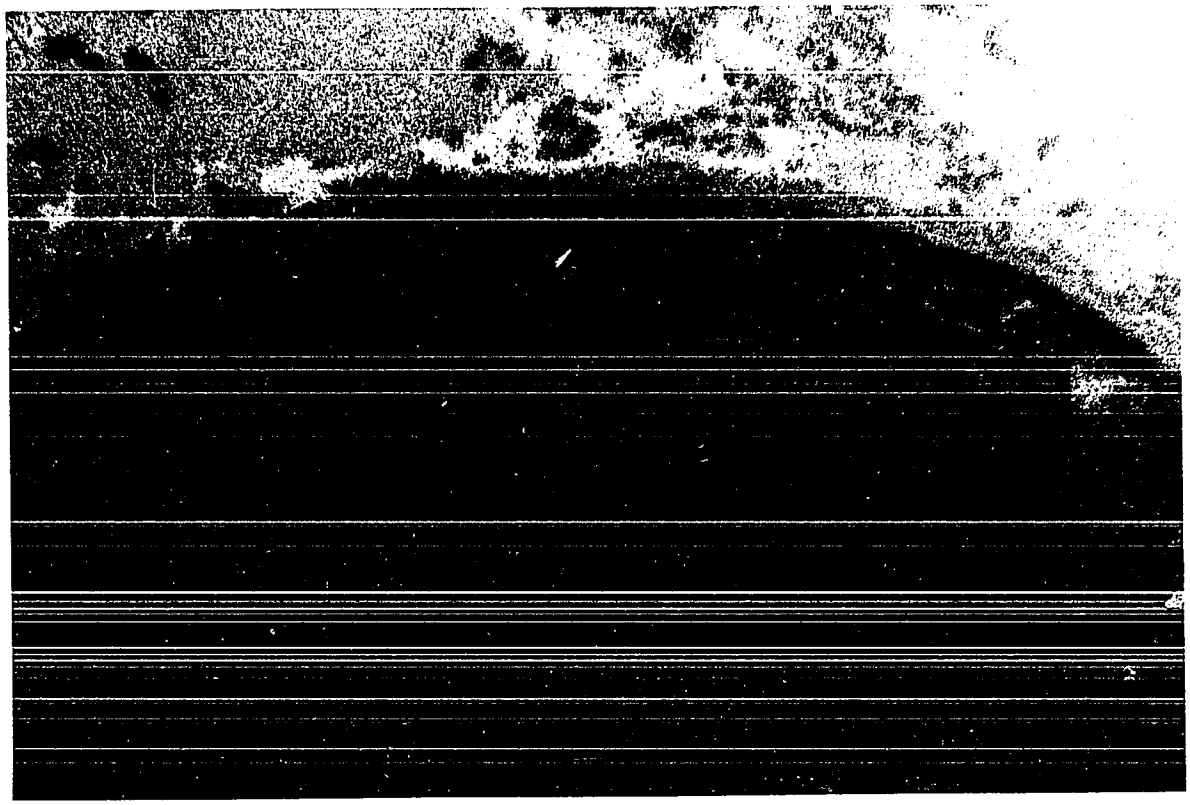
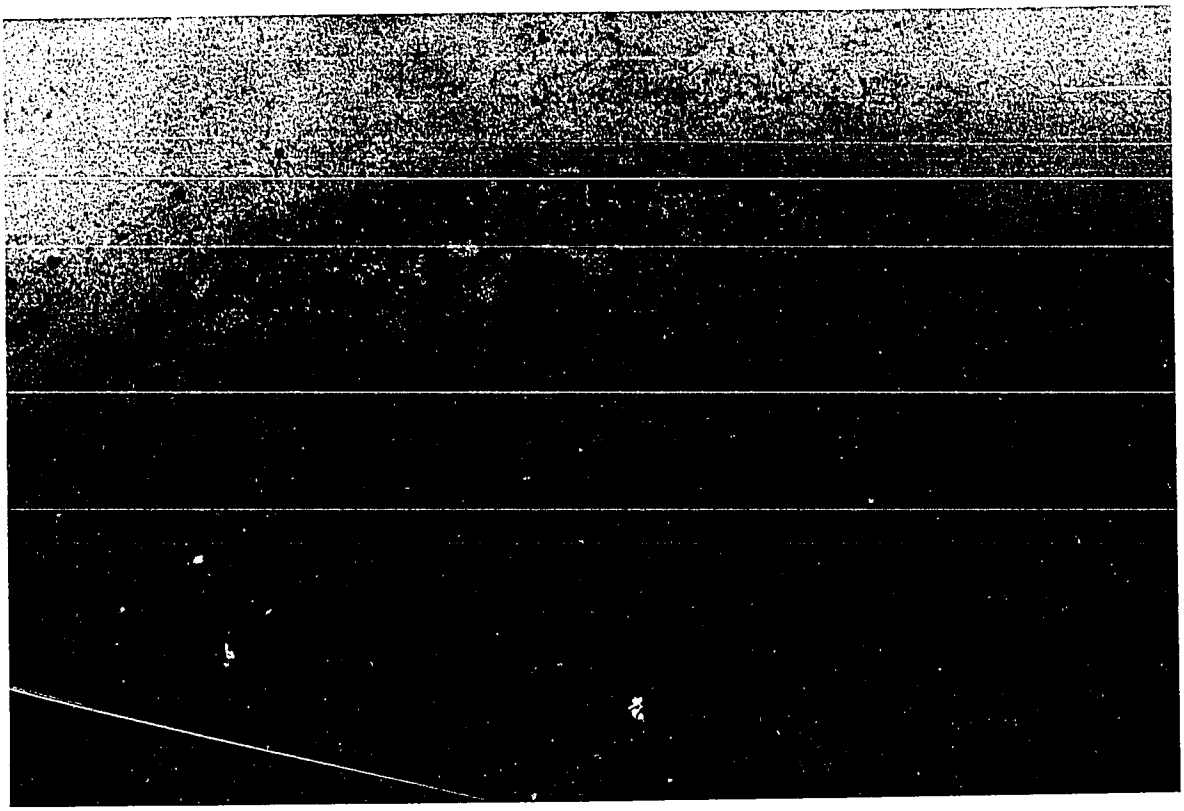
