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ULTRASTRUCTURAL AND BIOCHEMICAL STUDIES OF ZOOSPORES, CYSTS AND GERMINATING CYSTS OF *Phytophthora palmivora* (Butl.) Butl.

by

Christina E. Bimpong

Department of Plant Sciences

Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Canada

December, 1973

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ABSTRACT

Changes in ultrastructure, metabolic reserves and some enzymes of the glyoxylate and tricarboxylic acid cycles in Phytophthora palmivora (Butl.) Butl. zoospores during motility and germination have been investigated.

These changes were studied using freshly liberated zoospores, in zoospores after 6 h motility and, following this motile period, in cysts allowed to develop and germinate for 2 h.

Electron microscopic techniques were used to study changes in fine structure occurring during these developmental stages. Cytochemical identification of vesicle contents and marker enzymes of microbodies and mitochondria were also investigated. Quantitative microdetermination of metabolic reserves and enzymes were by spectrophotometric methods.

Zoospores and cysts of Phytophthora palmivora contain three major types of vesicles, distinguished on the basis of their contents. Amorphous non-membrane-bound inclusions are lipid, crystalline vesicles contain some lipid together with other unknown material, and granular vesicles contain protein.

After 6 h motility, no ultrastructural changes were observed in metabolic reserves. However, during germination all granular vesicles disappeared, most of the lipids were broken down and crystalline inclusions were partially utilised, leaving few osmiophilic bodies in vacuoles of germ tubes. The breakdown products of lipid bodies and crystalline inclusions appeared very similar.

A central vacuole formed inside each cyst during germination, when cytoplasmic material moved into the growing germ tube and inclusions coalesced and were broken down. After 1.5 h germination, few lipid bodies and little cytoplasm remained in the cyst body.

Microbodies were closely associated with lipid bodies and mitochondria, and they proved to contain α-hydroxy acid oxidase. Succinate dehydrogenase and catalase (peroxidatic) activity was localised in mitochondrial membranes.

Quantitative assays of metabolic reserves showed that lipids were the major energy source for motility and germination. Carbohydrates decreased slightly during motility but increased during germination. Protein remained unchanged during motility but increased during germination.

Isocitrate lyase activity decreased during motility and germination. NAD-isocitrate dehydrogenase and malate dehydrogenase activities showed no significant changes after 6 h motility but increased during encystment and germination. NADP-isocitrate dehydrogenase and succinate dehydrogenase decreased during motility but they increased during

germination.

The key enzymes of the glyoxylate cycle and the tricarboxylic acid cycle are present in zoospores and cysts. The possible roles of the two pathways during motility and germination are discussed.

ACKNOWLEDGEMENTS

I would like to thank Dr. C. J. Hickman for his advice, support and criticisms during the course of this work and in the preparation of the manuscript.

I also thank Drs. W. E. McKeen, F. S. Cook and D. G. Wilson for their assistance in the preparation of this manuscript. I gratefully acknowledge the assistance and advice of Dr. W. L. Magee in whose laboratory lipid analyses were carried out. I would also like to thank Drs. J. E. Cummins and R. Gittins for assistance with biochemical problems and statistical interpretation, respectively. Special thanks to Mrs. Diane Scott for assistance with collecting the large quantities of zoospores and the preparation of photomicrographs. The help of Mr. G. Turcon and Mr. R. Smith with electron microscopic techniques is gratefully acknowledged. Sincere thanks to my fellow graduate students especially Mr. D. C. Bignell and Mr. S. R. Rimmer for miscellaneous help.

The financial support of the Canadian Commonwealth Scholarship and Fellowship Administration and the National Research Council of Canada (grant to Dr. Hickman, A1103) is acknowledged.

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ABBREVIATIONS RELATING TO ULTRASTRUCTURE

CV	Central vacuole
D	Dictyosome or Golgi body
ER	Endoplasmic reticulum
ERt	ER, enlarged containing tubules
F	Flagellum
G	Groove
GR	Groove region
K	Kinetosome or Basal body
L	Lipid or Amorphous "vesicle"
Lo	Lomasome
M	Mitochondrion
Mb	Microbody
Ms	Mastigoneme
N	Nucleus
NE	Nuclear envelope
Np	Nuclear pore
Nu	Nucleolus
P	Plasmalemma
Rt	Rootlet microtubule
TF	Tinsel flagellum
U	Unidentified body
V	Vacuole
VC	Crystalline vesicle

VCd	VC, dense-mass form	VCf	VC, finger-print form
VCn	VC, network form	VCr	VC, ring form
VGf	Flattened vesicle with granular contents		
VG	Granular vesicle		
W	Cyst wall		
WF	Whiplash flagellum		

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CHAPTER 1

INTRODUCTION

There have been many studies of fungal zoospores both by mycologists and plant pathologists. Motility of zoospores and the absence of a cell wall, makes this cell type unique among the many and varied cell types in fungi. Zoospore motility resides in the possession of flagella, whip-like appendages having a lashing or undulating movement by means of which the zoospore is propelled in water. Two distinct types of flagellum are recognised on the basis of their major features(23).One,the whiplash flagellum, has a smooth surface whilst the other, the tinsel flagellum, bears a large number of exceedingly fine lateral projections, the mastigonemes, which are arranged in two opposite rows along the length of the flagellum.

Several kinds of zoospores are recognised in the sub-division Mastigomycotina on the basis of the number, type and orientation of the flagella they possess. Members of the Chytridiomycetes produce uniflagellate zoospores which possess a single, posterior whiplash-type flagellum and those of the Hyphochytriales possess a single, anterior tinsel-type flagelium. Biflagellate zoospores of the Plasmodio-

phorales have two whiplash flagella attached to their anterior end. The primary zoospores of Saprolegniales have a whiplash and tinsel flagella attached at the anterior end. The secondary zoospores of fungi of this order and the zoospores of the Peronosporales are laterally biflagellate, possessing one whiplash and one tinsel flagellum.

It is with this last group of zoospores that the present investigation is concerned and, in particular, with the laterally biflagellate zoospores of Phytophthora palmivora, a member of the Peronosporales and an important pathogen causing black-pod disease of cocoa (Theobroma cacao).

Zoospores have several important functions in the life cycles of fungi of this order. In several species, they initiate a new generation. This may be by germination of resting spores to produce zoospores exclusively as in some members of the Peronosporales, e.g. Albugo candida. Other species in this group, e.g. Pythium spp., produce zoospores from germinating oospores, or oospores germinate by hyphae.

Two other very important functions performed by zoospores of species parasitic on plants, are those of spore dissemination and of infection. The role of zoospores in disease dissemination is well documented, including review articles of Waterhouse (102) and Hickman and Ho (52). Blackwell (11) emphasised the importance of the ability of parasitic Phytophthora species, to live saprophytically for long periods as water moulds followed by dispersion in water to fields or green-houses where they may again parasi-

tise crop plants. Working with Phytophthora fragariae, Hickman (49) correlated the gradual increase in the distribution of strawberry red core disease with the direction of water drainage to lower lying areas. In subsequent studies on the effect of water drainage on infection of strawberries by zoospores, Hickman and English (51) were able to account for the occurrence of the disease in freely-draining land with high rainfall, as well as under conditions of impeded drainage and lower rainfall.

Evidence for water transport of inoculum, presumably as zoospores, has been reported by many other workers for soil-borne plant pathogenic members of the Peronosporales in soil, stream and irrigation water. Thus, for example, Phytophthora cryptogea and P. parasitica were isolated from surface drainage, polluted shallow wells, brooks and ponds supplying commercial tomato houses (9). Klotz et al. (62) isolated P. citrophthora, P. parasitica and P. syringae from water sources for large acres of citrus orchards. The importance of irrigation water as a method of dispersal is well documented for many species including P. parasitica var. nicotianae (70, 95), P. cactorum (67), P. cinnamomi (107) and other Phytophthora spp. (66). For example, Thomson's recent studies with P. parasitica and two other species (94) indicates that the fungus is spread by recycled irrigation water, and that zoospores can play a significant role as survival or dispersal units. Chlamydospores (thick-walled survival cells) and sporangia were isolated in field soil from

infected orange groves. The chlamydospores in field soil germinated to produce sporangia in irrigation water. The sporangia, on germination, released zoospores into irrigation water 5 min after the field soil was innundated. These zoospores remained motile in irrigation water for up to 20 h at 20°C.

In vivo studies on the potential of zoospores or cysts as inoculum units in soil, was investigated by Mehrotra (68). Some zoospores of P. drechsleri and P. megasperma var. sojae remained motile in soil for up to 30 and 20 h, respectively. With the use of a modified Atkinson's soil perfusion apparatus, the results showed that added to soil as zoospores or cysts, P. drechsleri retains infectivity for up to 15 days compared to P. megasperma var. sojae in which infectivity lasted for only up to 24 h in non-sterile soil. After encystment and germination of the zoospores, survival apparently occurred as hyphae or sporangia. These results indicate differences in rates and modes of survival among different species. Thus for example, Zan (105) reported that added to soil as zoospores, P. infestans survived for 10 days in the form of germ tubes. In P. cactorum, zoospores were demonstrated to survive for several weeks in soil as cysts (67). Turner (98, 99) reported that zoospores (or structures produced by them) of P. palmivora remained viable in soil for more than 6 months.

Although wind dispersal of caducous sporangia (or conidia) is the most important dispersal mechanism in most

Peronosporales attacking aerial parts of plants, zoospores may also be equally important in other species. Thus heavy infection of cocoa pods by P. palmivora occurred when high rainfall was combined with periods when night temperatures fall to 20°C (40). At this temperature, a high percentage of sporangial germination was by zoospore liberation instead of direct, germ tube germination (21). Drip or rain-splash distribution of inoculum also plays an important part in the development of diseases caused by these aerial pathogens. Examples are P. palmivora, and P. infestans under overhead irrigation (83). Whether this phenomenon is a result of sporangial or zoospore dispersal has not always been determined. However, Buddenhagen and Young (16) found zoospores of P. ilicis in drops of water hanging from holly leaves during rainy weather.

Leaf penetration experiments with P. infestans (76) have shown the success of zoospores as inoculum. After encystment, germination and appressorium formation, penetration of potato leaves occurred. On the other hand, directly germinating sporangia failed as inoculum, no penetration taking place even though long germ tubes were found growing on the surface of the leaves.

The efficiency of zoospores as inoculum under favourable moisture conditions has been provided by several field studies (63, 76), with an increase of infection correlated with increase in zoospore concentrations and duration of motility (28, 38, 51, 53, 63, 76).

Motility of zoospore inoculum is an advantage in comparison with other fungal spore types, for it enables them to collect in areas favourable for infection in response to plant exudates. This phenomenon of chemotaxis by plant pathogenic Peronosporales and Saprolegniales is well documented (10, 18, 28, 51, 77, 85, 92, 106).

Zoospores can remain motile in the absence of external nutrients for up to several days depending on the species and enviromental conditions (51). Long motile periods occur, *in vitro*, at relatively lower temperatures (within the range for that particular species) which may be associated with a lower rate of energy utilisation (51). Thus Salvin (86) reported increased speed of motility with increased temperatures, within the range for the species, in members of the Saprolegniaceae. Similar observations were made with zoospores of *P. palmivora* (10).

The extent of the influence of exogenous nutrients on motility is not clear. Gooding and Lucas (38) reported a lengthening of the motile period in zoospores of *P. parasitica* var. *nicotianae* with a 1% solution of D-glucose whereas the period of motility was shortened by sugars and amino acids (0.5%) in *P. palmivora* (10). In *P. drechsleri* (3), motility was either shortened or was not affected when sugars and amino acids (0.05 M) were added to zoospore suspensions. Some details of the metabolism of exogenous

pounds tested were utilised by zoospores and germinating cysts of P. drechsleri to some extent except D-ribose, L-malic acid and palmitic acid, which were poorly utilised. However, germinating cysts were reported to utilise more of these exogenous carbon sources than zoospores and this led these workers to suggest that zoospores may be less dependent on external nutrients than germinating cysts.

In recent years increasing interest has been shown not only in the biology of laterally biflagellate zoospores, but also in their structure, external and internal; and in the relationship of structure to their movement.

The laterally biflagellate zoospores of some members of the Peronosporales and Saprolegniales have been studied. These include several species of Phytophthora (47, 54, 80, 101) and Pythium (41), Sclerophthora macrospora (Sclerospora, 34) - Peronosporales; Aphanomyces (56, 87) and Saprolegnia spp. (37, 46) - Saprolegniales. Although there are some variations in the disposition of certain organelles in zoospores of different species, some morphological and ultrastructural generalisations are possible.

Zoospores are ovoid, bluntly pointed at the anterior end and rounded at the posterior end; viewed laterally they appear reniform in shape. There is a groove running along the length of the zoospore on one side. It is shallow at each end and relatively deep in the centre region. The two flagella arise from a point protruding from this deep part of the groove. The flagella [illegible] directed tin-

sel and posteriorly directed whiplash type, have the 9 + 2 organisation of internal fibrils characteristic of flagella and cilia in general (90). Within the zoospore itself the groove region is further characterised by a lack of ribosomes and other cell organelles, and by the possession of numerous microtubules (54, 56, 80) and in Aphanomyces euteiches (56) small golgi proliferated vesicles. Lying just below the groove, towards the anterior end of the zoospore, is a single large vacuole, referred to as a contractile vacuole in A. euteiches (56).

There is a large pear-shaped nucleus, with a conspicuous nucleolus. The narrow end of the nucleus is oriented towards the groove region. The nucleus is surrounded by several stacks of rough endoplasmic reticulum. Endoplasmic reticulum is also present elsewhere in the cytoplasm. In the cytoplasm of A. euteiches zoospores (56), enlarged cisternae of endoplasmic reticulum containing tubular elements, is reported to be common and between the nucleus and the groove region is a golgi complex.

A common feature of the cytoplasm of zoospores is the occurrence of many varied types of vesicles. In Phytophthora parasitica (47, 80) and Sclerophthora macrospora (34) vesicles with lipid contents were reported. In some of the species (47, 54, 56, 80) vesicles with crystalline, unidentified contents have been recognised. Hickman and his co-workers (54) suggested the possibility of vesicular contents serving as energy sources for motility, on the

basis of differences in vesicular (crystalline) contents in young and old zoospores.

When the zoospore encysts, several marked changes occur not only in vesicles, but in other cytoplasmic structures as reported for A. euteiches (56), P. parasitica (47, 80), Pythium aphanidermatum (41) and S. macrospora (34).

During encystment, the flagella are lost either by withdrawal into the zoospore itself or by detachment as in P. parasitica (47) or in A. euteiches (56), often after rolling up of the flagella axonemes to form bead-like bodies. The zoospore gradually becomes spherical and secretes a thin wall around itself. In this process, the groove disappears and so does the large vacuole associated with it in the zoospore.

The disappearance of flagella during encystment has been suggested to be linked by a triggering mechanism, which couples flagella disappearance to cyst wall synthesis in P. palmivora (96). In A. euteiches (56) the cyst wall is reported to be derived from preformed golgi vesicles and in P. parasitica (47) and Pythium aphanidermatum (41) from preformed vesicles at the cell periphery. Wall formation started within 20 sec of encystment in Phytophthora palmivora (96) and in A. euteiches (56) was completed within 30 min.

After encystment the nucleus may at first remain pear-shaped and eccentrically placed, as in the zoospore, but eventually it rounds up and takes up a central position. The kinetosomes regress into centrioles.

The onset of germination after encystment is marked by the appearance of a lateral cluster of small vesicles below the cyst surface. As these vesicles enlarge, the adjacent wall bulges outward to form a germ tube. As the germ tube elongates, the cluster of tiny vesicles is retained at the apex, characteristic of hyphal tip zonation and formation (13, 41, 42, 43, 47, 56).

Throughout encystment and germination other cytoplasmic organelles and inclusions - mitochondria, dictyosomes, endoplasmic reticulum and vesicles - also undergo changes. The contents of vesicles containing electron opaque inclusions decrease and become less ordered. The formation of a central vacuole commences in both the cyst and young hypha (germ tube). This central vacuole appears to originate from coalescing vesicles with electron opaque inclusions, which appear to be breaking down. The central vacuole of the cyst enlarges as cytoplasmic contents move into the elongating germ tube (41, 56).

Division of the nucleus occurs in _Pythium aphanidermatum_ (41) when the germ tube is about as long as the diameter of the cyst. One daughter nucleus along with some cytoplasmic material moves into the growing young hypha. In _A. euteiches_ (56), within 2 h following encystment, two mitotic nuclear divisions have occurred.

At this point the ultrastructure of the germ tube is typical of somatic hyphae (41, 56).

Compared with the environmental and ultrastructural

studies on laterally biflagellate zoospores, work on the biochemical events associated with the pattern of development outlined above, is almost lacking. Only in a few fungi, of groups unrelated to Phycomycetes, have such studies been made.

A common feature of zoospore-forming species, differentiating them from most other fungi, is the _de novo_ formation of cell walls. This obviously must be the case when naked cells (zoospores) encyst and produce a cell wall. Generally, fungal cell walls are 80 to 90% polysaccharide (from a variety of sugars) with the remainder composed of proteins and lipids. Slight variations may occur but wide departures are rare.

Recent studies on some Peronosporales including _Phytophthora_ and _Pythium_ spp. (4, 5) have shown that hyphal cell walls contain 80 to 90% glucans, 5 to 10% proteins, and 1 to 2% lipids. Cyst walls of _Phytophthora palmivora_ (96) showed marked structural differences but did not differ in qualitative composition, from hyphal walls. Glucans (β-1-6 and β-1-3) were present in both cysts and hyphal walls. However, the latter contained more protein, 1-6 linked glucosyl residues and/or non-reducing terminal residues but less 1-2 and/or 1-4 linked glucosyl residues than cyst walls.

Apart from the cyst wall analyses outlined above, the only other study on zoospores in the Peronosporales has been physiological in nature, namely oxygen uptake during the period of zoospore motility through to cyst germination

in *Pythium aphanidermatum* (19). Oxygen uptake in the absence of nutrients in zoospores and newly formed cysts was relatively low, but increased during germination. Added glucose and fructose (5 mM) had little effect on oxygen consumption of zoospores. Added nutrients had variable effects on germinating cysts. Pyruvate, fructose and fructose phosphate increased oxygen consumption as did sucrose. But glucose or glucose phosphate had no effect. Sucrose stimulation and failure of glucose to stimulate oxygen uptake during germination, agreed with earlier findings on the effect of various nutrients in pea (host) root exudate on cyst germination in the same species (18). Intermediates of the Entner-Doudoroff pathway also had no effect on oxygen uptake. Of the citric acid cycle intermediates tested, only pyruvate and α-ketoglutarate were effective in increasing oxygen consumption. Generally, the increase in oxygen consumption with added nutrients was about 30% but host root exudate caused a stimulation of more than 70%.

With the exception of the changes outlined above, no information is available on biochemical changes that occur during zoospore motility and cyst germination in members of the Peronosporales. Such changes could involve metabolic reserves, enzymes and metabolic pathways.

The present study was undertaken in an attempt to relate biochemistry to the morphological and ultrastructural changes occurring during motility, encystment and germination, with some emphasis on the role that the many varied

types of vesicles play during zoospore motility and cyst germination.

Phytophthora palmivora was chosen for study because during the period of July 1968 and June 1969, this fungus was used in studies on motility and chemotaxis. As a result, the author became familiar with this fungus and the culture techniques required to produce the enormous quantities of zoospores necessary for the present studies.

CHAPTER 2

MATERIALS AND METHODS

A CULTURE OF FUNGUS AND PRODUCTION OF ZOOSPORES

I The test organism:

The isolate of *Phytophthora palmivora* (Butl.) Butl. used in these studies was obtained from naturally infected cocoa (*Theobroma cacao*) pods. The cocoa pods were collected from the Experimental Farms of the Cocoa Research Institute in Ghana in 1968.

The isolate was maintained by periodic transfers on a V8-juice agar medium.

II Culture media:

The difficulties associated with producing the large numbers of zoospores (about 30 million zoospores for each experiment) of such minute size (approx. 10 µm), necessary for determining enzyme concentrations were numerous. Added to this was the need to produce final zoospore suspensions containing a minimum of external nutrients, which would be suitable for the experimental design. The experiments required holding zoospore suspensions for 6 h in the absence

of external nutrients and assessing the ultrastructural and biochemical changes that occurred during this period.

Production of such large quantities of zoospores meeting the above requirements was difficult, since the medium that produced maximum sporulation (uncleared V8-juice agar), also yielded suspensions containing relatively large amounts of nutrients (see Table I). Cleared V8-juice agar, on the other hand, did not produce as many sporangia, but yielded final suspensions with minimal nutrient content.

After consideration of all these factors, the methods of Ho and Hickman (53) and Mitchell (personal communication) were adopted with modifications. The method used for zoospore production ensured ease of handling large numbers of cultures (about 350 plates for each experiment) without contamination.

V8-juice agar medium was prepared by mixing 200 ml V8-juice (Campbell Soup Co.), 800 ml distilled water, 1.5 g calcium carbonate and 20 g of Difco Bacto-agar (uncleared medium).

Cleared V8-juice agar was prepared by boiling the mixture of V8-juice, distilled water and calcium carbonate for 10 min. The cooled mixture was then filtered once through cheese cloth and twice through Whatman number 3 filter paper. The resulting cleared juice was diluted (2:1, v/v) with distilled water, and the pH adjusted to 6.8 ± 0.2. Difco Bacto-agar (2%, w/v) was added and the medium autoclaved at 15 p.s.i. for 15 min.

TABLE 1

Nutrient content of water from zoospore suspensions (250 ml)* provided from V8-juice agar media with different treatments.

Treatment	Carbohydrate (μg)	Protein (μg)
UNCLEARED (unwashed)	3000	150
UNCLEARED **(washed)	1500	140
CLEARED **(washed)	500+	140+

* Suspension (250 ml) freeze dried, redissolved in 10 ml distilled water

** Medium was washed every 24 h for 4 days

\+ No significant difference in values for suspensions of 0 h or after 6 h motility

The fungus was grown on 20 ml cleared V8-juice agar plates (9 cm diam.) at 28°C for 6 days in the dark. The inocula for these agar plates were 3 mm diam. discs taken from the growing edge of 3 to 5 day old V8-juice agar plate cultures.

After 6 days when the colonies had covered the agar, the cultures were transferred to sterile plates (14 cm diam.), three 9 cm cultures per dish. Sterile distilled water (75 ml) at 25°C was added to each dish and the cultures were placed in an illuminated incubator, 80-90 ft-c (Percival Refrigeration and Mfg. Co., Model 1-36), at 25°C. The distilled water was aseptically replaced with fresh sterile distilled water at 25°C every 24 h for 4 days, so that the zoospore suspension finally produced would contain a minimum of nutrient. After the final water change, the cultures were washed and drained leaving practically no water. They were returned to the lighted incubator for 3 days during which aerial sporangiophores and sporangia were produced in great profusion.

III Zoospore production:

Cultures bearing abundant sporangia were rinsed once with sterile distilled water and resuspended in 60 ml distilled water. They were incubated at 5°C for 15 min and then transferred to room temperature (25°C). Sporangia readily liberated abundant zoospores within 1 h. This zoospore suspension was decanted from the cultures and gently

filtered through Whatman number 41 paper to remove sporangia, mycelium and pieces of agar. Table I shows the nutrient content in zoospore suspensions produced by different methods.

IV Experimental treatments:

The filtered zoospore suspension was used in various enzyme and metabolic reserve analyses and electron microscopic observation experiments, after separation into different fractions (see Fig. 1, p. 20), as follows:

i) Immediately after filtration, i.e. after a period of motility of 1 to 2 h (designated T1).

ii) After holding at 17°C (opt. temperature for motility) for 6 h, the suspension was centrifuged at 6000 x g for 10 min at 4°C, to synchronise encystment and to pellet the spores. The pellet was divided into two approximately equal portions with the aid of a small spatula. Half of this pellet (designated T2) was used immediately in experiments.