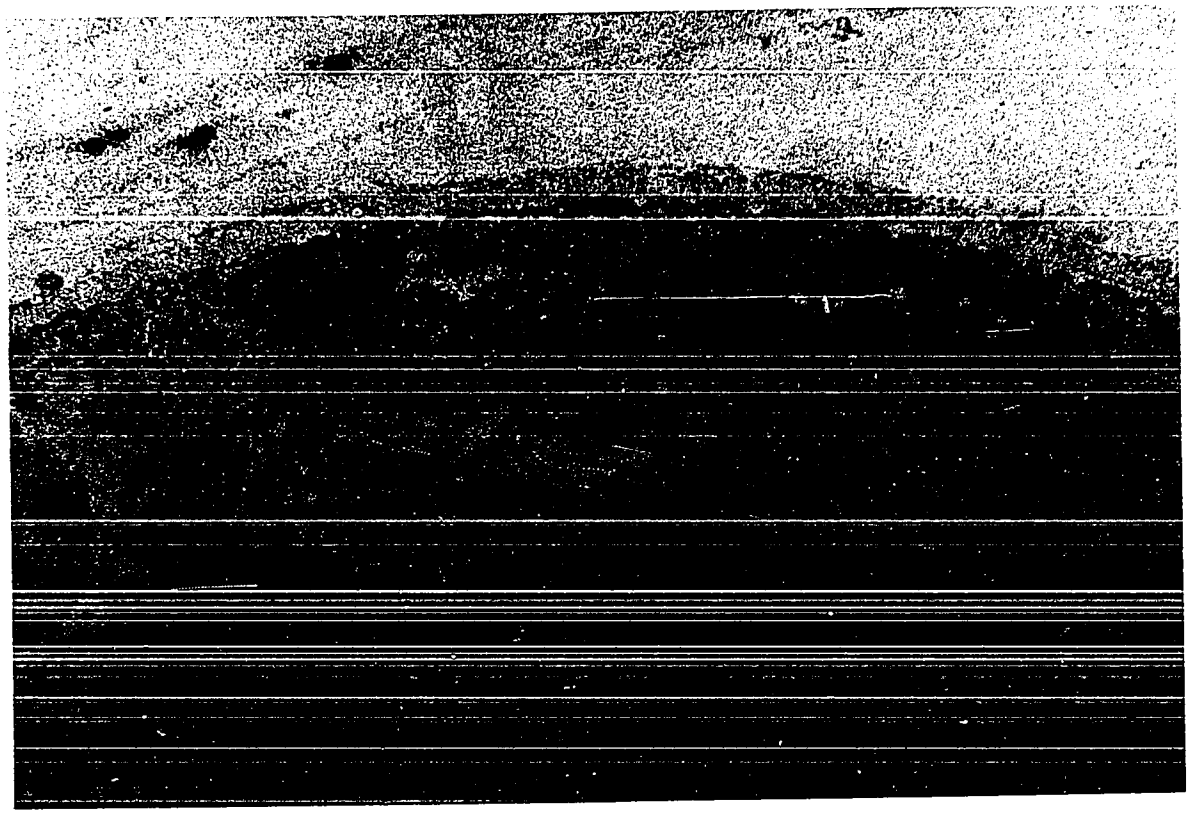


Fig. 4-5 Faintly-staining wall produced 5 hrs after zoospore release by cysts incubated in 50% papaya extract containing 10^{-3} g/ml cycloheximide. GOP. X 45,000

Fig. 4-6 Normal cyst wall produced within 1 hr after zoospore release by cysts germinating in 50% papaya extract. Dictyosome (D), microbody-like structures (M). GOP. X 44,400





CHAPTER VII

DISCUSSION

The ability of sporangia and cysts of Phytophthora parasitica to germinate by the production of germ tubes (direct germination) or to release zoospores (indirect germination) provided an ideal system to study the basic problems of wall formation and hyphal growth in fungi. The results will be discussed in three parts: (1) the physiology and control of the two modes of germination, (2) the ultrastructure of wall formation in direct sporangial germination and encystation of zoospores, and (3) the ultrastructure of hyphal tip growth.

(1) Physiology and control of germination. Attempts to induce uniform populations of directly and indirectly germinating sporangia and cysts for ultrastructural studies were successful. The system works efficiently and percentages for each type of germination surpassed ninety per cent. Previous reports had shown that the mode of germination was affected by temperature, sporangial age, nutrients, pH, and growth substances (Uppal, 1926; Katsura, 1961; Harvey, 1954; Blackwell and Waterhouse, 1931; Crosier, 1934). The effect of these parameters on germination in Phytophthora parasitica, as determined by our experiments, can be summarized as follows: (a) Sporangial age. Sporangial age plays a key role in respect to the number of germinating sporangia, but does not affect the mode of germination. Sporangia apparently have a viable life of approximately 144 hrs for germination on the culture plate. Sporangia which

germinate from cultures more than 144 hrs of age are ones which form after the initial exposure to light. This is in contrast to Blackwell and Waterhouse's findings (1931) with Phytophthora cactorum that under normal conditions the determining factor in method and time of germination is the age of the spore. Their sporangia gave an initial high percentage of indirect sporangial germination in distilled water up to two weeks of age with no direct germination. However, three week old sporangia gave a low percentage of direct germination in the same incubation medium.

(b) Temperature also appears to affect only the percentage of sporangia germinating and not the mode of germination. Aragaki et al. (1967) made an extensive study of temperature optima for direct and indirect sporangial germination in the same isolate of Phytophthora parasitica as used in this study and showed that direct germination has a narrow temperature optimum, but they, too, observed that the mode of germination is determined by the incubation medium and not the temperature.

(c) The pH, too, has an effect only on the percentage and extent of germination, but not on the type of sporangial germination.