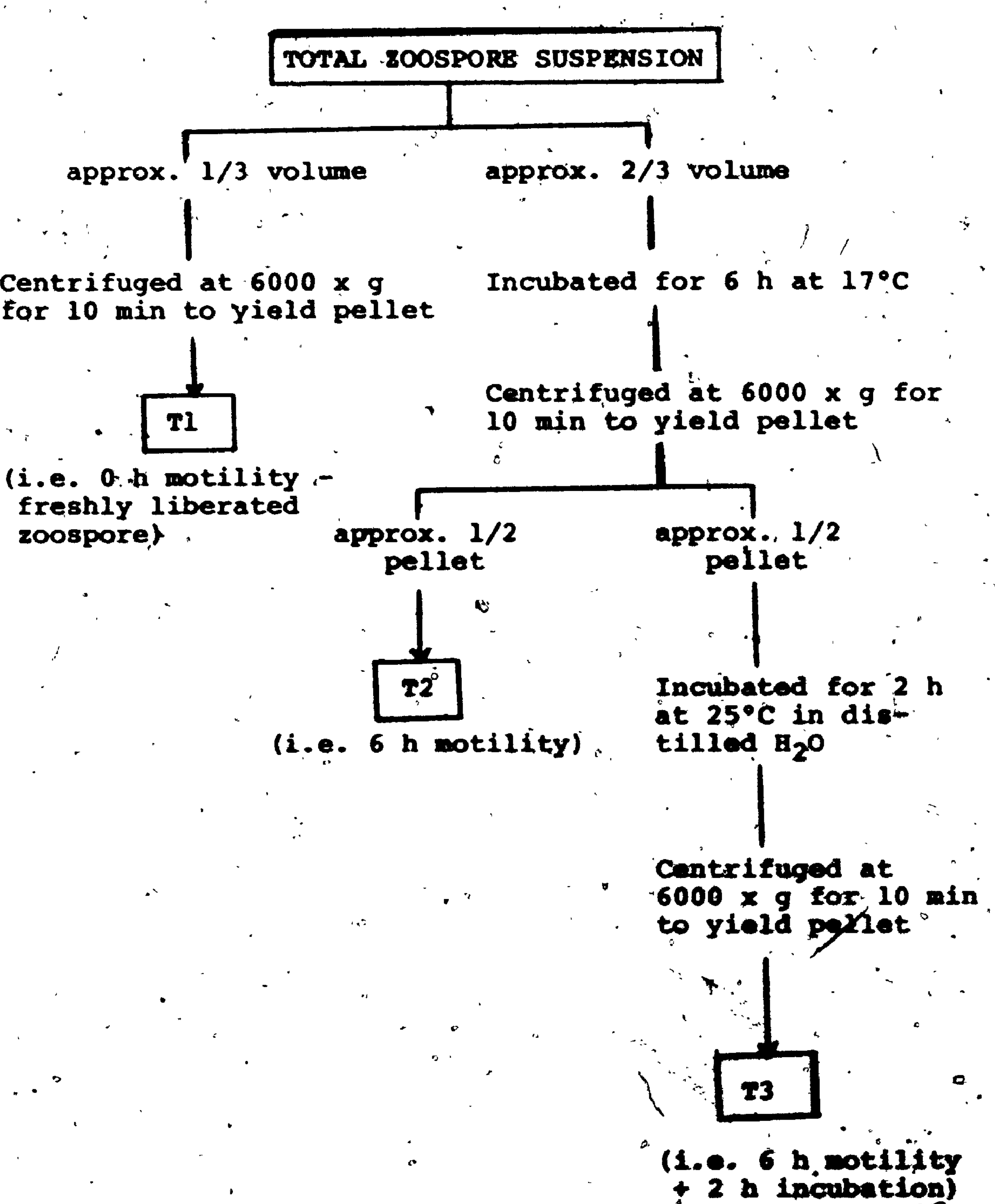
iii) The other half of above pellet was resuspended in 15 ml sterile distilled water and incubated at 25°C, the optimum temperature for germination. It was incubated for 2 h (T3, see Fig. 1), after which it was centrifuged and the resulting pellet was used immediately for metabolic reserve and enzyme analyses experiments.

For electron microscopic observations, aliquots of the suspension at 25°C were removed after varying periods

of time (15 min to 2 h) and fixed as described in Section B I, pages 21 to 22.

FIGURE 1

EXPERIMENTAL TREATMENTS OF ZOOSPORE SUSPENSIONS AND GERMINATING CYSTS USED IN CHEMICAL AND ENZYME ASSAYS



B ELECTRON MICROSCOPY

I General:

Zoospores and cysts were fixed in glutaraldehyde and osmium tetroxide using a slight modification of the method of Ho et al. (54), or in potassium permanganate: -

Gluaraldehyde-osmium tetroxide. Zoospore suspensions were rapidly mixed with an equal volume of fixative (4% glutaraldehyde in 0.1 M phosphate buffer and 2% (w/v) sucrose at pH 6.8). After 30 min the suspension-fixative mixture was filtered through a millipore filter disc (5 μm pore size) on which the zoospores were retained. The disc was coated with a thin agar film by dipping it quickly in 2% buffered (0.1 M phosphate) water agar at 45°C. The coated millipore disc was placed in fresh 3% buffered glutaraldehyde for 1 h.

Fixation of germinating cysts held in distilled water at 25°C for varying periods of time (15 min to 2 h) was carried out as for motile zoospores above.

Pelleted cysts were directly mixed and resuspended in 3% buffered glutaraldehyde and incubated for 1.5 h. They were then filtered onto a millipore disc and agar coated as described above.

After the glutaraldehyde fixation, millipore discs were washed overnight in three changes of buffer. They were then post-fixed in 2% (w/v) aqueous osmium tetroxide for 2 h, rinsed for 15 min in each of two changes of dis-

tilled water. The agar-coated discs were stained for 30 min in 0.5% (w/v) aqueous uranyl acetate. The millipore discs were cut up into small pieces (approximately 1 mm diameter) and dehydrated in an acetone series and embedded in an Epon-Araldite mixture.

Potassium permanganate. Zoospores and cysts were fixed by mixing with 2% (w/v) filtered aqueous potassium permanganate solution for 15 to 30 min. This was followed by filtration on to millipore discs, coating with thin agar film and dehydration in an acetone series. Small pieces of agar-containing spores were embedded in an Epon-Araldite mixture.

Sections were cut on a Porter-Blum microtome and stained with lead citrate (0.035 g in 10 ml double distilled water and 0.1 ml of 10.0 N NaOH solution; 82). Micrographs were taken using a Philips 75 or 200 electron microscope.

II Identification of vesicular contents:

To identify contents of vesicles, the extraction procedure of Eurenius and Jarskar (31) was tested. This method involved the removal of the epoxy resin and lipids from ultra-thin sections with sodium methoxide reagent and a methanol-benzene mixture (1:1, v/v). This procedure was found to be unsatisfactory since it yielded dirty sections, or vesicular contents were not always consistently removed.

Other methods were therefore used. Zoospores and cysts were fixed with glutaraldehyde as previously de-

scribed but before post-fixation in osmium tetroxide they received one of the following treatments:

For removal of lipid:

i) A 30 min immersion period in each solution of a graded acetone series - 20, 50, 70, 90, 100, 100, 70, 50 and 20% (modified after McKeen, 65).

ii) Immersion for 2 h in a chloroform-methanol mixture (3:1, v/v), followed by 1 h in chloroform.

iii) Immersion for 3 h in an ether-ethanol mixture (2:1, v/v).

For removal of protein:

iv) Buffered pepsin (1.0 mg/ml in 0.1 M HCl at pH 2.0) at 37°C for 15 min.

v) Treatment with α-amylase (250 μg/ml in 0.003 N $CaCl_2$ in a half-saturated $NaCl_2$ solution). After glutaraldehyde fixation, spores were pelleted by centrifugation and incubated with the α-amylase solution for 15 min at 37°C. The spores were then filtered on to a millipore disc, rinsed with buffer and coated with a thin film of agar as before.

Standard procedures described previously were followed to obtain electron micrographs.

III <u>Cytochemical localisation of marker enzymes in microbodies and mitochondria:</u>

<u>Microbodies</u>. Cytochemical demonstration of marker enzymes of microbodies, L-α-hydroxy acid oxidases and catalase, was

by the method of Shnitka and Talibi (88) and the 3,3'-diaminobenzidine (DAB) method of Novikoff and Goldfischer (71) as modified by Beard and Novikoff (6), respectively.

The demonstration of L-α-hydroxy acid oxidase activity involved the enzymatic reduction of ferricyanide to ferrocyanide. The ferrocyanide was then captured by cupric ions to yield insoluble, electron-opaque cupric ferrocyanide.

Catalase activity was investigated with the 3,3'-diaminobenzidine method which also produces electron-opaque reaction products in the presence of hydrogen peroxide.

Mitochondria. For the localisation of succinate dehydrogenase activity the method of Kerpel-Fronius and Hajós (60) was used. The procedure is based on a simultaneous coupling reaction in which the ferrocyanide produced was captured by copper at the site of enzyme activity as an electron opaque product.

Standard procedures as described previously were then followed to obtain electron-micrographs.

C CHEMICAL ANALYSES

I Buffer systems:

Crude cell-free extracts were prepared with 0.05 M phosphate buffer, 0.05 M Tris buffer or in 0.05 M sodium bicarbonate in 0.25 M mannitol buffer. The pH values of these buffers were adjusted according to the enzymes being assayed and details are given in section D.

Several buffers were used in individual enzyme assays depending on which enzyme was under investigation. Details of buffer systems used for each assay, and pH, will be given in section D.

II Cell-free homogenates:

Cells at the desired stage of development were collected by centrifugation. The pellets were weighed wet after drainage for 5 min and suspended in 5 ml of the appropriate buffer. This was then subjected to sonic disruption for 5 min at 5 μ for 22 Kc/s, with an MSE ultrasonic disintegrator. Crushed ice was used as coolant and the temperature was never allowed to rise above 10°C. The cellular debris was removed by centrifugation at 22,000 x g for 20 min at 4°C. The supernatant was designated as crude enzyme preparation, and used immediately for the analysis of enzymatic activity or metabolic reserve.

III Metabolic reserves:

It was very difficult to measure the minute quantities of endogenous nutrients present in zoospores and cysts with most biochemical methods. Therefore most of the methods adopted were colorimetric assays, which were capable of measuring the μg amounts present.

a) Carbohydrates. Determination of soluble carbohydrates was carried out by a slightly modified phenol-sulphuric acid method of Dubois et al. (27). For total carbohydrate content, samples were taken after 5 min sonication in 0.05 M phosphate buffer before centrifugation. Carbohydrate content both of the 22,000 x g supernatant and of the pellet (resuspended in 2.0 ml of buffer) were determined. Phenol (1.0 ml of 5%, v/v, solution) was added to the test tubes containing 0.5 ml sample and 0.5 ml of buffer. Concentrated sulphuric acid (5.0 ml) was added rapidly, the stream of acid being directed against the liquid surface to obtain good mixing. The tubes were allowed to stand at 25°C for 10 min, then they were shaken and placed for 10 to 20 min in a water bath at 25° to 30°C. The optical density at 488 nm was recorded with glucose (in buffer) being employed as a standard. The standard curve was linear from 0 to 50 μg/ml, and all measurements of the samples were made in this range. Values were expressed as μg carbohydrate/mg dry spores.

b) Lipids. Total lipids were extracted by serial sonication for 5 min in 10, 5 and 4 ml of chloroform-

methanol mixture (2:1, v/v). After each sonication period, the mixture was centrifuged at 22,000 x g for 10 min and the pellet resuspended in a fresh volume of chloroform-methanol mixture as indicated above. After the third sonication, the mixture was combined with supernatants from the two previous centrifugations. The mixture was made up to a volume of 20 ml and left at 25°C for 12 h in a stoppered Pyrex test tube. Folch's lipid extraction procedure as modified by Radin (80) was then used. Vacuum dried samples were finally resuspended in 5 ml of chloroform and designated as chloroform extracts.

Determination of lipid phosphorus. The analysis of phosphorus content in 1.0 ml of chloroform extract was by a method adapted from Rouser et al. (84). Samples were transferred to small Kjeldahl flasks, each containing 4 BDH boiling chips. The tubes were placed on ashing racks and heated to evaporate the solvent. To each tube was added 1.0 ml of 60% perchloric acid, and the mixture boiled for 20 to 25 min on ashing racks. After cooling the tubes and their contents, 7 ml of distilled water, 1 ml of 2.5% (w/v) ammonium molybdate solution and 1 ml of 10% (w/v) ascorbic acid solution were added. The tubes were heated in a boiling water bath for 5 min. Cooled samples were measured at 820 nm. Potassium phosphate (KH_2PO_4) was employed as standard.

Acyl ester linkage determination. The procedure of Stern and Shapiro (91) was used to estimate the content of

glycerides in 1.0 ml of the chloroform extracts. Recrystallised methyl palmitate was employed as a standard. Samples were measured at 520 nm.

Free Fatty Acids. Estimation of the total free fatty acids in sonicated cell-free extracts in 0.05 M phosphate buffer was by a slight modification of the method of Itaya and Ui (58). Chloroform (6 ml) was added to 1.0 ml of sample in buffer and 0.1 ml of 2% albumen in NaCl. Free fatty acids transferred from the aqueous phase into the chloroform, were mixed with copper triethanolamine (1.0 M triethanolamine: 1N acetic acid:6.45% $Cu(NO_3)_2.3H_2O$, 9:1:10 v/v/v). The Cu complex of free fatty acids in chloroform after separation and filtration was measured colorimetrically at 440 nm, after the addition of 2 drops of sodium diethyldithiocarbamate solution in n-butanol. Recrystallised palmitic acid in chloroform was used as a standard.

c) Protein determination. The amount of protein in cell free-extracts in 0.05 M phosphate buffer (before centrifugation, i.e. total protein), the 22,000 x g supernatant (designated CFE) and pellet (resuspended in 2.0 ml of buffer) was estimated with the procedure of Bramhall et al. (14). This method involved spotting samples onto pieces of filter paper (Whatman 42), air drying and transferred to trichloroacetic acid (7.5%, w/v) and heated at 80°C for 30 min in a water bath. After removal of the acid and lipids with ether-ethanol (1:1) and ether, the dried paper was stained with dye solution (xylene brilliant cyanin G, 10 mg/ml in

7% acetic acid). Excess dye was removed with 7% acetic acid and the dyed sample spots were eluted with destain solution (66 ml methanol, 34 ml water and 1 ml concentrated ammonium hydroxide). The dye released into the solution was measured at 610 nm. Bovine serum albumin was used as a standard.

D ENZYME ASSAYS

I Spectrophotometric determination of enzyme activities:

All enzymes were assayed spectrophotometrically using the crude enzyme fraction previously described. Colorimetric methods were used to maximise the chances of monitoring the low enzyme activities present in such small samples (approximately 5.0 mg dry weight of spores).

The reaction systems and the conditions of each assay are given below. The enzymes assayed were isocitrate lyase (L_S-Isocitrate glyoxylate-lyase, EC 4.1.3.1)*, malate synthase (L-Malate glyoxylate lyase, EC 4.1.3.2), NAD-isocitrate dehydrogenase (D_S-Isocitrate : NAD oxidoreductase (decarboxylating), EC 1.1.1.41), NADP-isocitrate dehydrogenase (L_S-Isocitrate:NADP oxidoreductase (decarboxylating), EC 1.1.1.42), malate dehydrogenase (L-Malate:NAD oxidoreductase, EC 1.1.1.37) and succinate dehydrogenase (succinate:(acceptor) oxidoreductase, EC 1.3.99.1). The activity of α-ketoglutarate dehydrogenase could not be detected with several assay techniques (59, 73, 97).

Assays were conducted in a Beckman DB spectrophotometer with a recorder and a Unicam 1800 spectrophotometer equipped with a high speed recorder. Specific activity is expressed as units of enzyme per mg protein.

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* For enzyme nomenclature see Florkin and Stotz (33).

a. SPECTROPHOTOMETRIC DETERMINATION OF ACTIVITY OF KEY ENZYMES OF THE GLYOXYLATE CYCLE - ISOCITRATE LYASE AND MALATE SYNTHASE

Isocitrate $\rightleftharpoons$ succinate + glyoxylate 1

Acetyl Coenzyme A + glyoxylate $\longrightarrow$ malate 2

Spectrophotometric assays of isocitrate lyase (reaction 1) and malate synthase (reaction 2) were made by a modification of the methods of Dixon and Kornberg (25) and Olson (76).

The isocitrate lyase assay depends on the measurement of the rate of increase in optical density consequent upon the formation of glyoxylate phenylhydrazone at 324 nm. After a lag of about 1 min after addition of isocitrate to start the reaction, the increase in optical density was linear for 4 to 5 min; this rate is proportional to the enzyme concentration.

Malate synthase activity was followed by measuring the decrease in optical density at 232 nm, consequent upon the breakage of the thio-ester bond of acetyl coenzyme A in the presence of glyoxylate. Glyoxylate was added to start the reaction. However, before its addition, the recorder was allowed to run for several minutes to detect the possible presence of acetyl coenzyme A deacylase. The initial rate of decrease in optical density is proportional to the amount of malate synthase present.

The enzyme extract was prepared in 0.05 M Tris buffer, pH 7.0, containing 2 µmoles/ml of dithiothreitol. Reac-

tions were carried out in a Unicam 1800 spectrophotometer at 28°C.

For both enzyme assays, the crude enzyme (0.4 ml) was added to a reaction mixture (0.5 ml), the components of which are given in Table II. Table III shows the assay mixtures used to determine enzyme activities.

A unit of activity was defined as the amount of enzyme causing a change in optical density of 0.001 per min.

TABLE II

REACTION MIXTURES FOR GLYOXYLATE CYCLE ENZYMES

REACTION	REACTANTS	STOCK SOLUTION	VOLUME (ml)
* ICL	CYSTEINE-HCl	0.03M	0.2
	PHENYLHYDRAZINE-HCl	0.216% (w/v)	0.5
	$MgSO_4$	0.05M	0.3
	BUFFER	0.2M phosphate, pH 6.0	1.0
	DOUBLE DISTILLED WATER		0.5
+ MS	ACETYL CoA	0.025 μmoles	0.02
	$MgCl_2$	2.860% (w/v)	0.07
	BUFFER	0.2M Tris, pH 7.6	0.5
	DOUBLE DISTILLED WATER		2.0

* Isocitrate lyase (ICL).

\+ Malate synthase (MS).

TABLE III

SPECTROPHOTOMETRIC ASSAY OF ISOCITRATE LYASE (ICL) AND MALATE SYNTHASE (MS) ACTIVITIES

REACTION	REACTANTS	STOCK SOLUTION	VOLUME (ml)
ICL	REACTION MIXTURE	pH 6.0	0.5
	ENZYME		0.4
	*DL-SODIUM ISOCITRATE	0.2M	0.1
MS	REACTION MIXTURE	pH 7.5	0.5
	ENZYME		0.4
	*SODIUM GLYOXYLATE	2.0 µmoles	0.1

* Reactions were started by the addition of isocitrate and sodium glyoxylate for isocitrate lyase and malate synthase, respectively.

Controls accompanying each assay were without isocitrate or glyoxylate.

b. SPECTROPHOTOMETRIC DETERMINATION OF ISOCITRATE DEHYDROGENASE ACTIVITY

d-Isocitrate + $NAD^+ \longrightarrow \alpha$-Ketoglutarate + NADH + H^+ + CO_2 1

d-Isocitrate + $NADP^+ \longrightarrow \alpha$-Ketoglutarate + NADPH + H^+ + CO_2 2

Isocitrate dehydrogenase activities were assayed spectrophotometrically by following the reduction of NAD^+ (reaction 1) and $NADP^+$ (reaction 2) at 340 nm. All assays were performed at 25°C in cuvettes of 1 cm light path, in a spectrophotometer equipped with a recorder, by the method of Plaut and Sung (75).

A unit of activity was defined as the amount of enzyme causing an increase in optical density of 0.01 per min under conditions for which the rate of optical density increase remained linear for at least 5 min.

The reaction systems used in these assays are shown in Table IV. In all cases a control containing all reaction components except isocitrate accompanied the sample.

The enzyme preparation used was in a 0.05 M sodium bicarbonate in 0.25 M mannitol buffer at pH 8.0.

TABLE IV

SPECTROPHOTOMETRIC ASSAY OF ISOCITRATE DEHYDROGENASE ACTIVITIES

REACTION	REACTANTS	STOCK SOLUTION	VOLUME (ml)
1	NAD	0.01M	0.1
	ENZYME		0.2
	BUFFER	0.1M cacodylate, pH 6.5	0.5
	AMP	0.01M	0.1
	$MnSO_4$	0.02M	0.1
	*DL-ISOCITRATE	0.80M	0.1
2	NADP	0.0015M	0.2
	ENZYME		0.2
	BUFFER	0.1M Tris, pH 7.4	0.5
	$MnSO_4$	0.02M	0.1
	*DL-ISOCITRATE	0.08M	0.05

* Reactions were started by the addition of DL-isocitrate, sodium salt.

c. SPECTROPHOTOMETRIC DETERMINATION OF MALATE DEHYDROGENASE ACTIVITY

Oxaloacetate + NADH + H^+ $\rightleftharpoons$ L-Malate + NAD^+

Malate dehydrogenase was assayed spectrophotometrically by following the oxidation of NADH at 340 nm. Assays were conducted in a spectrophotometer equipped with a recorder. Initial rates of reaction were determined at 25°C in cuvettes with 1 cm path-length and a total volume of 3.0 ml.

The reaction system used in these assays is shown in Table V. Controls containing all reaction components except NADH (replaced with buffer) were included in each assay.

One unit of enzyme is defined as that amount of enzyme causing a decrease in optical density of 0.01 per min.

The enzyme preparation used in these assays was prepared in a buffer of 0.05 M sodium bicarbonate in 0.25 M mannitol at pH 8.0.

TABLE V

SPECTROPHOTOMETRIC ASSAY OF MALATE DEHYDROGENASE ACTIVITY

REACTANTS	STOCK SOLUTION	VOLUME (ml)
NADH	0.00128M (in buffer)	0.2
ENZYME		0.2
BUFFER	0.25M phosphate, pH 7.4	2.6
*OXALOACETATE	0.0076M (in buffer)	0.1

* Reactions were started by the addition of oxaloacetate to the assay mixture. Controls had oxaloacetate added before establishing a base line tracing for NADH absorption at 340 nm.

d. SPECTROPHOTOMETRIC DETERMINATION OF SUCCINATE DEHYDROGENASE ACTIVITY

Succinate $\rightleftharpoons$ Fumarate + $2H^+$ + 2e

Several manometric and spectrophotometric methods were tested for the activity of succinate dehydrogenase (1, 2, 8, 12, 17, 30, 79, 89, 97). Finally the assay method adopted was modified after those of Arrigoni and Singer (1), Baginsky and Hatefi (2) and Ells (30).

Succinate dehydrogenase activity was measured by following the oxidation of succinate and subsequent reduction of an N-alkylphenazonium salt coupled to a terminal electron acceptor. The N-alkylphenezonium dye used was phenazine methosulfate (PMS) coupled to a terminal acceptor 2,6-dichlorophenol indophenol (DCIP). The reaction rate was measured by following the reduction of DCIP at 600 nm. The concentration of PMS was varied (0.2, 0.1, 0.07, 0.05, 0.03 ml) to overcome the possibility that at fixed dye (PMS) concentrations, the affinity of the dye for the enzyme may vary depending on source or age of enzyme preparation (1, 79).

Enzyme extract was prepared with 0.05 M potassium phosphate buffer, pH 7.6. The assay mixture of 0.4 ml enzyme extract and 0.4 ml of reaction mixture (the components are given in Table VI) were allowed to equilibrate for 7 min in cuvettes at 38°C. The cuvettes were incubated in a Unicam 1800 spectrophotometer equipped with a rapid recorder. After temperature equilibration, 0.01 ml of DCIP

(0.002 μmole) and varying concentrations of 1.0% (w/v) PMS were rapidly added and mixed. Controls were run with each assay, the mixtures were without sodium succinate.

The initial rate of change in optical density per min at 600 nm, following addition of DCIP and PMS, was calculated from the 30 to 90 sec period following addition of the dyes. A reciprocal concentration of PMS was plotted against the reciprocal of initial rate of change in optical density and extrapolated to give V max. (maximum velocity) values which were then used for calculating specific activities.