These findings leave nutritional and growth substances to be considered as key factors determining the mode of germination, and, indeed, these parameters appear to be of primary importance in the production of direct germination in both the sporangia and cysts of Phytophthora parasitica. It has been concluded previously (Aragaki et al., 1967) that papaya extract contains factors promoting direct germination. Since the factor which promotes direct germination was

found to be dialyzable and heat stable in our experiments, simple sugars, small peptides and amino acids, metal ions, and other smaller molecules could be suspected. The artificial medium prepared containing sucrose, calcium ions, and amino acids proved successful in stimulating 90% direct sporangial germination. No level of sucrose, calcium ions, or amino acids alone could induce direct germination. Each component had an additive affect. However, a specific concentration of these components was required to prevent a percentage of the sporangia from reverting to indirect germination. It appears that this artificial medium has the osmolarity sufficient to prevent sporangial cleavage and also has the nutritional or stimulatory components for hyphal growth.

(2) Ultrastructure of direct sporangial germination. In many respects direct germination by germ tubes in Phytophthora parasitica follows a pattern comparable to that of indirect germination via zoospores. Flagella form and some of the cleavage vesicles fuse, but then the flagella disappear and cleavage is very rarely complete. The protoplast remains within the confines of the sporangial wall, where it surrounds itself with a new wall layer, the germination wall, just as the free zoospores in indirect germination surround themselves with a cyst wall after they have settled and retracted their flagella. In this sense then, direct germination of Phytophthora parasitica may be regarded as an intrasporangial encystation of partially formed zoospores. The observation that occasionally cleavage is completed, but the zoospores are not released but encyst and subsequently germinate within the sporangium, lends support to this interpretation.

Based solely on ultrastructural evidence no definite statements can be made on the role of various cell organelles during direct sporangial germination. Judging from their relative abundance and their peripheral location during germination the vesicles (probably derived from the dictyosomes), the microbodies, the endoplasmic reticulum, the lomasomes, and possibly the plasma membrane itself may function, in a manner only poorly understood, as part of the wall-forming machinery. Part or all of these components have been implicated in cell wall formation in a variety of fungi and higher plants. Roland (1967) describes a complex of endoplasmic reticulum as "corps denses", dense bodies, corresponding to the microbodies, and dictyosome derived vesicles located at the site of collenchyma wall formation in Daucus carota and Sambucus nigra. The plasmalemma itself as a possible site of polymerization of fibrillar polysaccharides has been discussed for two cellular slime molds, Acytostelium leptosomum (Hohl, Hamamoto, and Hemmes, 1968), and Dictyostelium discoideum (George, 1968). The importance of the dictyosomes and the endoplasmic reticulum in wall synthesis of meristematic and xylem cells has been demonstrated by radioautographic techniques (Pickett-Heaps, 1967). A possible relationship between endoplasmic reticulum and wall formation in fungi is described by Marchant, Peat, and Banbury (1967) and Marchant (1968), and the question of Golgi apparatus and endoplasmic reticulum participation in wall formation is discussed by Bracker (1967) for fungi and of plants in general by Mollenhauer and Morré (1966). Finally, the possible involvement of lomasomes in wall formation has

been suggested by Wilsenach and Kessel (1965). Esau et al. (1966) concluded that the presence of lomasomes indicated passage of materials toward the wall as part of the mechanism of wall growth in higher plants.

The functions and contents of the various vacuoles found in Phytophthora species are poorly understood. Chapman and Vujičić (1965) suggested that the central sporangial vacuole and surrounding vacuoles of Phytophthora erythroseptica contained glycogen, since Yamamoto and Tanino (1961) had shown that sporangia of Phytophthora infestans were rich in glycogen. The electron transparent vacuole content would also fit this hypothesis, since glycogen does not reduce osmium tetroxide nor permanganate. The disappearance of the large central vacuole together with the decrease in number and change in appearance of the smaller vacuoles in germinating sporangia would coincide with Yamamoto and Tanino's evidence that glycogen content decreased and respiration increased during germination of Phytophthora infestans.

The presence of numerous pockets of vesicular material in the germination wall may give a clue to the nature of the wall-forming process. The vesicular aggregates are intermingled with small fragments of cytoplasmic material such as ribosomes and are, therefore, cytoplasmic in nature. This, together with the observation that wall-like material forms in and around vesicular areas of the cytoplasmic periphery, suggests that the peripheral areas are liberated from the sporangial protoplast as they become encased in wall material. This mechanism would again stress the

importance of the peripheral cytoplasmic region for wall formation (Hohl et al., 1968). A mode of wall formation as proposed here for Phytophthora has previously been suggested for higher plants by Wardrop (1965).

The question remains: why are numerous vesicular inclusions found in the basal plug of the sporangium and in the germination wall, but only a few are in the original sporangial wall and none in the wall at the hyphal tip? For a possible answer we have to consider that the wall material of the basal plug and that of the germination wall forms in the absence of cell expansion. The wall at the hyphal tip, on the other hand, forms as the cell elongates rapidly. This suggests that the rapidly expanding plasma membrane at the tip of the hypha might readily incorporate the membranes of the vesicles arriving at the cell periphery. In the basal plug and the germination wall, on the other hand, the plasma membrane does not expand as the wall is deposited, and vesicles arriving at the cell periphery might create a large membrane surplus. This membrane surplus, together with an appositional mode of wall growth as observed in both the basal plug and the germination wall, could possibly explain entrapping of large amounts of membranous material.

According to this interpretation, the numerous vesicular elements in the walls would be a reflection of wall formation in the absence of cell expansion, a situation creating the favorable conditions of a membrane surplus coupled with appositional growth. Further support for such a mechanism is provided by a study on wall

deposition in pea stem internodes (Bouck and Galston, 1967). Treatment of the internodes with the auxin analog 2,3,5-triiodobenzoic acid (TIBA) prevents growth of the cells without inhibiting wall synthesis. As a result irregular cell wall deposits are produced containing large numbers of profiles of flattened sacs, similar in appearance to the vesicular inclusions described for Phytophthora.

Ultrastructure of encystation. There are few reports in the literature on the encystment of zoospores of aquatic Phycomycetes. Cantino et al., (1963) and Fuller (1966) discuss the redistribution of organelles and flagellar retraction upon encystment, but there are no observations on cyst wall formation. There appears to be two stages in the production of the cyst wall of Phytophthora parasitica, a poorly staining initial wall and the mature wall associated with the appearance of vesicles and lomasomes at the cyst periphery. The initial wall can be barely detected in sections stained with lead citrate and uranyl acetate, but can be visualized by staining with $BaMnO_4$.

The disappearance of the flattened vesicles upon encystment makes them prime candidates for a role in wall formation. These flattened vesicles do not join to the plasmalemma but appear to break down leaving fibrillar areas, devoid of ribosomes near the plasmalemma. One might speculate that the fibrillar material is then translocated across the plasmalemma for the initial wall. Dictyosomes at this stage are morphologically similar to those observed in the zoospore and lomasomes are not associated with the initial wall.

In samples taken one hour after zoospore release, the wall is readily stainable with lead citrate alone. Many of these cells have initiated germination and contain at the cell periphery accumulations of vesicles morphologically similar to those at the hyphal tip. At the plasmalemma the vesicles appear to break up or join to the plasmalemma creating an excess of membranous fragments which, in our tentative interpretation, might give rise to lomasomes. The increase in cyst wall density accompanying this vesicular activity indicates that these vesicles are possibly involved in wall formation.

(3) Ultrastructure of hyphal tips. Originally, Reinhardt used external surface markers to demonstrate that hyphae of young sporangiophores of fungi elongate by tip growth. The same results were obtained recently by the use of fluorescent antibodies (Marchant and Smith, 1968). Bartnicki-Garcia and Lippman (1969) have gone one step further and have shown that structural polymers of the cell wall are apically deposited in Mucor rouxii by the use of labeled N-acetyl-D-(GL-^{3}H) glucosamine, a hyphal wall component.

Hyphal tip growth implies that a mechanism exists in this region to account for directing growth and the deposition of new wall material. Fibril free regions at the hyphal apex of Polystictus versicolor surrounded by concentrically arranged microfibrils have been reported by Strunk (1963). Hawker (1963) suggested that mitochondria which accumulated at hyphal tips and then moved backward might be involved in apical extension. More recently, two different systems of organelles found in hyphal tips of fungi have been reported and related to apical growth. The first is the apical

corpuscle reported in germ tubes of Mucor rouxii (Bartnicki-Garcia et al., 1968). Evidence for the participation of this organelle in apical extension is the demonstration of a steep gradient of active synthesis at the hyphal tip relative to the minute (less than 1 μ) apical corpuscle seen in thin sections of hyphal tips of Mucor rouxii.