TABLE VI

REACTION MIXTURE FOR SPECTROPHOTOMETRIC ASSAY OF SUCCINATE DEHYDROGENASE ACTIVITY

REACTANTS	STOCK SOLUTION	VOLUME (ml)
SODIUM SUCCINATE	0.02M	0.5
PHOSPHATE BUFFER	0.05M, pH 7.6	5.0
POTASSIUM CYANIDE	0.01M, pH 7.6	0.5

CONTROL MIXTURE FOR SPECTROPHOTOMETRIC ASSAY OF SUCCINATE DEHYDROGENASE ACTIVITY

REACTANTS	STOCK SOLUTION	VOLUME (ml)
DOUBLE DISTILLED WATER		0.5
PHOSPHATE BUFFER	0.05M, pH 7.6	5.0
POTASSIUM CYANIDE	0.01M, pH 7.6	0.5

II Distribution of enzymes in cell fractions:

To determine the relative quantities of all the enzymes assayed earlier in Section I (except succinate dehydrogenase), in various fractions of spores, all the zoospores produced from 350 plates were used. The zoospores (1 to 2 h motility) were centrifuged to synchronise encystment and pellet the spores. The spore pellet was divided into two with the aid of a small spatula.

One half of the pellet was suspended in 5.0 ml of 0.05 M Tris buffer at pH 7.0 containing 2 μmoles/ml of dithiothreitol (for glyoxylate enzymes) and the other half in 5.0 ml of 0.05 M sodium bicarbonate in 0.25 M mannitol buffer at pH 8.0 (for isocitrate dehydrogenase and malate dehydrogenase assays). The spores were sonicated for 5 min as previously described.

Sonicated cell-free extracts in the appropriate buffer were centrifuged at 6000 x g for 15 min (pellet designated as P 1), 22,000 x g for 30 min (pellet P 2), and 46,000 x g for 90 min (pellet P 3) (see Fig. 2, p. 44). After each centrifugation step, the resulting pellet was washed once with 2.0 ml buffer and centrifuged again at the same speed. The two resultant supernatants were then combined and the centrifugation procedure repeated at the next level. The final two supernatants (after 46,000 x g) were combined and designated S 4. Each of the pellets were subsequently resuspended in 5.0 ml of buffer.

Relative specific activities of isocitrate lyase and

malate synthase in the different fractions were assayed as previously described (pages 31 to 34).

Isocitrate dehydrogenase (NAD and NADP-linked enzymes) and malate dehydrogenase activities were also determined on the various fractions. The total volume of assay mixtures in these three tests was 1.0 ml.

Isocitrate dehydrogenase - NAD linked. This was assayed by incubating 0.2 ml of enzyme extract with 0.7 ml of reaction mixture containing NAD, cacodylate buffer pH 6.5, AMP and $MnSO_4$ as in Table IV (reaction 1). DL-isocitrate, 0.1 ml was added to start the reaction.

Isocitrate dehydrogenase - NADP linked. This was assayed by incubating 0.2 ml of enzyme extract with 0.7 ml of reaction mixture containing NADP, Tris buffer pH 7.4, and $MnSO_4$ in quantities as given in Table IV (reaction 2). DL-isocitrate 0.05 ml was added to start the reaction.

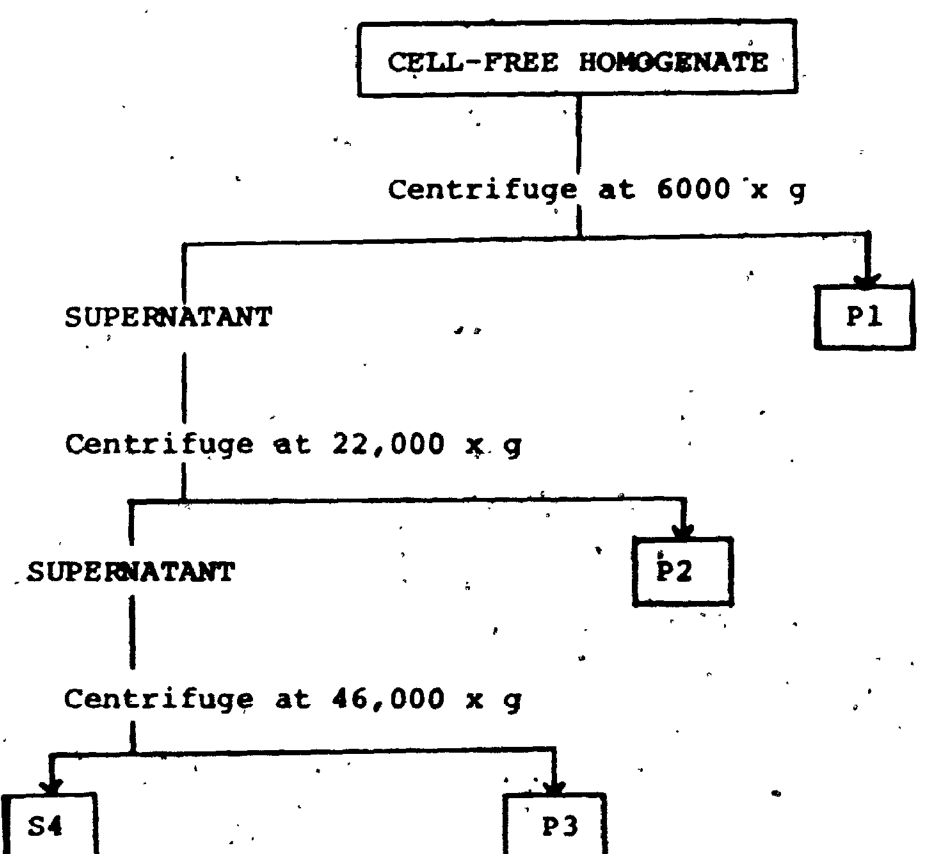
Malate dehydrogenase activity was assayed by incubating 0.2 ml of enzyme extract with 0.2 ml NADH and 0.5 ml phosphate buffer (0.25 M pH 7.4). The reaction was started by the addition of 0.1 ml of oxaloacetate (0.0076 M).

All other assay conditions (temperature, wavelengths, unit of enzyme) were as previously described for each enzyme in Section I.

With fractions S 4, enzyme extract was doubled in all assays since total volume of S 4 was twice as much as the other fractions.

FIGURE 2

DIFFERENTIAL CENTRIFUGATION FOR CELL FRACTIONS



E DRY WEIGHT DETERMINATION

Dry weight determinations of zoospore or cyst material were made for each of the enzyme and chemical assays, since enzyme activities and quantities of metabolic products were finally calculated per unit of dry spores. A portion of the spores in each of the incubation periods or stage (T1, T2 and T3) was weighed wet after 5 min draining. It was then dried in an oven at 80°C for 48 h, cooled in a desiccator and then weighed again.

Changes in dry weight with time of incubation or stage of development were also investigated. In this case all the spores for each incubation period were weighed wet, then dried as above and weighed again.

F CHEMICALS

All chemicals used in the enzyme assays, chemical analyses and cytochemical localisation studies were of reagent grade. They were obtained from Sigma Chemical Co. and Nutritional Biochemical Corp.

Epoxy resins for electron microscope studies were from Ladd Research Industries Inc.

CHAPTER 3

OBSERVATIONS AND RESULTS

A ULTRASTRUCTURAL OBSERVATIONS

I ULTRASTRUCTURE OF ZOOSPORES

The general morphology and fine structure of zoospores of *Phytophthora palmivora* are illustrated in Plates 1 to 5 and diagrammatically in Fig. 3 (for abbreviations relating to ultrastructure see page xix).

Zoospores are surrounded by a plasmalemma (plasma membrane) (Plates 1, 3 and 4). The plasma membrane is generally indented or wavy and in several places, it is pushed outwards by vesicles with granular contents (Plates 4 and 5).

Running longitudinally along one side of the zoospore is a groove (Plate 1) shallow at each end, deep in the centre (Plates 2 to 4). Below the groove is a single large vacuole, about 2 to 3 µm in diameter (Plates 3 and 4). The vacuole is in an anterior position in relation to the nucleus. The area around the groove and vacuole - the groove region - possesses certain distinctive features. Among these is the lack of organelles and few ribosomes. Another characteristic feature of the groove region is the

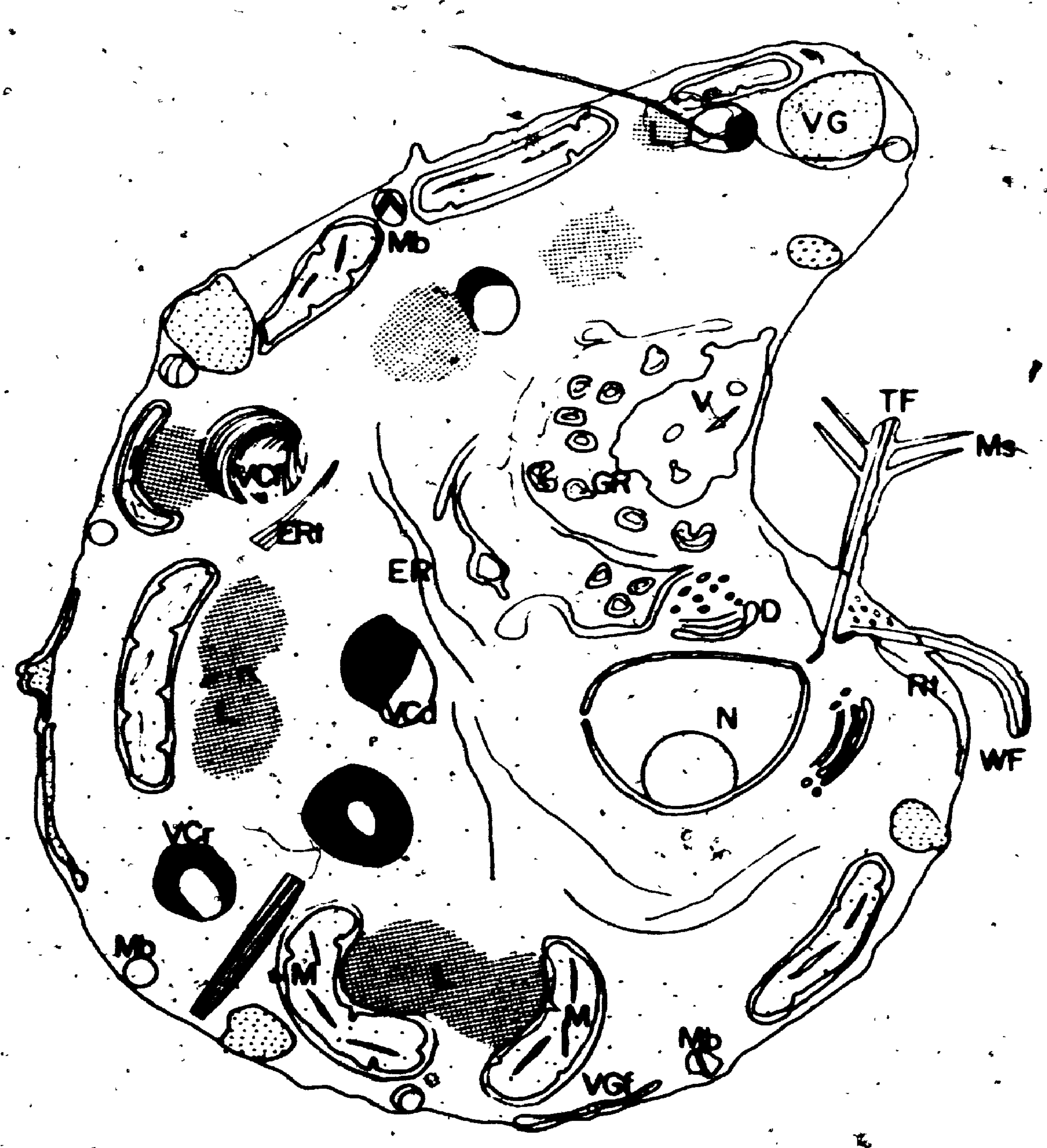
presence of several small, golgi proliferated vesicles (about 15 nm diam., bounded by single membranes); and of long, parallel membranes (with rough surfaces) which loop in several places to delimit double membrane-bound vesicles (Plates 2, 5, 11, 16, 17 and 18). The latter vesicles are about 100 to 150 nm in diameter, and are either spherical or cup-shaped (Plates 5 and 15). The smooth, inner membranes of these vesicles are thinner than the rough outer membranes (Plates 5, 15 and 17). These vesicles are almost electron-transparent. Plates 3, 5 and 15 show several vesicles at various stages of merging with the tonoplast of the contractile vacuole. This suggests that these vesicles migrate and coalesce with the vacuole (thus serving a water expulsion function).

The flagella arise from a protuberance (Plate 7) which is located in the deepest part of the groove. The flagella, one tinsel and one whiplash (Plate 12) contain the characteristic 9 + 2 fibrils, surrounded by a sheath (Plates 13 and 14) which is continuous with the plasmalemma of the zoospore body (Plates 6 and 9). The two flagella are anchored inside the zoospore by fibre bundles or rootlets. The rootlets originate from the kinetosomes (blepharoplasts) and radiate into the cytoplasm below the plasmalemma and into the protuberance (Plates 6, 7 and 8).

The large pyriform nucleus with a prominent nucleolus occupies a more or less central position in the zoospore. Its narrow end is pointed towards the kinetosomes of the flagella (Plates 9 and 10).

FIGURE 3

DIAGRAM TO ILLUSTRATE THE GENERAL STRUCTURE OF A ZOOSPORE OF *P. palmivora*.



The most prominent feature of zoospores is the occurrence of several different types of large vesicles and other inclusions (Plates 1 to 5). These are described in detail below.

Vesicles:

Several types of large vesicles are distributed throughout zoospores except in the groove region (Plates 1, 3 and 5). These vesicles are named according to the appearance of their contents in material fixed with glutaraldehyde and OsO_4 but they were also examined after $KMnO_4$ fixation. Three major types of vesicles are recognised (Fig. 4).

Amorphous "vesicles". These lack limiting membranes and contain electron-dense, evenly dispersed material (OsO_4 fixation). In $KMnO_4$ fixed zoospores, they appear empty. Amorphous "vesicles" are distributed throughout the zoospore cytoplasm. Here and there, closely adjacent vesicles coalesce (Plates 1 and 16). The vesicles range in size from 0.5 - 1.5 x 0.8 - 1.5 µm.

Crystalline vesicles. Of all the different vesicles in the zoospore, these are the most numerous and also most variable in size, ranging from 0.6 to 2.0 µm diam. Several forms of crystalline vesicles occur. Their appearance is illustrated in Plates 1 to 5 and diagrammatically in Fig. 4. The contents of these membrane-bound vesicles are highly osmiophilic and show distinct crystalline structure except when fixed in $KMnO_4$. In the latter case, they are less electron-dense and show little or no crystalline structure (Plate 10).

Four forms of crystalline vesicles are distinguished (these represent different arrangements in the lumen of the vesicle).

i) Finger-print: This form of vesicle (VCf) must have provided the historical basis for naming these vesicles "crystalline". Similar crystalline inclusions were reported in sporangia of *Phytophthora erythroseptica* (20, 101).

The osmiophilic contents display a regularity in electron-transparent zones running from one end of the vesicle to the other. These highly regular electron-transparent zones have the appearance of crystalline structures (Plates 6, 8, 15, 19 and 20). The finger-print contents occupy the lumen partially or completely (Fig. 4).

ii) Dense-mass: The contents of these vesicles (VCd) uniformly occupy the lumen as a dense osmiophilic body (Plates 1, 5 and 7 and Fig. 4). Variations of this basic form observed are those with an amorphous-looking or highly osmiophilic centre and a cortex of the finger-print form. Both types may be present (Plates 6, 8 and 19).

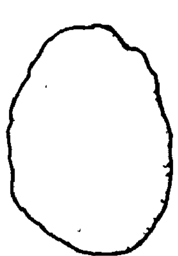
iii) Network or reticulate: The lumen of this type of vesicle (VCn) is occupied by an irregular network of highly osmiophilic material (Plates 4 and 18). At high magnifications, some of the vesicles also show the regularity of electron-transparent zones, described above for the finger-print forms (Plates 8 and 17).

iv) Ring: The osmiophilic contents form a ring lining the membrane of the vesicle (VCr). In some cases, there are

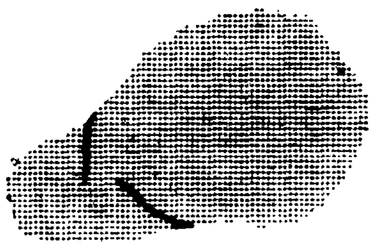
FIGURE 4

DIAGRAM TO ILLUSTRATE THE GENERAL STRUCTURE OF MAJOR VESICLES IN ZOOSPORES AND CYSTS OF *P. palmivora*.

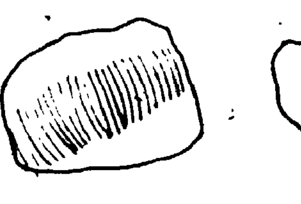
GRANULAR

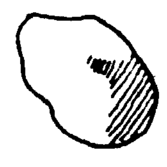


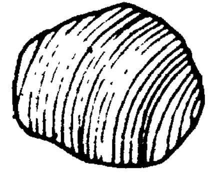
AMORPHOUS OR LIPID



CRYSTALLINE

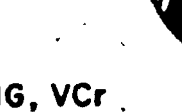




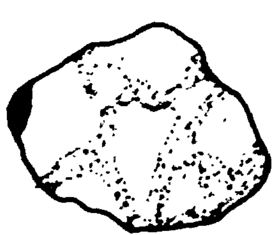


FINGER-PRINT, VCf

DENSE - MASS, VCd



RING, VCr



NETWORK, VCn

electron-transparent regions outside the ring (Plate 2). The contents are similar to those of the finger-print (Plate 6) and dense-mass forms (Plates 1, 3, 5 and 15).

Granular vesicles: These vesicles are membrane-bound structures containing evenly-dispersed, finely granular lightly staining material (Plates 3, 4, 5, 8, 15 and 19 and Fig. 4). Characteristically they are located immediately below the plasmalemma and frequently result in bulging of the zoospore surface (Plate 5). In size they range from 0.5 to 0.8 μm in diameter.

Elsewhere, below the plasmalemma several narrow flattened vesicles (VGf) about 0.6 x 2.0 μm, containing similar granular material also occur (Plates 1, 3 and 9 and Fig. 3).

Other organelles in the zoospore, described in detail below, include microbodies, mitochondria, dictyosomes and endoplasmic reticulum. These organelles undergo the most changes in size and distribution during zoospore encystment and subsequent germination.

Dictyosomes:

In most sections of zoospores, the most commonly observed dictyosome (Golgi body) lies between the nucleus and the groove region (Plates 4 and 5). Occasionally three dictyosomes were observed in sections of zoospores (Plate 10).

Endoplasmic reticulum:

Few endoplasmic reticulum (ER) cisternae were found in the cytoplasm. When present, the ER may be arranged in layers

(Plate 16) and except when associated with the nuclear envelope, ribosomes are attached to the membranes, as well as being freely distributed elsehwere in the cytoplasm. Occasionally, cisternae of expanded ER with squared ends, approximately 0.2 x 1.35 μm, are observed. These enlarged cisternae contain tubules (Plate 20) and up to about 10 such tubules may be clearly distinguished.

Microbodies:

These are densely-stained spherical bodies bound by a unit membrane. They are about 0.4 to 0.6 μm in diameter and distributed around the periphery of zoospores (Plates 1 and 2). Two types of microbodies are observed. One form contains evenly dispersed material in the matrix (Plates 1, 2 and 8), whereas the second type contains one to three "bars" of material denser than the general matrix. In the latter, the areas around the highly osmophilic bars are electron-transparent (Plates 5, 7 and 15). These densely stained bars sometimes adhere closely to the limiting membranes of the microbodies, but are distinctly thicker than unit membranes (Plates 7 and 15).

Mitochondria:

Mitochondria in zoospores are generally arranged around the periphery (Plates 1 and 2) and also in close association with crystalline and amorphous "vesicles" (Plates 1 and 5). Around the latter, curved mitochondria are frequently seen (Plate 16).

Mitochondria range in size from 0.5 - 1.0 x 0.8 - 2.0

μm, and contain thin or slender sparsely distributed cristae (Plates 5, 15 and 16). The average number of mitochondria in zoospore sections is about 12. Mitochondrial granules are scattered at random in the matrix (Plates 6, 16 and 19).

Unidentified body:

Membrane bound bodies about 0.7 μm in diameter containing ribosome-like particles occasionally occur just below the plasmalemma (Plates 5 and 17).

One of these bodies which appears to have been lost from a zoospore is illustrated in Plate 17A. This unidentified body bounded by three membranes appears to have a cortex of ribosome-like particles and an amorphous matrix.

The fate of these bodies during encystment and subsequent germination is unknown; they are seen only occasionally in sections of non-germinating cysts (Plate 26).

Ultrastructure of zoospores after 6 h motile period

The ultrastructure of zoospores after 6 h of motility is similar to that described above for freshly liberated zoospores. The fine structure of a zoospore after a 6 h motile period is illustrated in Plate 21.

PLATE 1 Longitudinal section of a zoospore, after 1 to 2 h motility, fixed in glutaraldehyde and osmium tetroxide. It shows most of the components of a zoospore. Note the groove with a small part of the vacuole below it, and ribosomes distributed throughout the cytoplasm except in the groove region (GR). Also note large nucleus (N) and nucleolus (Nu), mitochondria (M) and microbodies (Mb) around the periphery of zoospore. Note various forms of crystalline vesicles (VCf and VCr) and lipid bodies (L) throughout the zoospore except in the groove region. Observe irregularity of the plasmalemma surrounding the zoospore. x 21,000.

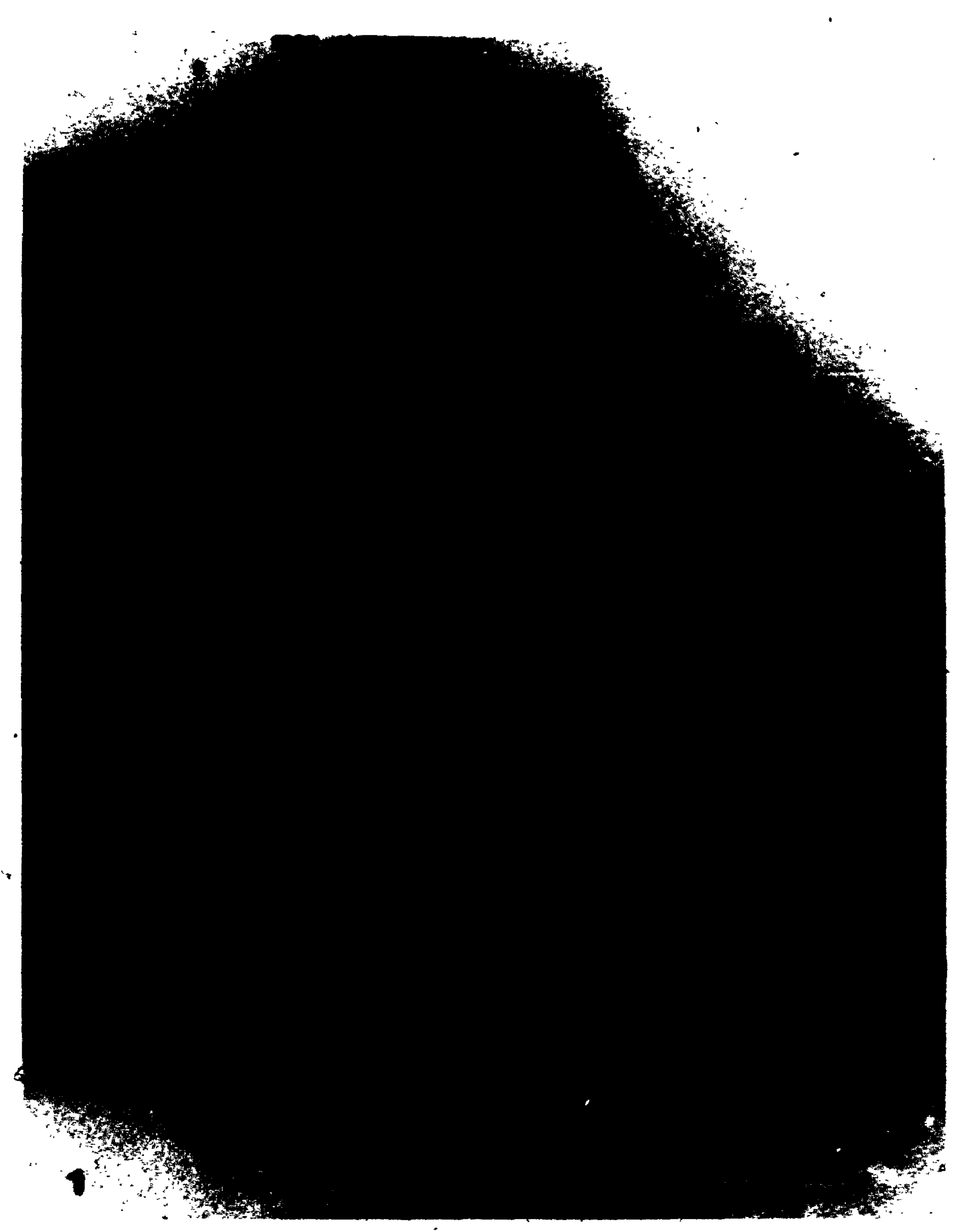


PLATE 2 Transverse section through the central part of a zoospore, after 1 to 2 h motility, showing the characteristic deep groove (G). Note mitochondria and microbodies (mostly with a uniform matrix) around the periphery of the zoospore. Observe the granular vesicles (VG) below the plasmalemma and the crystalline vesicles, mostly of the ring form (VCr). x 18,000.