The second candidate is the Spitzenkörper discovered in growing hyphae of higher fungi (Brunswik, 1924) and recently studied in fungal species of different systematical origin by Girbardt (1969). This apical body represents at the electron microscope level an area of ground cytoplasm of various size and shape, containing apical vesicles, microvesicles, and ribosomes. Dictyosomes are not found in this area.

The origin of apical vesicles differs with the fungal species studied. Grove, Bracker, and Morré (1967) observed two distinct types of vesicles, 0.04-0.12 μ and 0.2-0.3 μ in size. They suggested that the small vesicles are formed from dictyosomes and, while migrating to the apex, fuse to form the larger vesicles. However, dictyosome vesicles will not account for wall extension in the many species that contain no morphologically recognizable Golgi apparatus, such as Aspergillus niger and Fusarium oxysporum. McClure, Park, and Robinson (1968) in a study of these fungi observed apical vesicles 0.07-0.12 μ in size, but could not determine their origin. Girbardt (1969) studying several different fungal species lacking dictyosomes, observed single cisternae that produced apical vesicles. These cisternae were not rough endoplasmic reticulum as the middle lamella of these cisternae membranes measured 41.5 Å as compared to 28 Å for

the endoplasmic reticulum. There is no evidence for the involvement of microtubules in orienting or depositing fungal wall materials.

Manocha and Colvin (1968) found at least two distinct layers in the cell wall of mature hyphae of Pythium debaryanum. These layers differ in the arrangement of the microfibrils, whereby the outer layer contains randomly dispersed microfibrils and the inner has a parallel longitudinal arrangement of thin elements. Studies on the biochemistry of morphogenesis in Mucor rouxii revealed pronounced alterations in cell wall composition during sporogenesis and spore germination indicative of a major reorganization in wall metabolism (Bartnicki-Garcia and Nickerson, 1962; Bartnicki-Garcia and Reyes, 1964 and 1968). Metal-shadowed specimens of Phytophthora parasitica cyst walls and germ tube walls also differ in microfibrillar organization (Tokunaga and Bartnicki-Garcia, 1968). The cyst wall consists of a thin layer of loosely packed, randomly oriented microfibrils; whereas, no microfibrillar pattern was discerned on the germ tube walls. It was suggested that these differences in the orientation of microfibrils may well be the result of two differing modes of wall deposition, one for cyst walls and one for hyphal walls. It appears from our study of wall deposition in Phytophthora parasitica that dictyosome-derived vesicles function in the transfer of wall materials to the periphery of the cyst and to the germ tube tip, but the details of translocation and deposition are not yet known.

Our observations on hyphal tip growth in Phytophthora parasitica are in close agreement with those obtained for Pythium ultimum (Grove,

Bracker, and Morré, 1967), for Ascodesmis sphaerospora (Brenner and Carroll, 1968), and for Aspergillus niger (McClure, Park, and Robinson, 1968). In each case the importance of the vesicular zone at the hyphal apex has been stressed.

The results of inhibition were preliminary, but added support for our tentative ideas on the importance of dictyosome-derived vesicles in wall formation and hyphal tip growth. Cycloheximide's ability to inhibit protein synthesis is well documented (Lin et al., 1966). The effect of its inhibition is also apparent at the ultrastructural level in the disruption of the formation of normal dictyosome vesicles in germinating cysts of Phytophthora parasitica. It is not known if enzyme systems for normal dictyosome function are inhibited, but the result is the absence of vesicles transported to the cell periphery and the incompletion of the cyst wall. The absence of lomasomes again fits our scheme for their formation.

In summary, in direct germination and hyphal tips, dictyosome-derived vesicles appear to function as a transport system for wall precursor material. The contents of these vesicles is probably a glucan, since the walls of Phytophthora contain up to 90% glucan-like material (Bartnicki-Garcia, 1966). In indirect germination, on the other hand, vesicles occur which are also most likely produced by dictyosomes, but their main function is to form the plasmalemma of the developing zoospores in the sporangium. The dictyosome thus probably performs a dual role in Phytophthora: during direct germination it might serve primarily as a transport system for wall material; during indirect germination it appears to function as a membrane donor.

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