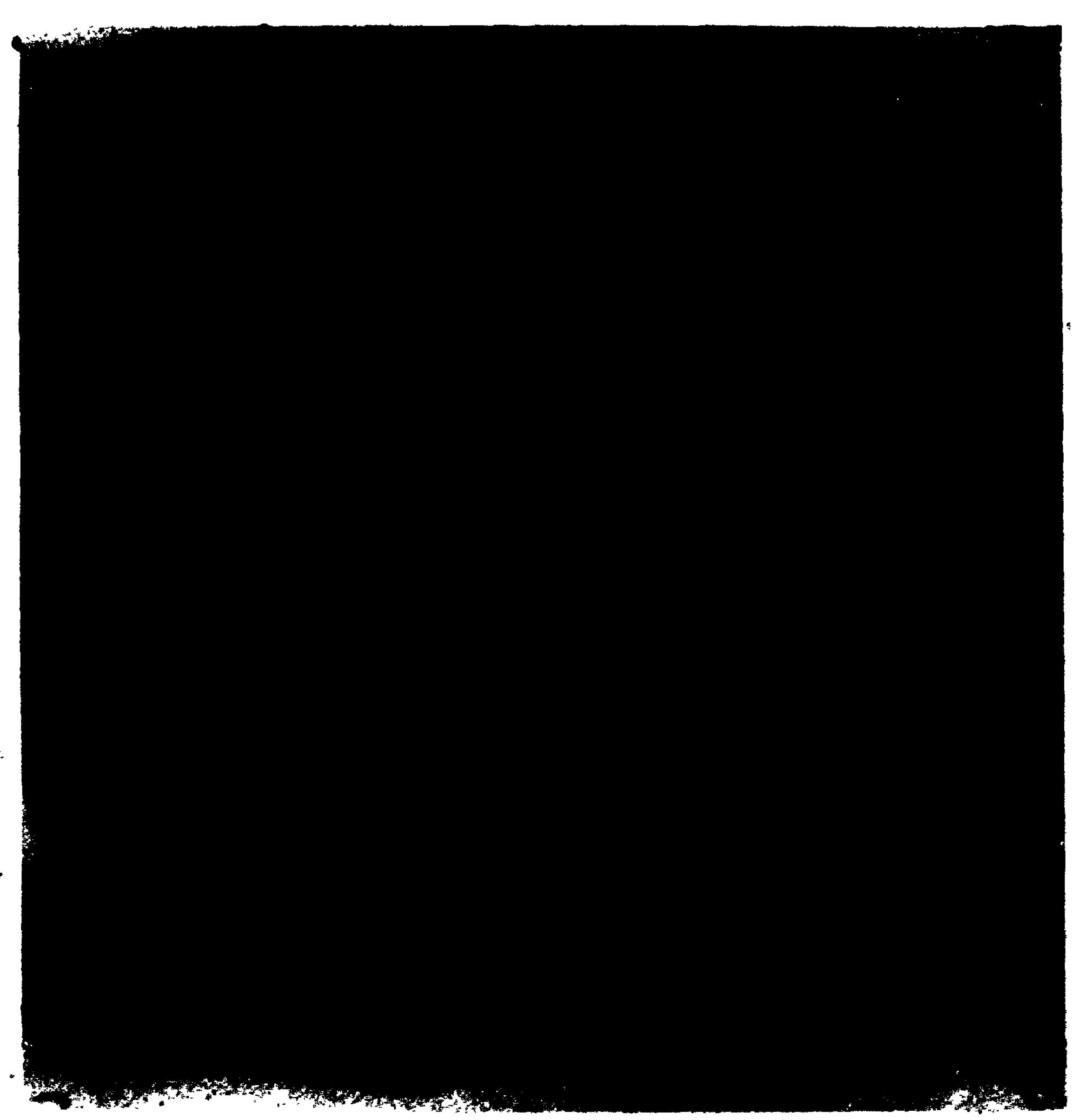


PLATE 3 Longitudinal section of a zoospore, after 1 to 2 h motility, showing a large vacuole. Note looping membranes and several small vesicles surrounding the vacuole (V) and the remains of vesicles inside the vacuole. Also note granular vesicles and flattened vesicles below plasmalemma (arrows). x 21,000.

PLATE 4 Longitudinal section of a zoospore, after 1 to 2 h motility, showing outgrowths of zoospore body overarching the groove. Note several small vesicles around the vacuole; and the dictyosome (D) below the vacuole. Granular vesicles (VG) are well-preserved and in some places plasmalemma bulges outwards. Crystalline vesicles are of the network form (VCn). (The discontinuity apparent in several areas of the cytoplasm is due to inadequate fixation and the effect of dehydration). x 18,000.

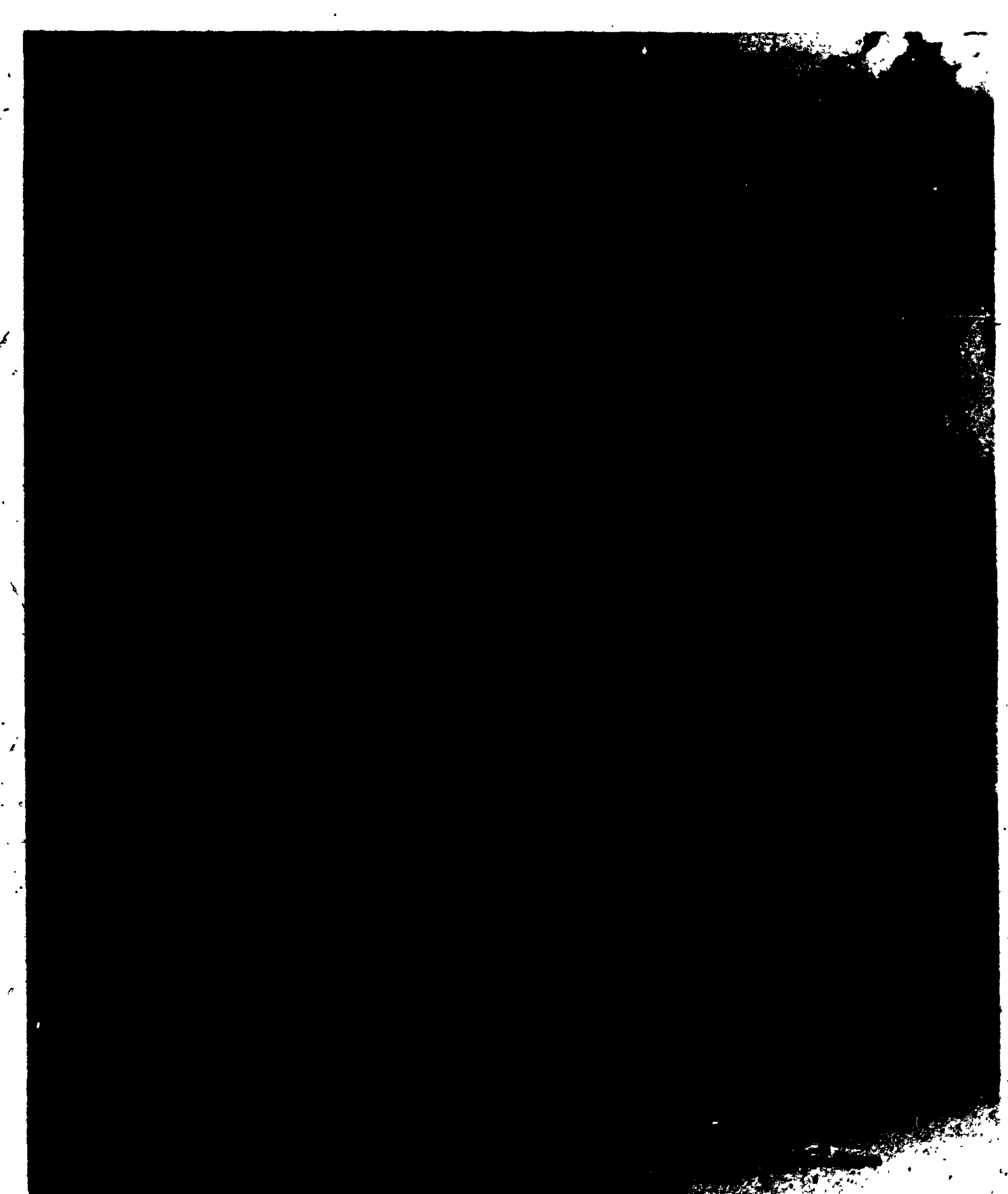


PLATE 5 Transverse section of a zoospore, after 1 to 2 h motility, with several granular vesicles projecting above the zoospore body. Crystalline vesicles are of the ring and dense-mass forms. Note numerous small vesicles (with thin inner membranes, arrows) surrounding the vacuole. There is an unidentified body (U) containing ribosome-like particles. Two forms of microbody are present. Mitochondria contain thin cristae. Note relative absence of ribosomes in the area around the vacuole. x 22,000.

7

PLATE 6 Longitudinal section through whiplash flagellum and kinetosome and a portion of the other kinetosome. Part of the protuberance in the groove, from the base of which flagella arise, is shown on the right. Note the rootlet microtubules under the plasmalemma (Rt) and microtubules running from kinetosome into the protuberance (arrows). Also note the continuity of the flagellum sheath with plasmalemma of the zoospore. Observe the finger-print structure of the dense-mass and ring forms of crystalline vesicles. x 35,000.

PLATE 7 Section of the complete protuberance showing a part of flagellum and incompletely portions of the kinetosomes. Organelles in the protuberance include microbodies (Mb), mitochondria, microtubules (arrows) and crystalline vesicles - ring and dense-mass forms. x 26,000.

F
0.5μm
VG
Cr
F
7
VG

PLATE 8 Longitudinal section through kinetosomes showing rootlet microtubules (Rt) running from the kinetosomes into the zoospore cytoplasm, below the plasmalemma and into the base of the protuberance. Note electron-dense fibre bundle connecting the kinetosomes (arrow). Crystalline vesicles are of finger-print and ring forms. Granular vesicles are well preserved. x 24,000.

PLATE 9 Section of a portion of zoospore showing orientation of the narrow end of pear-shaped nucleus towards kinetosomes. Note the prominent nucleolus and the electron-dense zone separating a less dense area at the apex of the nucleus, the nuclear cap, from the rest of the nucleus. Adjacent to the nucleus, below the plasmalemma, are rootlet microtubules and a flattened vesicle (VGf). x 25,000.

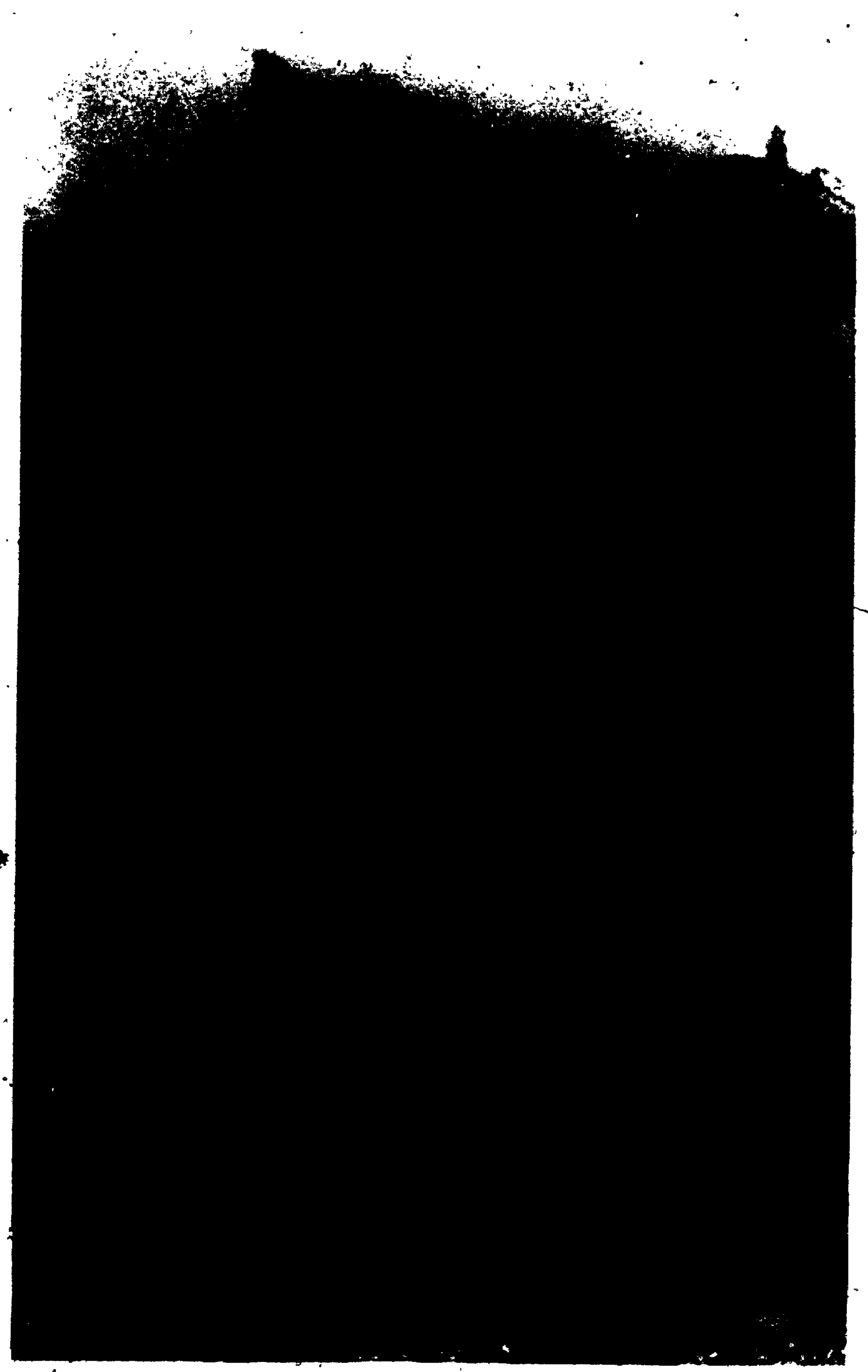


PLATE 10 Section of a zoospore fixed with potassium permanganate. Observe part of kinetosome subunit directly opposite one of the many pores (Np) in the nucleus envelope (NE). Other features of interest are the well-preserved membranes including two dictyosomes and mitochondria. Crystalline vesicles are less electron-dense with some finger-print structure still visible.

Stained with lead citrate. x 20,000.

PLATE 11 Transverse section of a portion of a zoospore showing kinetosome triplet subunits. Note looping membranes in the groove region.

x 66,000.

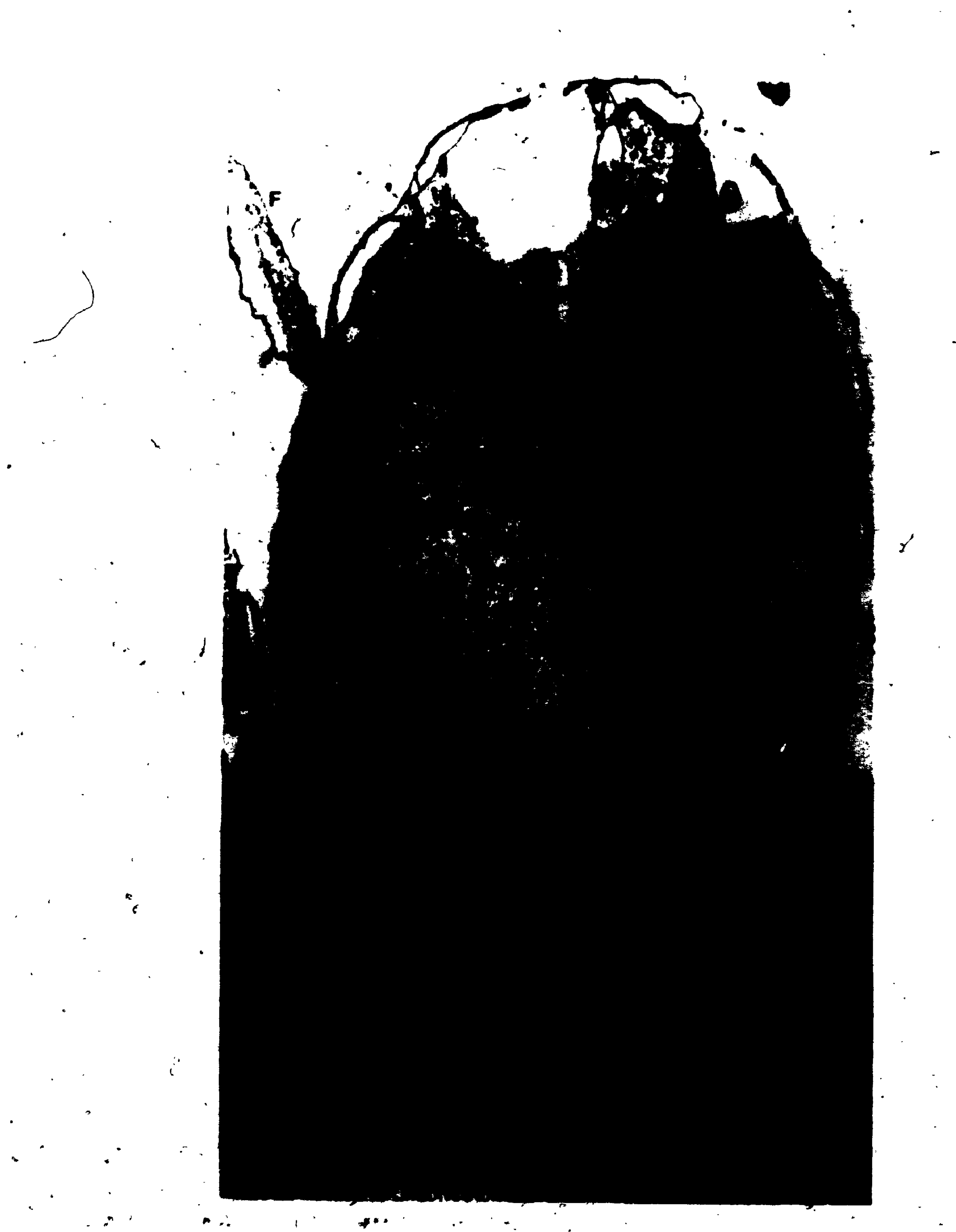

F

PLATE 12 Light micrograph of a zoospore showing the tinsel flagellum (TF) with mastigonemes and the whiplash flagellum (WF). Fixed in osmium tetroxide. Stained with Löffler's flagella stain (22). x 2,200.

PLATE 13 Transverse section of a flagellum showing two central microtubules surrounded by nine pairs of adjoined microtubules. x 138,000.

PLATE 14 Longitudinal section of the tinsel flagellum showing portions of two mastigonemes. Notice continuity of sheath membrane and membrane around the mastigonemes, which appear to lack any internal structure. x 54,000.

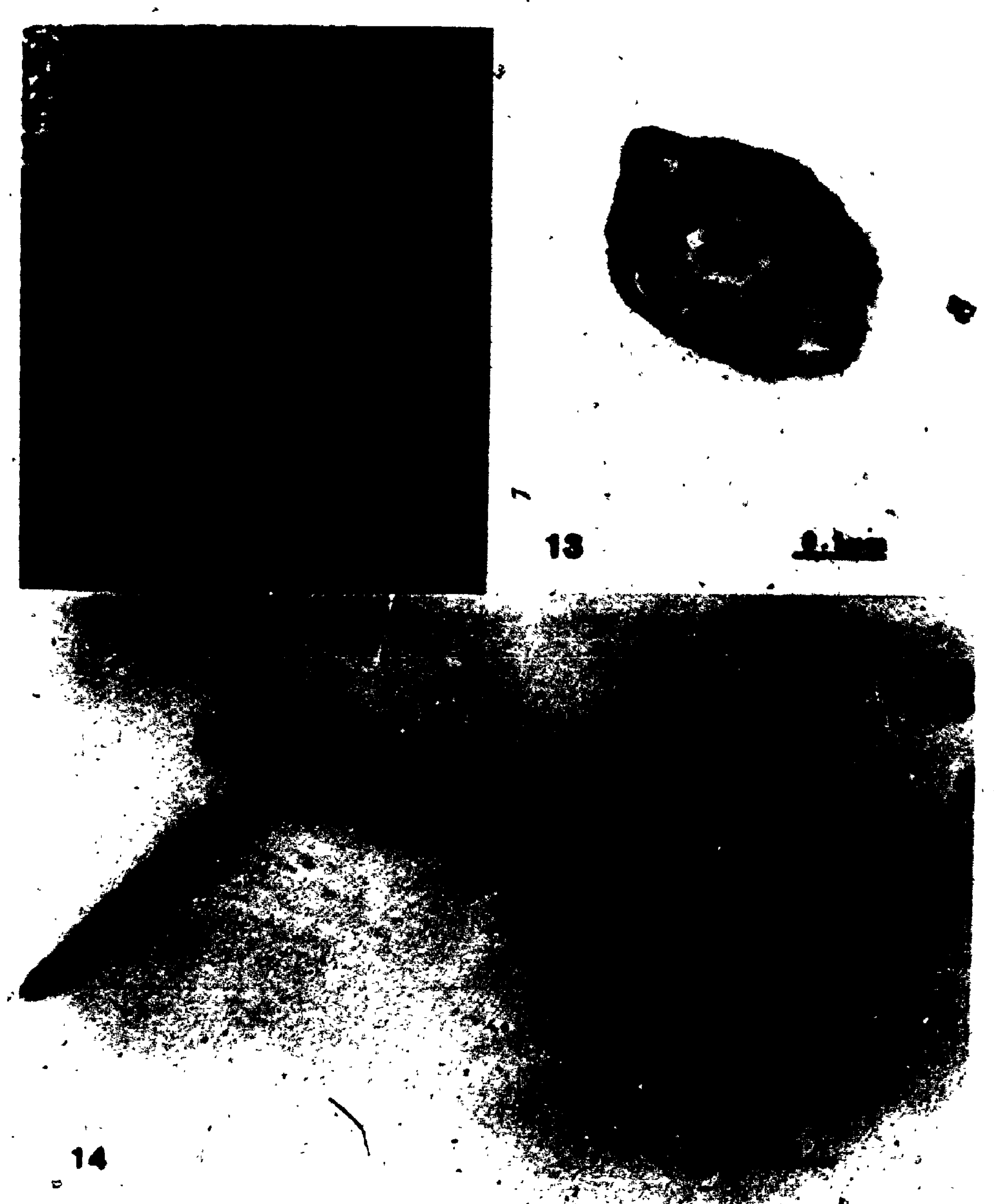

13
14

PLATE 15 Section of a zoospore showing groove region and vacuole. Note numerous vesicles around the vacuole and vesicles coalesced with tonoplast of the vacuole (arrows). Crystalline vesicles are of the dense-mass, finger-print and ring forms. All microbodies contain densely-stained material surrounded by narrow electron-transparent areas. x 23,000.

PLATE 16 Section of a zoospore showing looping membranes in the groove region. Note layers of endoplasmic reticulum (ER), coalesced lipid bodies and the long mitochondrion (associated with lipid) containing sparsely distributed, thin cristae. Also note the absence of ribosomes in the groove region (GR). x 42,000.

VG

2

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PLATE 17 Section of groove region (GR) of a zoospore showing several looping membranes. Note thin inner membranes (arrows) of double membrane-bound vesicles and the unidentified body (U) near the groove region. Crystalline vesicles are of the network and ring forms. x 34,000.

PLATE 17A Section through unidentified body lost from zoospore. Note the central amorphous matrix surrounded by ribosome-like particles and an outer envelope consisting of three distinct membranes. x 30,000.

PLATE 18 Section of a zoospore showing network forms of crystalline vesicles. Looping membranes are visible in the groove region; also note part of vacuole (V). x 24,000.

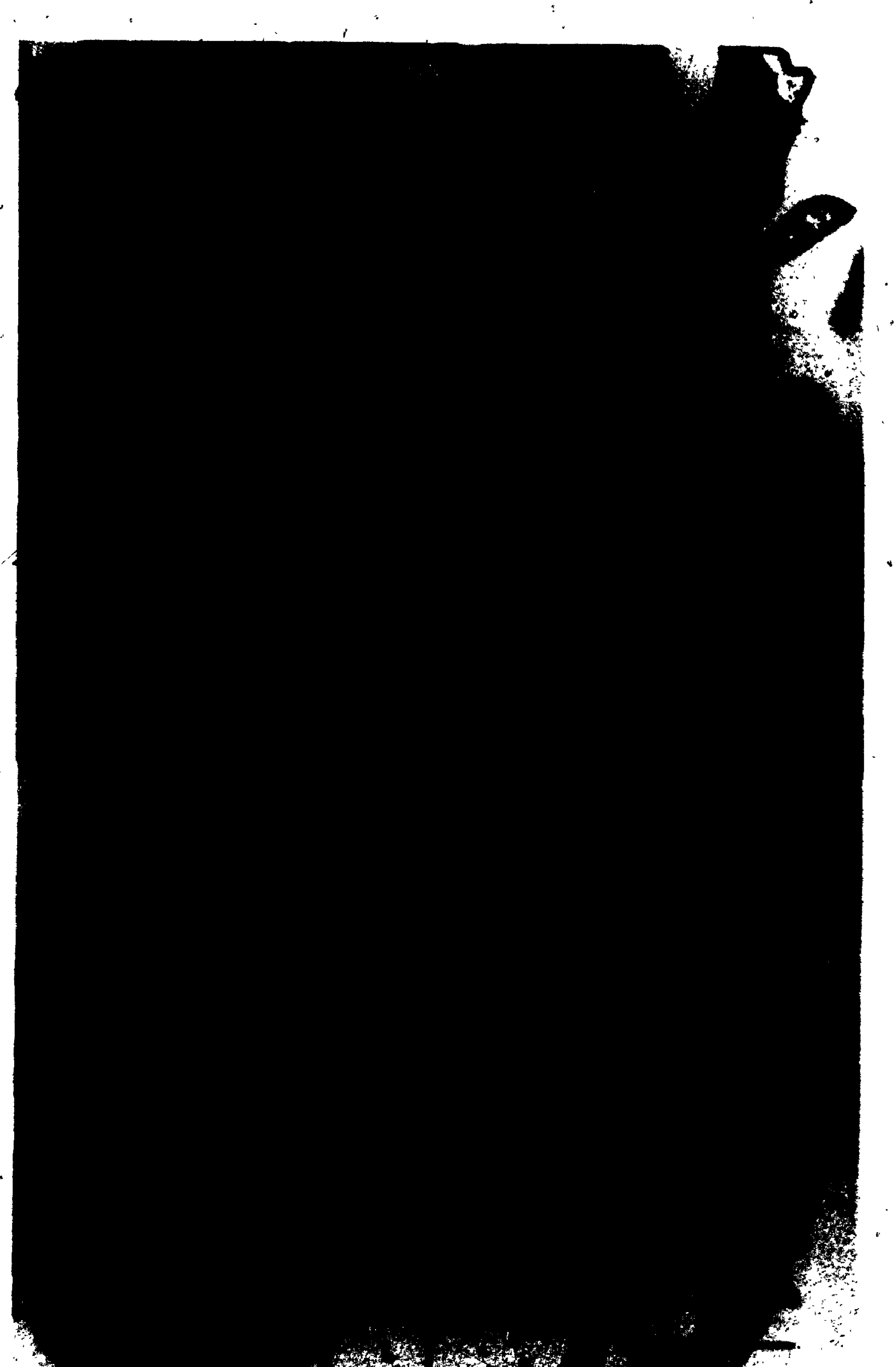
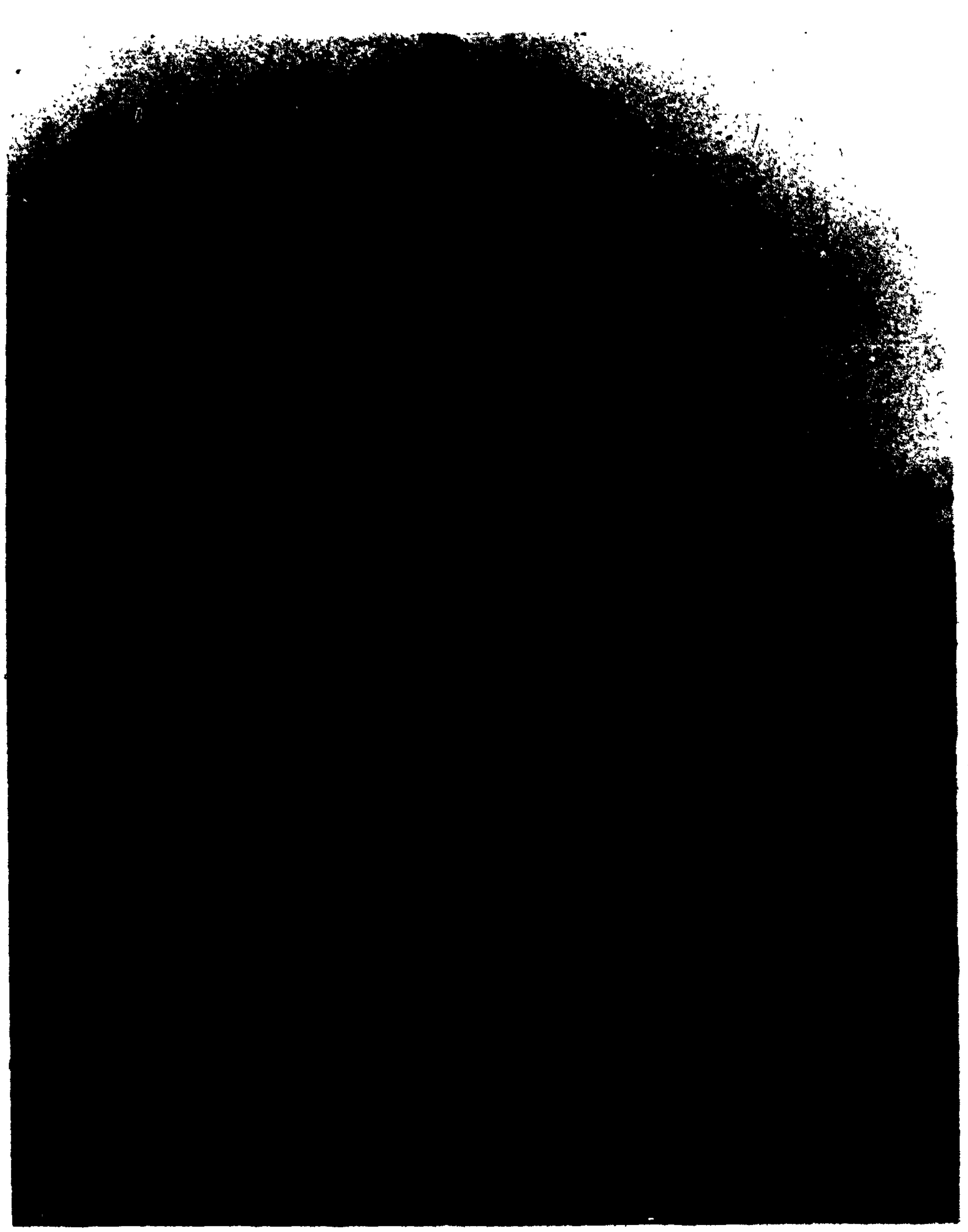


PLATE 19 Section of a portion of zoospore showing crystalline vesicles. Note all crystalline vesicles contain some finger-print structure. Also note mitochondrial granules. x 35,000.

PLATE 20 Longitudinal section of enlarged endoplasmic reticulum containing tubules. x 46,000.

PLATE 21 Longitudinal section of a zoospore, after 7 to 8 h motility, showing lipid bodies, crystalline and granular vesicles. Note that lipid bodies and vesicle contents differ little from corresponding structures after 1 to 2 h motility (see Plates 1 to 5). Also note microtubules (arrows) radiating from area of flagella origin towards centre of zoospore. x 31,000



II ULTRASTRUCTURE OF ENCYSTED ZOOSPORES

Several changes occur when zoospores of P. palmivora encyst. These include the shedding of the flagella; there is no evidence for flagella retraction in this species, i.e. no axonemes are observed in sections of newly encysted zoospores. On encystment, zoospores round up into roughly spherical cysts (about 11.74 ± 0.44 μm diam.) with well-defined walls, the groove gradually disappears and the spore loses its surface irregularities (Plate 22). Concurrent with the disappearance of the groove is the disappearance of associated internal structures - vacuole, small vesicles and microtubules. The pyriform, eccentrically placed zoospore nucleus gradually rounds up and assumes a central position. The kinetosomes regress into centrioles, closely associated with the nucleus.

The fine structural features of non-germinated cysts, up to 30 min after encystment (Plate 23 and Fig. 5), are as follows:

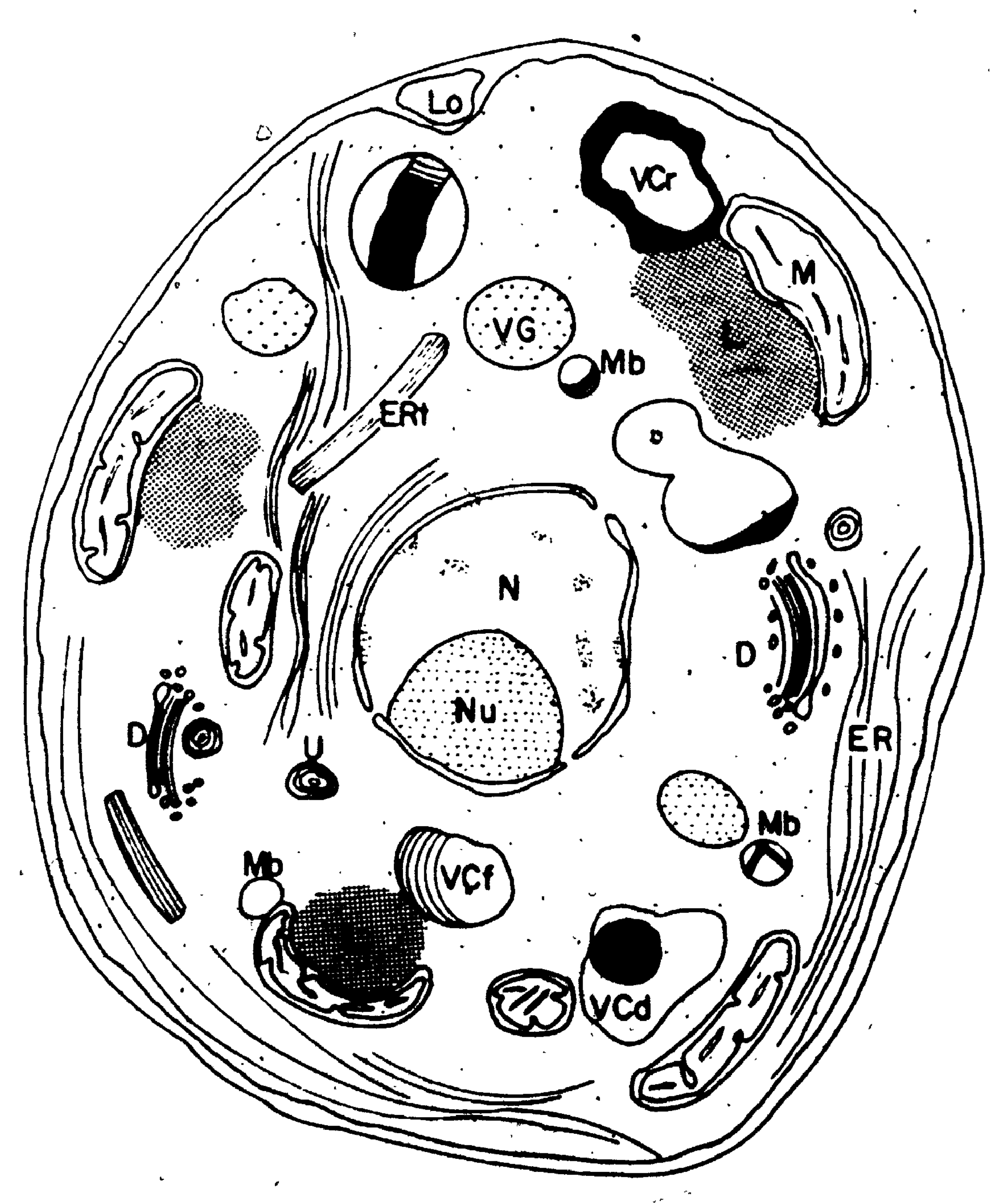
Cell wall:

The cell wall of the cyst consists of two amorphous layers. An outer less electron-dense layer is observed only in extremely well-preserved cysts. The inner layer is more electron-dense and is the only layer preserved in most cysts examined. Cell walls are about 15 to 20 nm (OsO_4 fixation). In $KMnO_4$ fixed cysts only a single electron-dense cyst wall is visible (Plate 49).

Enzymic and organic solvent digestion (p.23) demon-

FIGURE 5

DIAGRAM TO ILLUSTRATE THE GENERAL STRUCTURE OF AN ENCYSTED ZOOSPORE OF P. palmivora.



strated that the wall is composed mostly of polysaccharide with some lipid and protein.

Plasmalemma:

This is a unit membrane located immediately inside the cyst wall and, as in the zoospore, is wavy or indented; but with the formation of the cell wall, the plasmalemma loses the large protrusions characteristic of zoospores.

Lomasomes:

These aggregations of membranes in a matrix, not seen in zoospores, lie between the cell wall and plasmalemma which is pushed inwards at these points (Plate 24). The membranes are vesicular and some appear to be continuous with the plasmalemma.

Nucleus:

The nuclei of cysts are roughly spherical, with a large electron-dense nucleolus. The nucleus in Plate 23 is still pear-shaped as in the zoospore. Chromatin is scattered throughout the nucleus and is also attached to the nuclear envelope.

Dictyosomes:

At least three dictyosomes are observed in sections of cysts, about 30 min after encystment. These are either adjacent to the nucleus or occur elsewhere in the cytoplasm (Plate 23). Several small golgi proliferated vesicles surround the dictyosomes (Plates 23 and 24). Closely adjacent to dictyosomes, and also to endoplasmic reticulum, are organelles of unknown nature containing several concentric

rings (Plates 23 and 27). Some of these organelles are also found elsewhere in the cytoplasm, close to the major types of vesicles and to mitochondria (Plate 23).

Endoplasmic reticulum:

Endoplasmic reticulum (ER) present as layers of cisternae, increases during the 30 min post-encystment period. Several layers of ER occur throughout the cytoplasm and also around the nuclei (Plates 23 and 49). Plate 50 shows a connection between ER and the nuclear envelope in a cyst.

The number of enlarged ER cisternae with internal tubules show no increases in cysts, compared to what was observed in freshly liberated or immediately encysted, zoospores.

Mitochondria:

Mitochondria in newly encysted zoospores are similar to those described in motile zoospores, both in their form and in their peripheral location (Plate 22). However, in older (30 min) cysts, they are more variable in size, ranging from 0.5 - 0.7 x 0.7 - 2.1 μm, and there is a slight increase in their number probably associated with the onset of cyst germination. About 17 were counted in various sections of non-germinating cysts (30 min post-encystment) compared to about 12 in motile zoospores. The shapes ranged from round to ellipsoidal to dumbell. Some of the latter, strongly curved, appear as in Plate 24 to have a doughnut appearance with cytoplasmic material in the centre.

About 30 min after encystment, mitochondria are distributed throughout the cytoplasm (Plate 23) and adjacent

to the major types of vesicles. There are more cristae than in mitochondria in zoospores. The cristae are mostly thin folds, with some tubular forms.

<u>Microbodies</u>:

Like mitochondria, the microbodies in non-germinating cysts are no longer restricted to the periphery of the cells but are distributed throughout the cytoplasm and close to mitochondria and the major vesicles (Plates 23 and 26). Both forms (p. 54) are observed in cysts.

<u>Vesicles</u>:

All the major vesicle types seen in zoospores are also observed in cysts. No recognisable changes in their contents had occurred after 6 h motility.

<u>Amorphous "vesicles"</u>. In newly encysted zoospores, amorphous vesicles are similar in size to those present in zoospores. However, with time (about 30 min following encystment) adjacent vesicles coalesce forming larger ones while other, smaller vesicles are located elsewhere in the cytoplasm (Plates 23 and 24).

<u>Crystalline vesicles</u>. These show little or no change in their location or contents after 30 min encystment.

<u>Granular vesicles</u>. These remain peripheral in newly encysted zoospores (Plate 22) but in older (30 min) cysts, they are distributed throughout the cytoplasm (Plates 23 and 24).

The elongated, flattened granular vesicles just below the zoospore plasmalemma, remain for a short time after encystment (Plate 22) but disappear after about 30 min (Plate

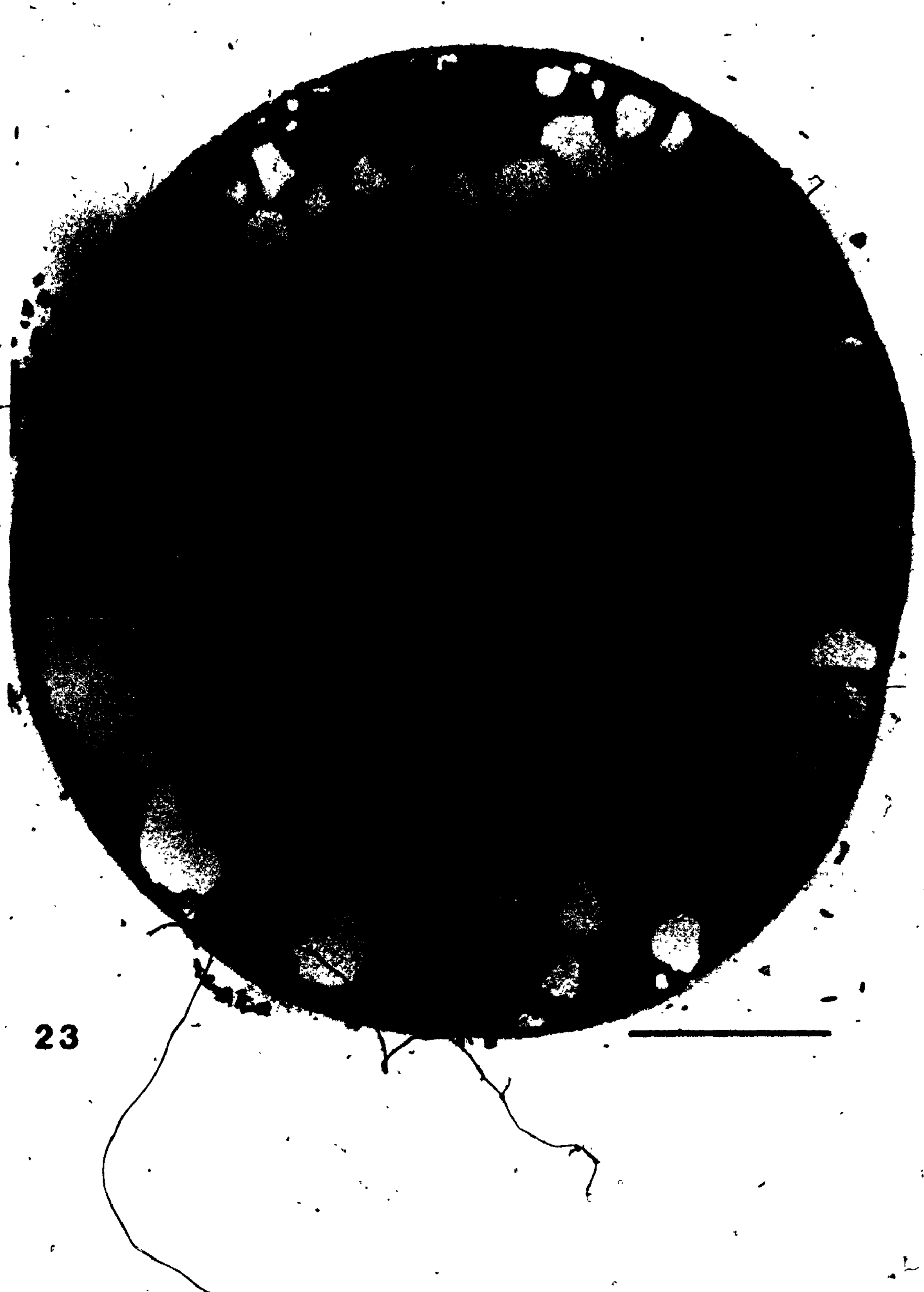
23).

Other vesicles. After about 30 to 45 min following encystment, in preparation for germination, a cluster of several small vesicles accumulates at a particular point below the surface of the cyst and at this point, the germ tube subsequently emerges. Most of these vesicles are almost electron-transparent but some contain finely granular electron-dense material (Plate 24).

PLATE 22 Section of a cyst, 10 to 15 min after encystment. Note granular vesicles and flattened vesicle (arrow) just below the plasmalemma (P). Also note the presence of the vacuole, lipid bodies, crystalline vesicles and mitochondria in positions similar to those they occupy in the motile zoospore (see Plates 1 to 5). x 23,000.

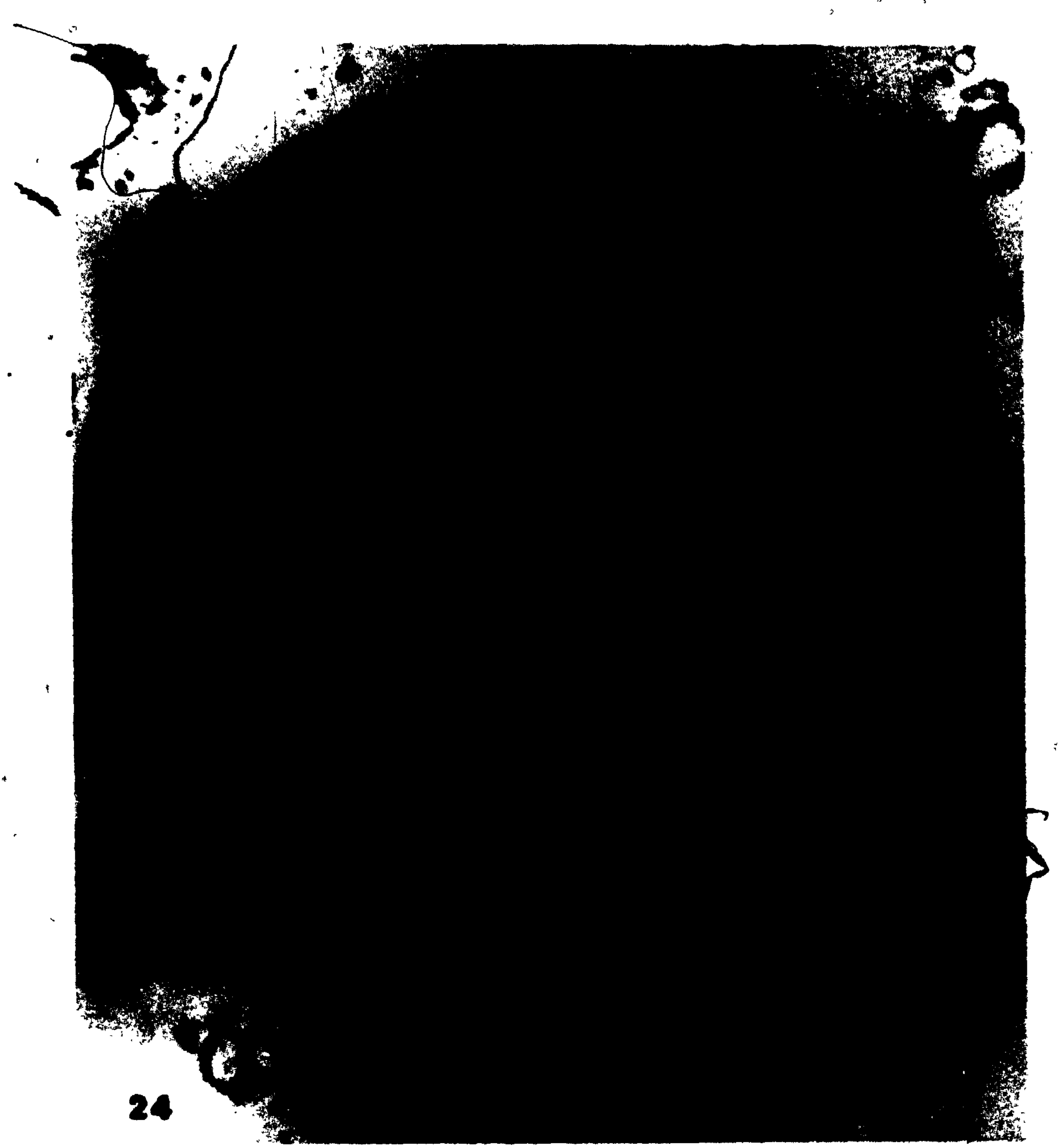
P
V

PLATE 23 Transverse section of a cyst, approx. 30 min after encystment. Granular vesicles are distributed throughout the cyst, endoplasmic reticulum has increased and three dictyosomes are present in the section. Note bodies with internal concentric membranes (arrows) and the association of two of these bodies with dictyosomes, to the left of the nucleus. x 25,000.



23

PLATE 24 Transverse section of a cyst, 30 to 45 min after encystment, showing a cluster of small vesicles below the plasmalemma, some of which contain finely granular electron-dense material. Note numerous mitochondrial cristae and "doughnut" mitochondrion. Also note that several lipid bodies have coalesced to form a large body (L). x 22,000.



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III ULTRASTRUCTURE OF GERMINATING CYSTS

Encysted zoospores were studied for varying periods from about 45 min to 2 h following encystment and incubation in distilled water. The 45 min to 2 h post-encystment or incubation period covers the period from the accumulation of small vesicles, which occurs after about 30 min, to the actual emergence of the germ tube.

During the early stages of germination (45 min), the organelles and inclusions in cysts were similar to those described for 30 min non-germinating cysts (pages 82 to 87). However, after about 1 h, when recognisable germ tubes have formed, distinct changes occur within the cysts (Figs. 6 and 7).

Germ tubes:

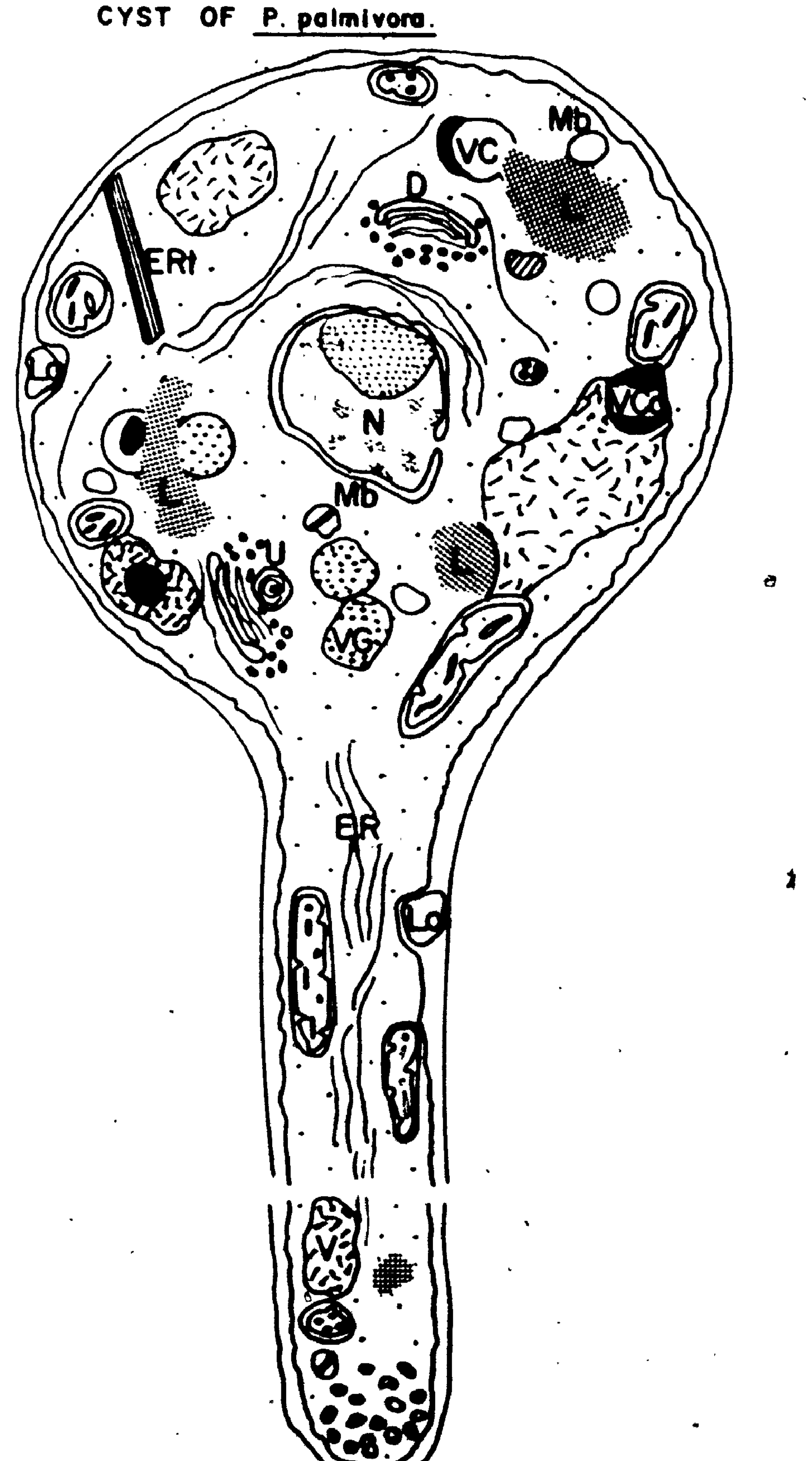
As already described (p. 87), germ tube formation is preceded by the development of a cluster of small vesicles at a particular point below the surface of the cyst wall. Following this, the germ tube emerges and the cluster of small vesicles remains at the tip of the germ tube (Plate 25) as it continues to elongate, and is followed by cytoplasm containing organelles and inclusions from the cyst (Plate 45).

Dictyosomes:

During the 45 min to 1.5 h post-encystment period (i.e. 15 min - 1.0 h germination), the number of dictyosomes in sections of germinating cysts increases. In several germinating cysts, up to about four dictyosomes are observed

FIGURE 6

DIAGRAM TO ILLUSTRATE THE GENERAL STRUCTURE OF GERMINATING CYST OF P. palmivora.



(Plate 28). Several Golgi-proliferated vesicles surround the dictyosomes (Plates 25, 28, 29 and 32). Plates 41 and 43 show a distinctly different structure, with internal concentric membranes, which occurs frequently adjacent to dictyosomes and to ER (Plates 28 and 32) in germinating cysts (see 30 min cyst, Plate 23).

Cell walls:

The cell walls of cysts and germ tubes are continuous (Plates 44 and 45). Germ tube walls are also composed of an outer less electron-dense and an inner more dense layer (Plate 45), similar to cell walls of non-germinating cysts. However, germ tube walls are slightly thicker (Plates 44 and 45) than cyst walls, about 30 nm and up to 20 nm diam., respectively.

Endoplasmic reticulum:

There is continued increase in ER, from the 45 min to 1.5 h post-encystment period, (Plates 25, 28, 29 and 32). During the same period, there is no increase in expanded ER cisternae containing tubules. Plate 38 shows a cross section through two ER cisternae with internal tubules.

After about 1.5 h period, there is little further increase in ER.

Lomasomes:

During germination, lomasomes increase in number and can be seen between the plasmalemma and walls of germ tubes (Plate 45) as well as in the cysts. These lomasomes are similar to those in non-germinating cysts (Plate 24).

Microbodies:

Three forms of microbody occur in germinating cysts. Two of these are similar to those observed earlier in zoospores and non-germinating cysts, namely those with amorphous matrix and/or densely staining bars (Plates 28, 29, 32, 41 and 42). A third form of microbody possesses both densely staining bars and fine striations or lamellae (Plates 39 and 40).

Microbodies remain closely associated with mitochondria and also with the major types of vesicles (described earlier), whose contents are now breaking down (Plates 28, 31, 39 and 41).

Mitochondria:

The number of mitochondria in various sections of germinating cysts is not substantially different from those in non-germinating cysts. There are about 20 in each section. Though some long mitochondria are observed (Plates 28, 29 and 46), in contrast to non-germinating cysts, small mitochondria about 0.7 μm diameter are most common (Plates 30, 31 and 47). The number of cristae increases and they are almost exclusively of the swollen form (Plates 28, 29, 45 and 46) compared to the sparsely distributed thin forms of zoospore mitochondria.

During germination, mitochondria remain adjacent to the amorphous, crystalline and granular vesicles (Plates 28, 29, 32 and 41) and microbodies.

Nucleus:

Nuclei of germinating cysts divide (Plate 44) when the germ tubes are about 20 μm long. By 2 h after encystment (i.e. 1.5 h germination), both nuclei and cytoplasm have moved into the young hypha.

Vesicles:

Amorphous "vesicles". As germination proceeds, adjacent "vesicles" continue to coalesce forming larger bodies. They occur in close association with crystalline and granular vesicles (Plates 28, 29, 30 and 31) and when adjacent to each other, no membranes separating them can be observed (Plates 28, 30 and 31).

After about 30 min germination (1 h post-encystment), the contents of amorphous "vesicles" start to break down (Plates 28, 30 and 31) and this process increases with time (Plate 47). The breakdown products are electron-dense, network-like, scattered throughout the lumen of amorphous "vesicles" and central vacuoles (p. 99) in cysts (Plates 30, 31, 46, 47 and 48).

By 2 h after encystment (approx. 1.5 h after beginning of germination), little of the contents of these vesicles remain (Plate 48).

Crystalline vesicles. The contents of these vesicles begin to break down in germinating cysts about 30 min after germination starts. The electron-dense breakdown products appear very similar to those of the amorphous vesicles (Plates 31 and 44).

Dense-mass, electron-dense contents are subsequently observed in vacuoles of germ tubes and these are believed to be the remains of contents of crystalline vesicles.

Granular vesicles. In contrast to the other vesicles, the breakdown of contents of granular vesicles is characterised by their disappearance. The disappearance of granular contents begins about 30 min to 1 h after germination has started (Plate 32). By 1.5 h after germination has begun (2 h post-encystment) granular vesicles are completely empty. Subsequently, the vesicle membranes also disappear (Plates 44 and 46).

Central vacuole:

As a result of breakdown of vesicles and movement of materials into the young hypha, described above, a virtually empty space forms inside the cyst, gradually enlarging with time (Plates 46 to 48 and Fig. 7). Most of the cysts examined after 1.5 h of germination contain only peripheral pockets of cytoplasm (Plate 48).

Unidentified bodies:

Plates 33 to 36 show several types of unidentified organelles which are observed in germinating cysts. The multivesicular body in Plate 34 was frequently observed in germinating cysts about 30 min after germination had started (see also Plate 30).

FIGURE 7

DIAGRAM TO ILLUSTRATE THE GENERAL STRUCTURE OF A CYST OF P. palmivora, (1.5h. germination).

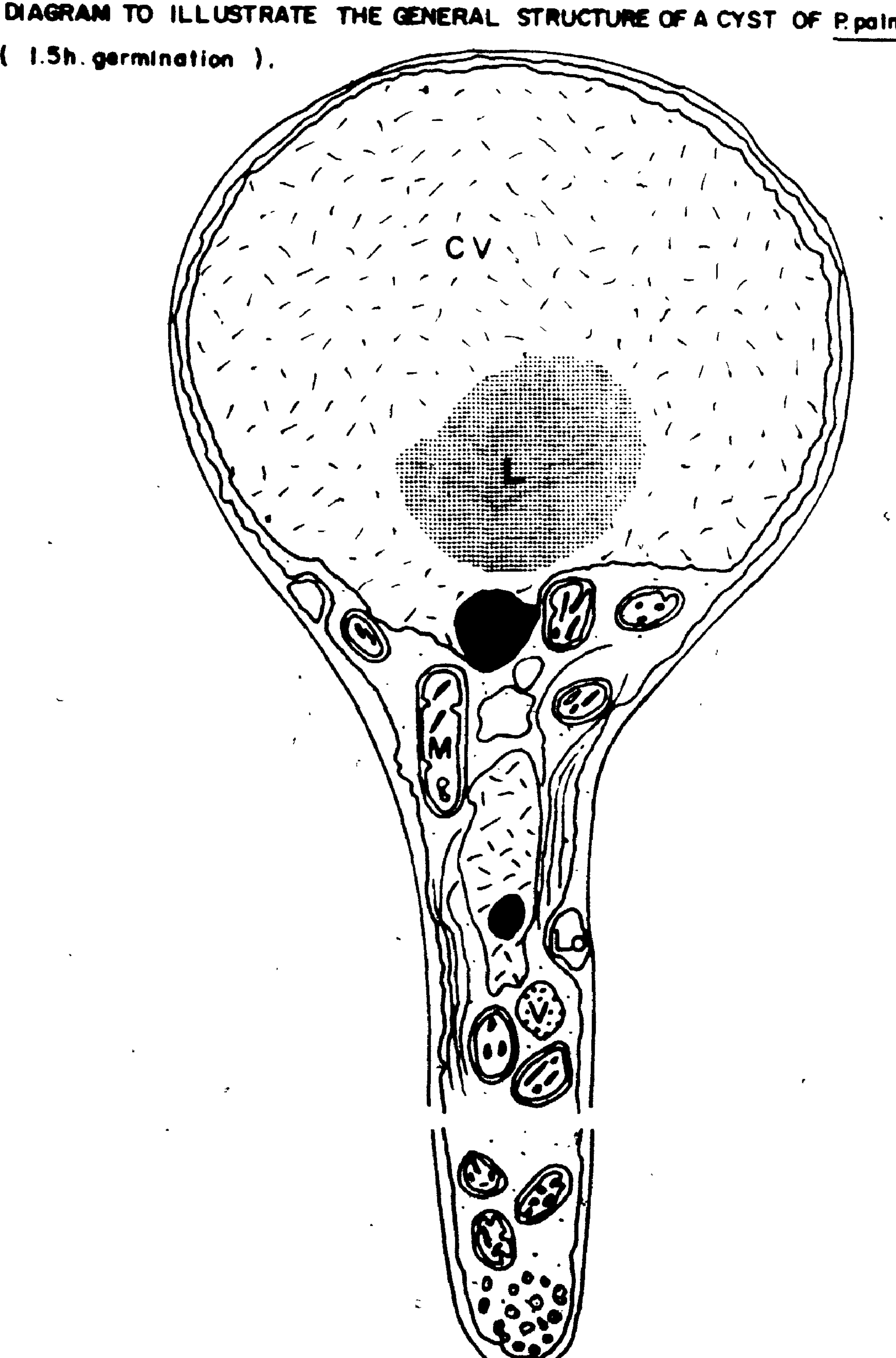


PLATE 25 Section of a germinating cyst, 45 min to 1.0 h after encystment (i.e. 15 min after germination has begun). Note numerous small vesicles at the tip of the emerging germ tube (also see Plate 24); and two dictyosomes with several associated vesicles. x 25,000.

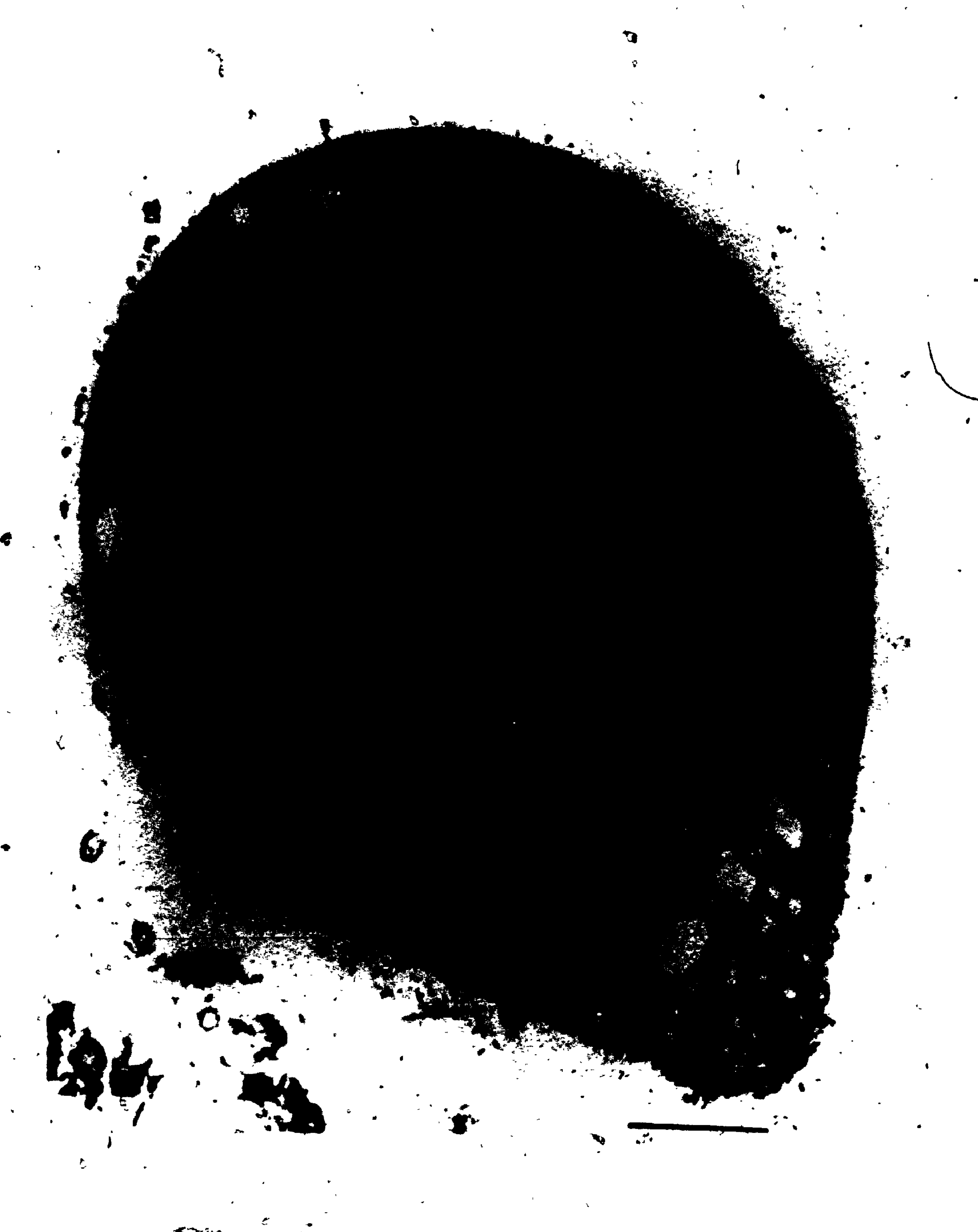
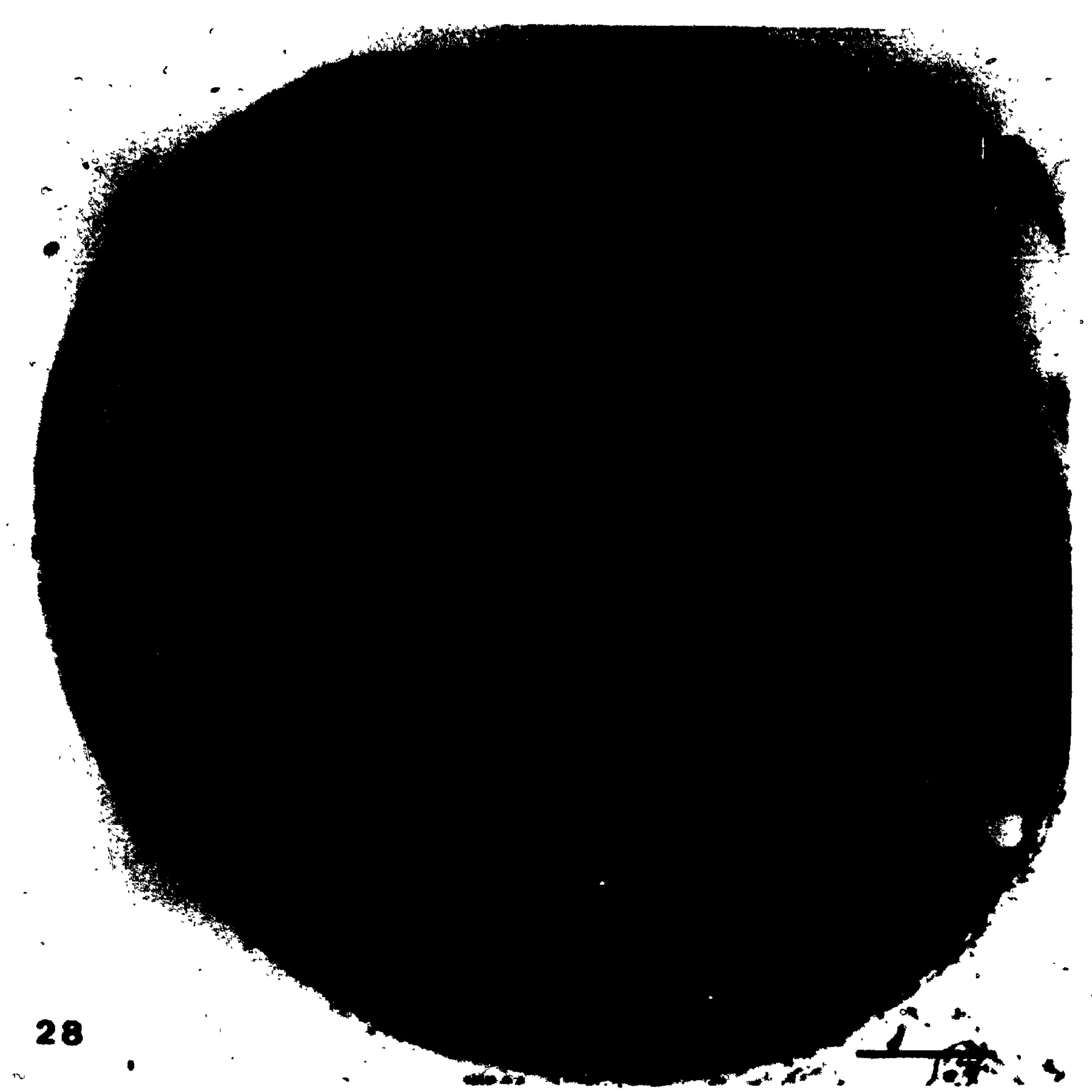


PLATE 26 Section of unidentified body and microbody in a cyst, 30 to 45 min after encystment. The unidentified, membrane-bound body possesses a cortex of ribosome-like particles and an amorphous matrix. x 53,000.

PLATE 27 Section of an unidentified body containing several membranes, in a cyst 30 to 45 min after encystment (see Plate 23). x 67,000.

PLATE 28 Transverse section of a germinating cyst, section does not include germ tube, 1.0 h after encystment (i.e. 30 min after germination started). Endoplasmic reticulum is greatly increased (compare with Plates 22 to 25), mitochondrial cristae are numerous and inflated and crystalline vesicle contents have started to disintegrate. Microbodies with densely stained material and mitochondria are adjacent to vesicles. Note dictyosomes and associated vesicles. x 20,000.



28

PLATE 29 Transverse section of a germinating cyst, germ tube not visible, about 1.0 h after initiation of encystment (i.e. 30 min after germination started). Note the contents of crystalline vesicles beginning to break down. Four dictyosomes surrounded by vesicles are seen in this section. The distribution of mitochondrial cristae is denser than in motile zoospores (Plate 19) or cyst, 30 min after encystment (Plate 23). Mitochondria are adjacent to lipid bodies and crystalline vesicles. x 22,000.

PLATE 30 Transverse section of a germinating cyst, germ tube not included, 1.5 h after encystment (i.e. 1.0 h after germination commenced). Note the absence of membranes between closely adjacent crystalline vesicles and lipid bodies. Also note mitochondria adjacent to disintegrating lipid and crystalline vesicles; and the enlarged endoplasmic reticulum (ERt). x 20,000.

30

PLATE 31 Transverse section of a germinating cyst, germ tube not visible, 1.5 h after encystment (i.e. 1.0 h after germination commenced). Note the similarity between breakdown products of crystalline vesicles (VCd) and lipid bodies (L). Also note mitochondria adjacent to disintegrating lipid and crystalline vesicles. x 21,000.

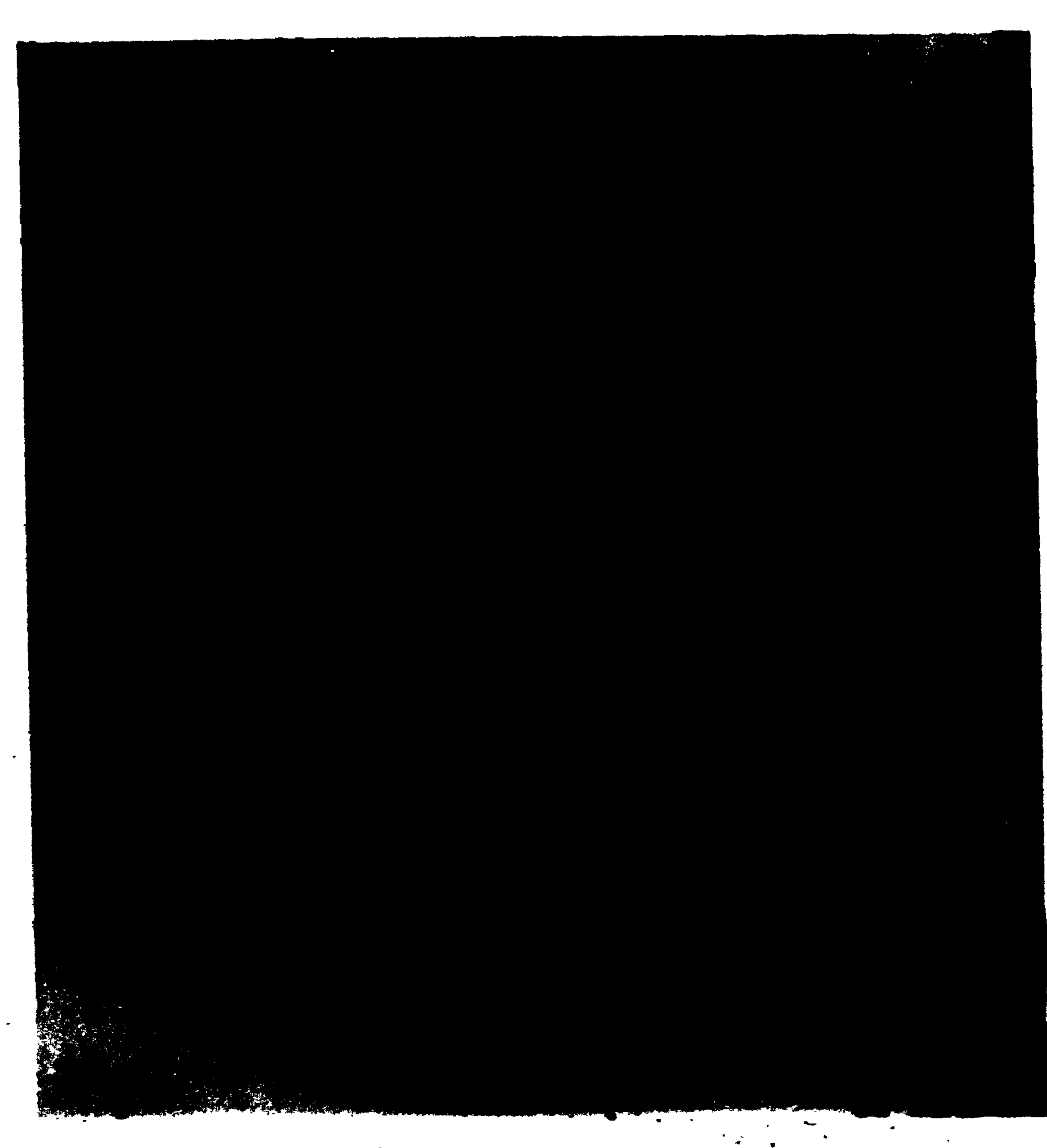


PLATE 32 Transverse section of a cyst, germ tube not shown, 1.5 h after encystment (i.e. 1.0 h after germination has begun). The contents of granular vesicles have almost all disappeared. Note numerous mitochondria in section. x 21,000.

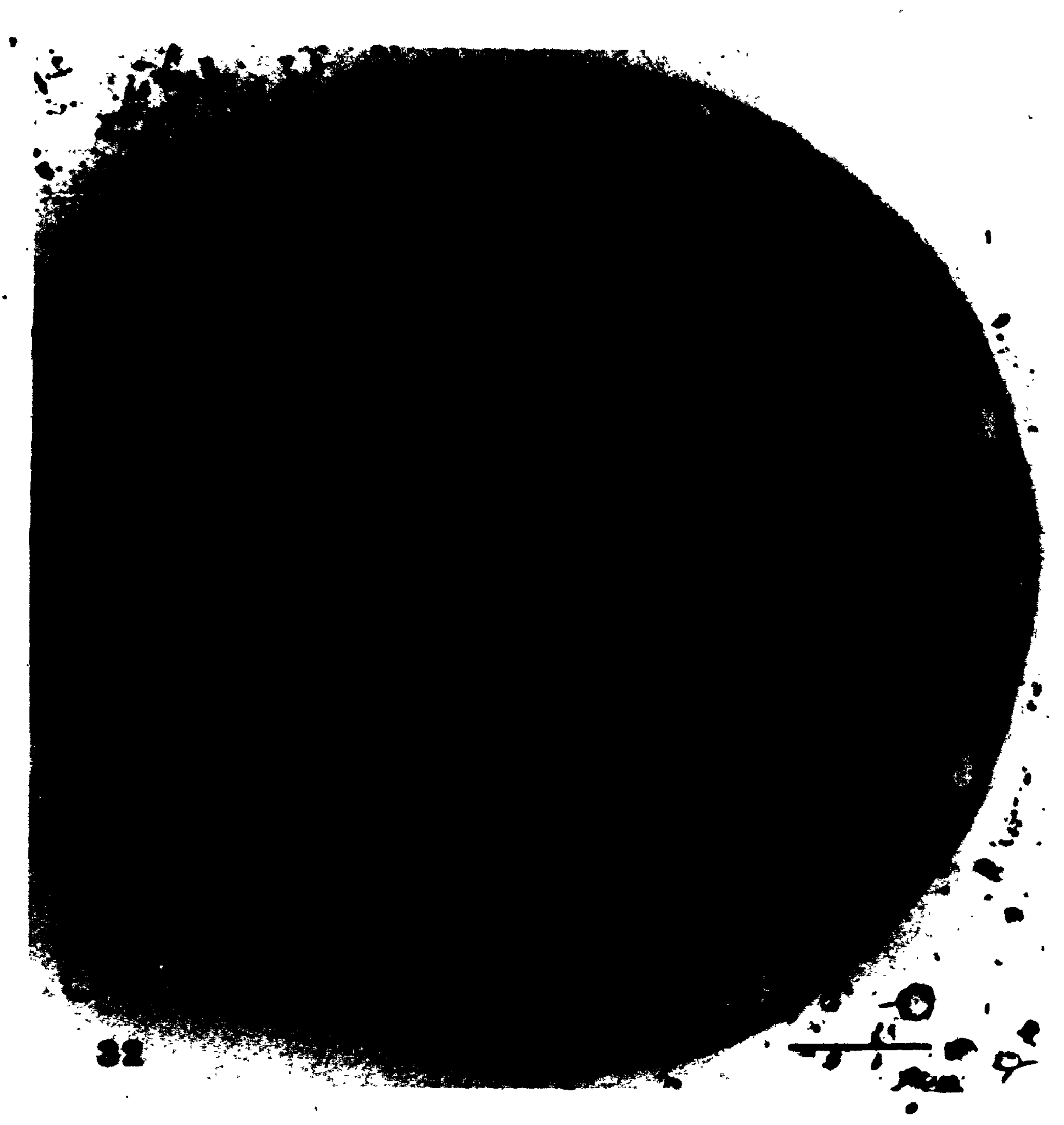

32

PLATE 33 - 36 Ultrastructure of unidentified bodies in germinating cysts.

The body in Plate 33 is bound by a double membrane with an electron-dense matrix. x 70,000.

Plate 34 shows a multivesicular body frequently observed in germinating cysts. x 70,000.

Unidentified body in Plate 35 contains concentric membranes and a granular matrix. x 38,000.

The body in Plate 36 is adjacent to endoplasmic reticulum. x 38,000.

PLATE 37 Longitudinal section of enlarged endoplasmic reticulum containing poorly defined material, in a germinating cyst. x 35,000.

PLATE 38 Transverse section of enlarged endoplasmic reticulum containing well-preserved tubules (ERt) in germinating cyst. x 44,000.

PLATES 39-40 Ultrastructure of microbodies in germinating cysts. Three types of microbodies are represented here, one with a uniform matrix, one with a ring of densely-stained material and two with a combination of densely-stained material and fine striations or lamellae. x 18,000.

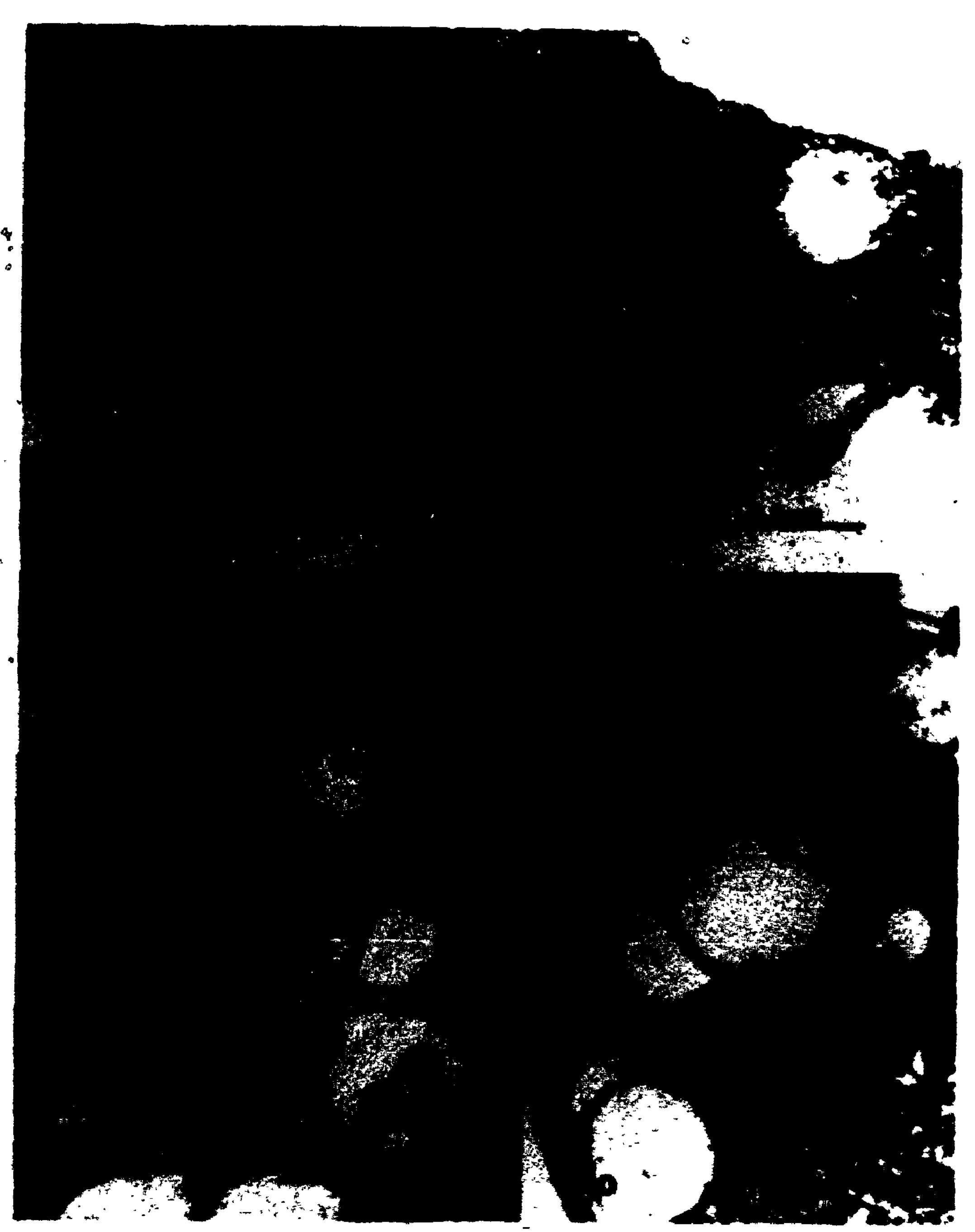


PLATE 41 Portion of a section of a cyst showing type of association generally observed between mitochondria, microbodies, lipid bodies and vesicles. Note unidentified body with internal membranes. x 100,000.

PLATES 42-43 Sections of microbody with bars and unidentified body with internal membranes, from germinating cysts. Note similarity between the outer membranes of the unidentified body and those of the dictyosome (D). x 100,000.

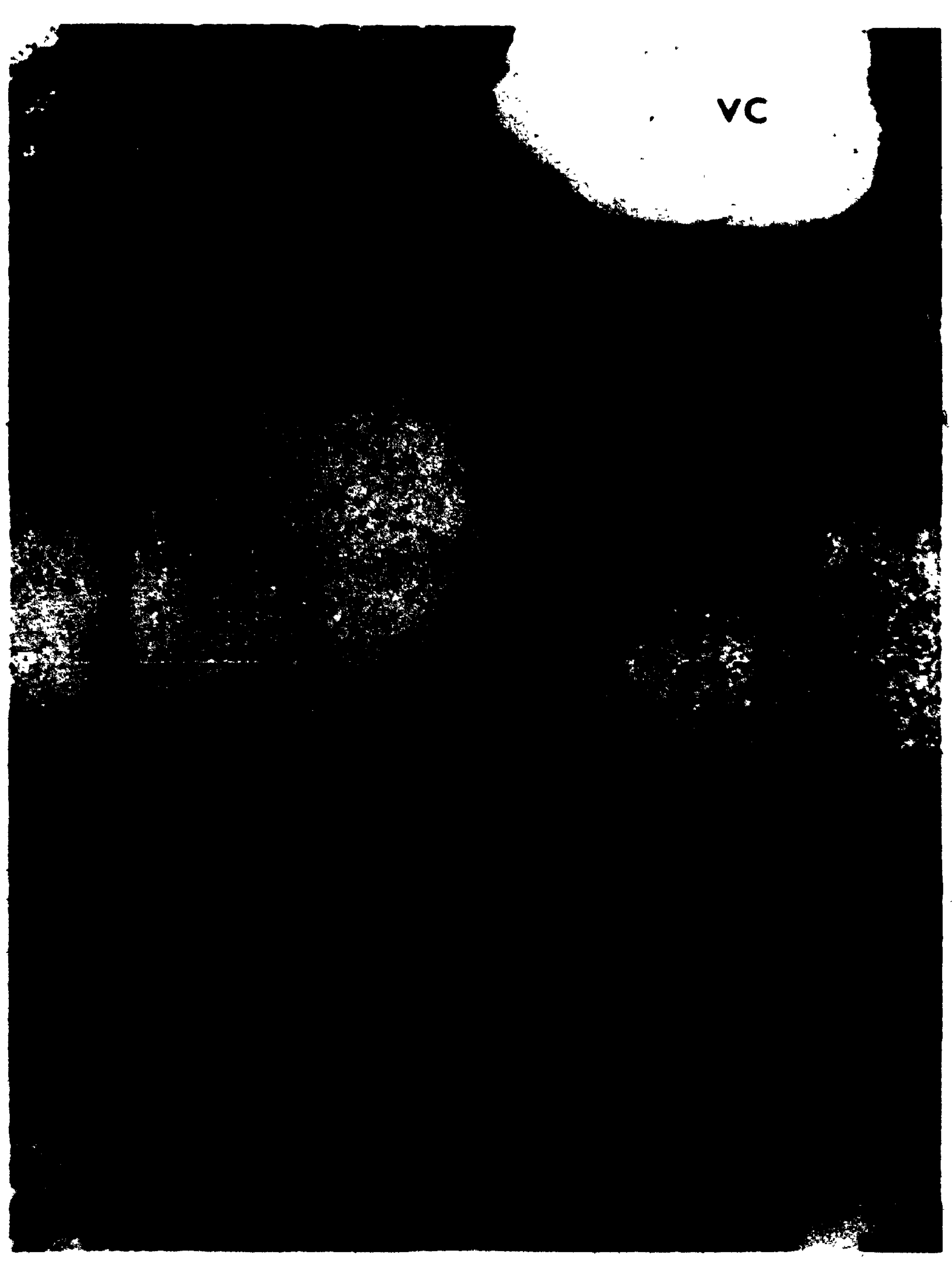

VC

PLATE 44 Longitudinal section of a germinating cyst, approx. 1.5 h after encystment (1.0 h after germination has started), showing two daughter nuclei. Note continuity of cyst wall and germ tube wall. x 11,000.

PLATE 45 Longitudinal section of a germ tube showing a nucleus and cytoplasm in the germ tube. Observe continuity of cell walls and plasmalemma of the cyst and germ tube. Also note lomasome between wall and plasmalemma of germ tube. x 19,500.

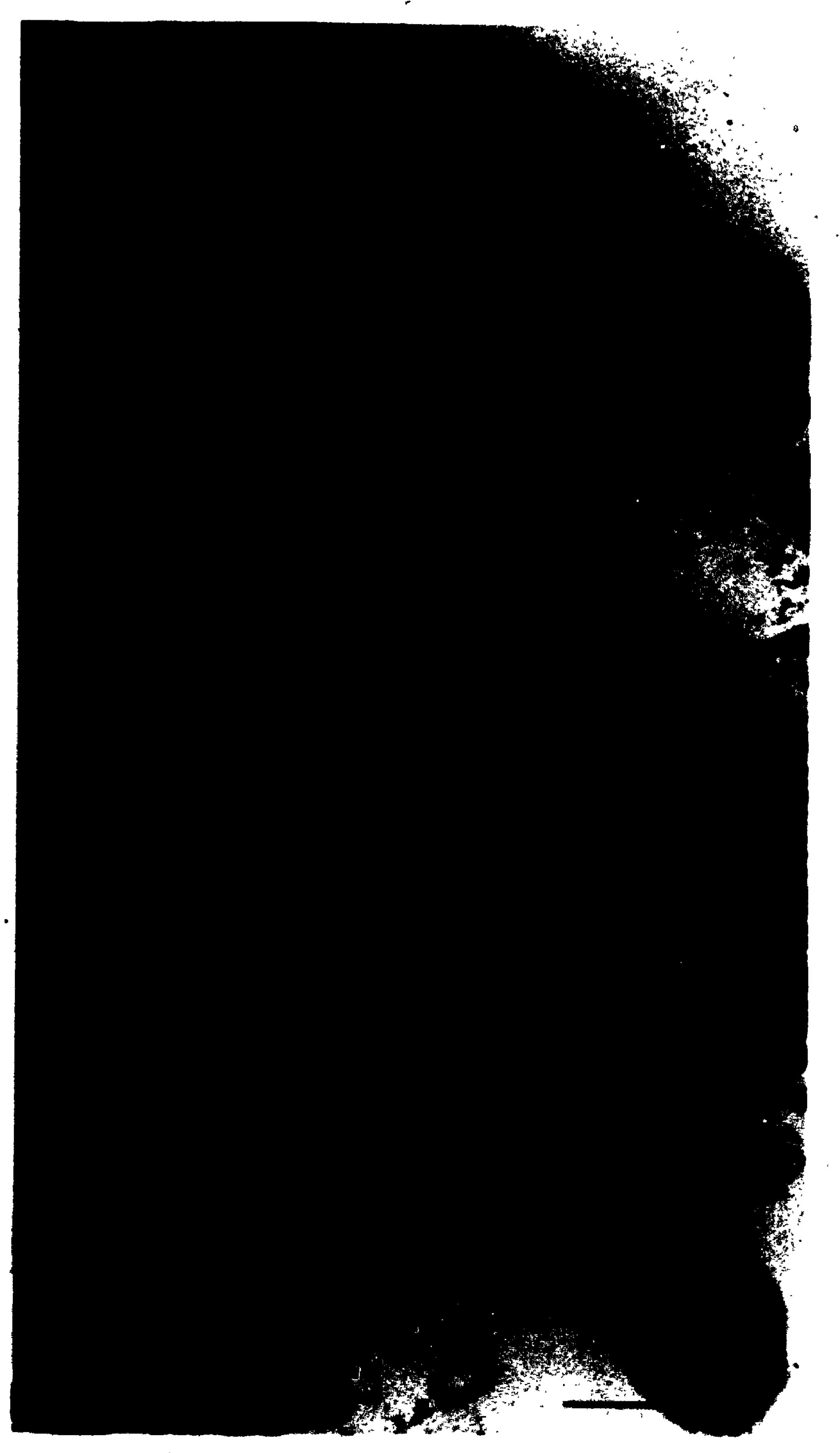


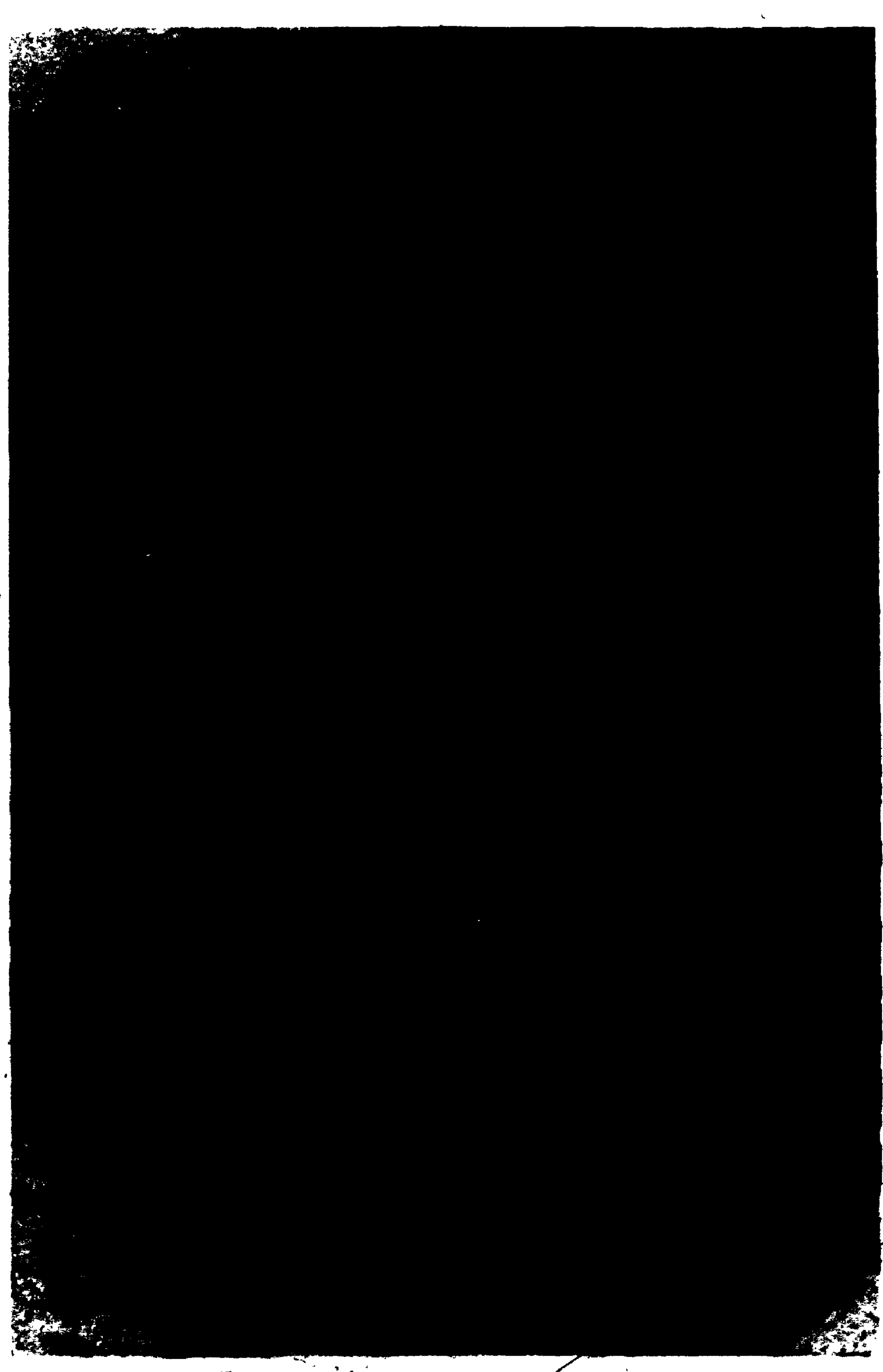
PLATE 46 Section of a germinating cyst, germ tube not shown. Note lipid bodies and crystalline vesicles have coalesced (with no membranes separating them) to form larger vesicles - beginning of a central vacuole. Mitochondria are closely associated with disintegrating vesicles. x 21,000.

PLATE 47 Section of a germinating cyst showing a larger central vacuole than in Plate 46, containing breakdown products of lipid bodies and crystalline vesicles. Observe many small mitochondria with numerous, swollen cristae, the dictyosome and nucleus near the developing germ tube (not shown in section). x 20,000.

PLATE 48 Section of a germinating cyst, approx. 2.0 h following initiation of encystment (1.5 h after start of germination). Note large central vacuole containing breakdown products of lipid bodies and crystalline vesicles (see Plates 46 and 47), and remains of cytoplasmic material at the junction of the cyst and germ tube (not included in section). x 20,000.

PLATE 49 Section of a non-germinating cyst fixed with potassium permanganate, post-stained with lead citrate, showing well-preserved membranes. Notice layers of endoplasmic reticulum around the prominent nucleus and the enlarged endoplasmic reticulum (ERt). x 14,000.

PLATE 50 Section of a cyst fixed with potassium permanganate, post-stained with lead citrate, showing endoplasmic reticulum around nucleus and connected to nuclear envelope (arrow). x 18,000.



IV NATURE OF VESICLE CONTENTS

The cytochemical identification of contents of the three major vesicles in zoospores and cysts was carried out with the aid of various organic solvents and enzymes as previously described in detail under Materials and Methods (pages 22 and 23).

Amorphous "vesicles":

The contents of amorphous "vesicles" are completely extracted by organic solvents (acetone, ether/ethanol and chloroform/methanol). Plates 51 to 54 show empty amorphous "vesicles" following treatment. Amorphous "vesicles" are evidently large lipid bodies.

Crystalline vesicles:

Of all the solvents and enzymes used, the chloroform-methanol mixture removes some contents of the crystalline vesicles. As a result, the contents are less electron-dense or "bleached" (Plates 53 and 54). The decrease in electron-density is especially noticeable in network and dense-mass forms of crystalline vesicles (Plates 53 and 54). In both cases, most but not all of the striations (fingerprint effect) have disappeared.

Digestion with pepsin (Plate 55) or α-amylase (Plate 56) does not affect the contents of crystalline vesicles.

These observations indicate that the contents of crystalline vesicles are partly lipid together with unidentified material.

Granular vesicles:

Granular vesicles are completely digested with pepsin (Plate 55) after 15 min incubation at 37°C. The contents are digested first, followed in the later stages, by the vesicle membranes.

Chloroform and methanol treated spores have granular vesicles which are less dense than normal or when they are treated with acetone or ether-ethanol.

It is concluded that the contents of granular vesicles are pepsin-digestible protein.

PLATE 51 Section of a cyst treated with acetone. Note lipid has been extracted, leaving empty spaces (L); crystalline and granular vesicle contents are still present. Also note the distortion in cytoplasm. x 11,000.

PLATE 52 Section of a cyst treated with ether-ethanol showing extraction of lipid bodies leaving empty spaces (L). Observe absence of vesicle membranes and cyst wall and presence of crystalline contents. x 13,000.

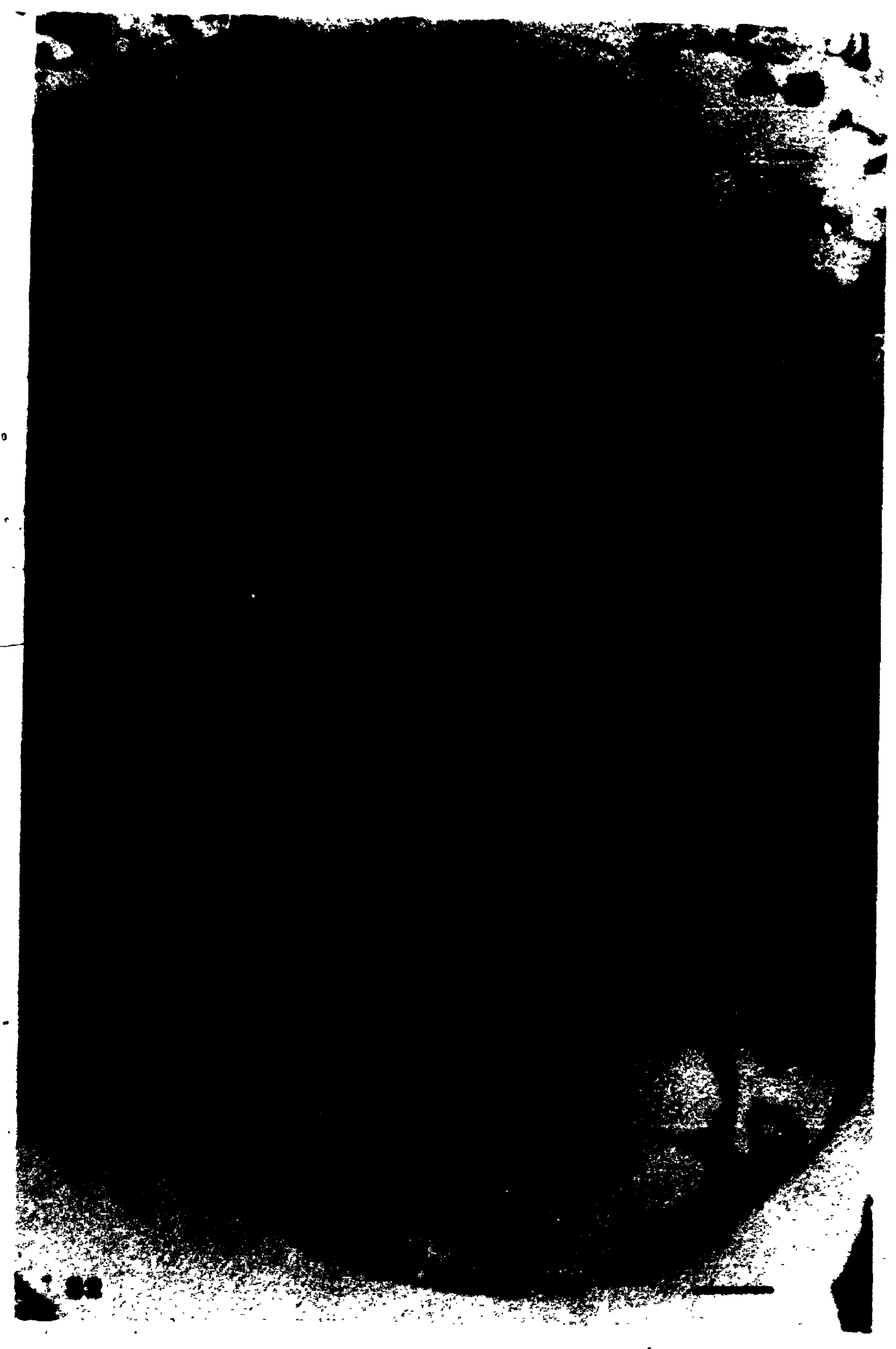


PLATE 53 Section of a zoospore treated with chloroform-methanol mixture showing results of extraction of lipid bodies (L). Note reduction in electron-density of dense-mass contents of crystalline vesicles (VCd). Granular vesicles are still well-preserved. x 15,000.

PLATE 54 Portion of a section of a binucleate zoospore extracted with chloroform-methanol mixture showing empty spaces (L) from which lipid has been removed. Note crystalline vesicles (dense-mass, network and ring forms) are less osmophilic. x 30,000.

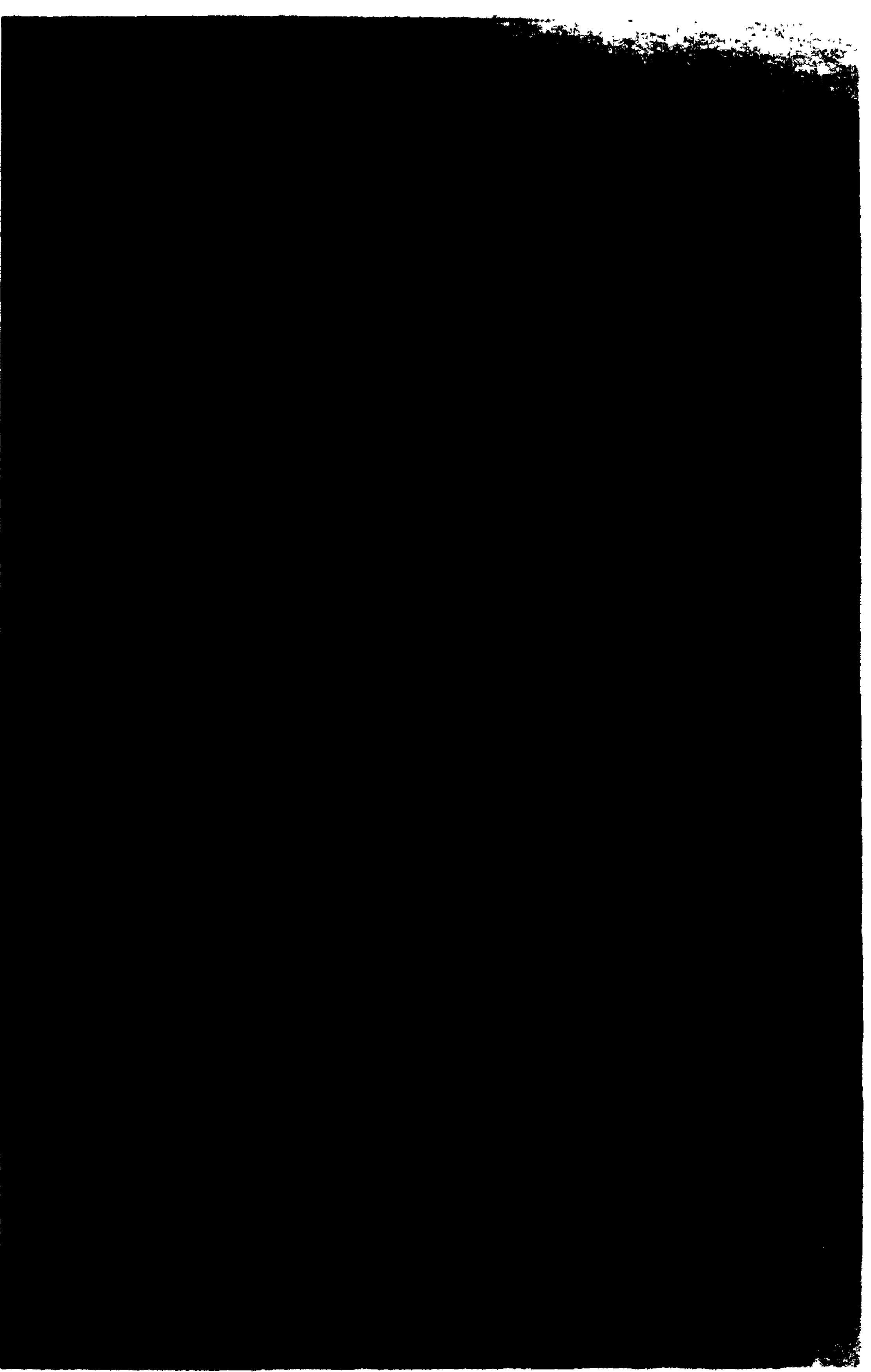
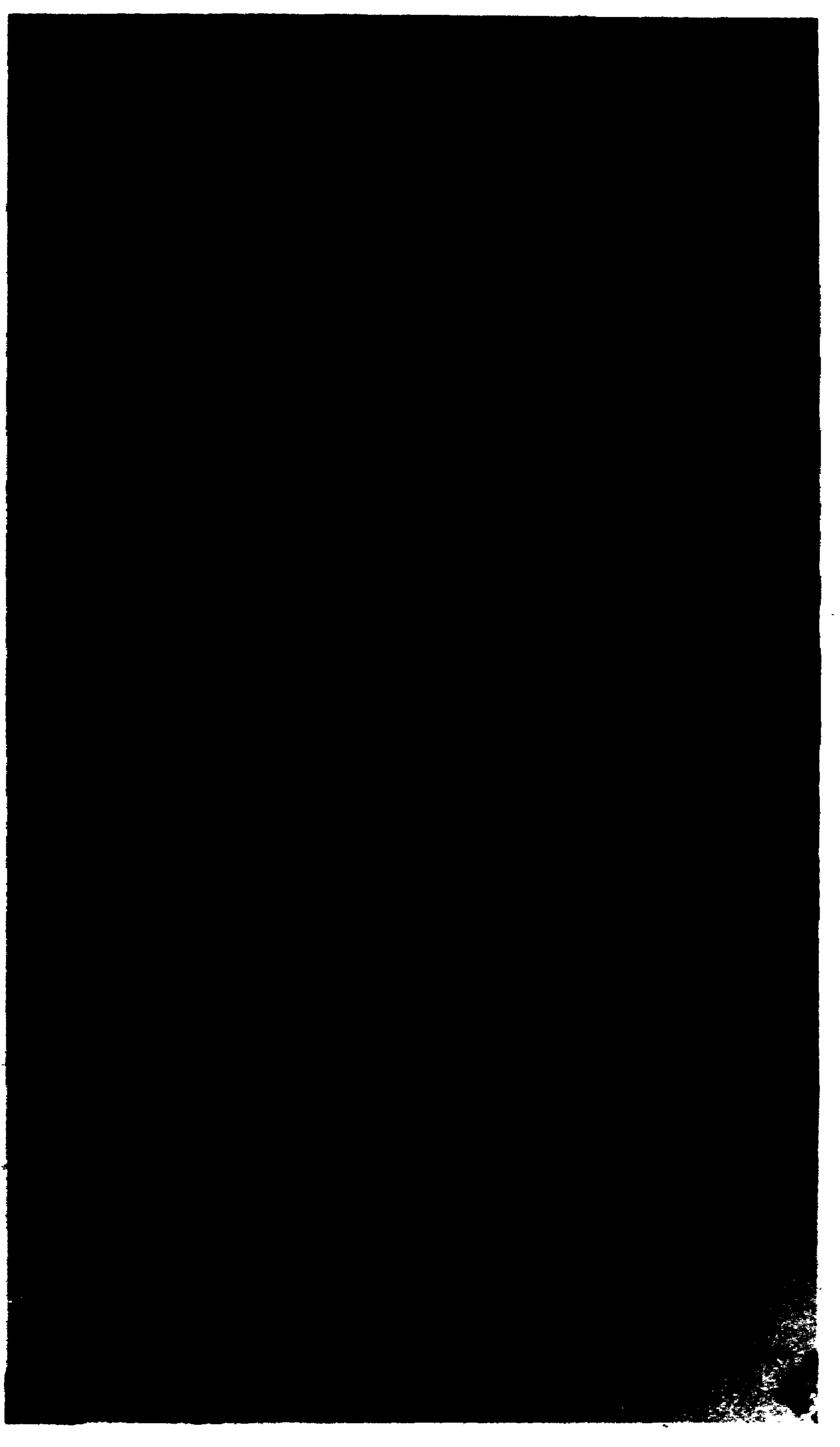


PLATE 55 Section of a cyst treated with pepsin, showing disorganization of the cytoplasm and removal of the cyst wall and plasmalemma. Mitochondria and the nucleus are still present but somewhat disorganized. Note the absence of granular vesicles and the preservation of crystalline vesicle contents and lipid bodies. x 18,000.

PLATE 56 Section of a cyst treated with α- amylase, showing lipid bodies and crystalline vesicles. x 12,000.



V MICROBODIES AND MITOCHONDRIA - LOCALISATION OF ENZYMES

The contents of microbodies were investigated by cytochemical methods as outlined on page 23. This was because of the difficulty in obtaining the enormous quantity of spores necessary to produce active enzyme sources in fractions, after the required separation methods. The presence of succinate dehydrogenase in mitochondria was also investigated similarly, for the same reason.

Microbodies

Microbodies in zoospores and cysts incubated in a standard medium (containing D, L -α- hydroxybutyric acid, see Materials and Methods section, page 23) show a pronounced deposition of electron-dense ferricyanide reduction material - copper ferrocyanide (Plates 57 and 58). The difference in electron-density is readily apparent when these microbodies are compared with those in zoospores and cysts incubated without hydroxybutyric acid (Plate 59).

This enhanced electron-density provides evidence of the presence of α-hydroxy acid oxidase activity in microbodies of zoospores and cysts.

Mitochondria

Catalase:

Cytochemical localisation of catalase peroxidatic activity was by the DAB method (p. 24). Incubation of zoospores and cysts in medium containing 3,3'diaminobenzidine and H_2O_2 results in deposition of electron opaque material

in the membranes of mitochondria (Plate 60). In the absence of H_2O_2 (Plate 61) or where catalase activity is inhibited by aminotriazole (Plate 62), deposition of electron opaque material in mitochondria does not occur.

Succinate dehydrogenase:

Mitochondria of zoospores and cysts incubated in standard medium (p. 24) with succinate showed highly electron-dense membranes and cristae (Plate 63) indicating deposition of copper ferrocyanide (the reaction product). Spores incubated in standard medium without succinate, show no such reaction (Plate 64).

PLATES 57-58 Sections of microbodies in cysts incubated in D,L-α-hydroxy butyrate/ferricyanide medium. Microbodies show deposition of copper ferrocyanide, the reaction product, indicating the presence of α-hydroxy acid oxidase.

Fixed with paraformaldehyde-glutaraldehyde and osmium tetroxide. Stained with uranyl acetate and lead citrate. x 18,000 and x 97,500.

PLATE 59 Ultrastructure of microbody incubated in ferricyanide medium without D,L-α-hydroxybutyrate. There is no deposition cf reaction product (compare with Plates 57 and 58).

Fixed with paraformaldehyde-glutaraldehyde and osmium tetroxide. Stained with uranyl acetate and lead citrate. x 22,000.

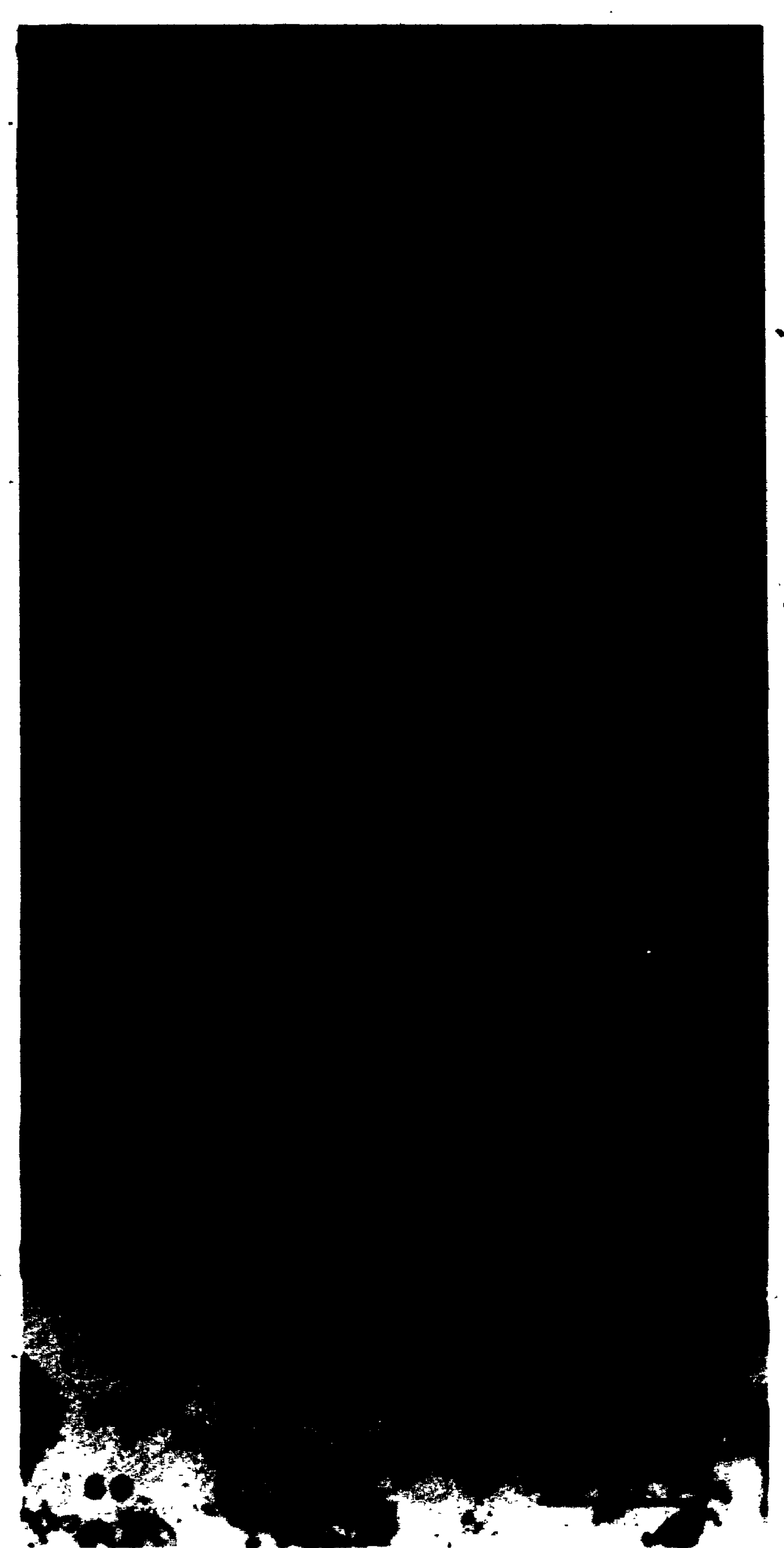


PLATE 60 - Section of a zoospore incubated in 3,3'-diaminobenzidine (DAB) medium for the demonstration of catalase activity. Note electron-dense mitochondrial membranes and also electron-density of areas where mitochondria and lipid bodies are adjacent. Observe absence of reaction product in microbodies. x 10,000.

PLATE 61 Section of microbodies and mitochondria in a zoospore incubated in the DAB medium without hydrogen peroxide. Notice the absence of electron-dense reaction products in mitochondrial membranes (compare with Plate 60). x 32,000.

PLATE 62 Section of microbody and mitochondria in a zoospore pre-incubated in 3-amino-1,2,4-triazole and in DAB medium with aminotriazole added to inhibit catalase activity. Mitochondrial membranes show no deposition of reaction products (compare with Plate 60). x 32,000.

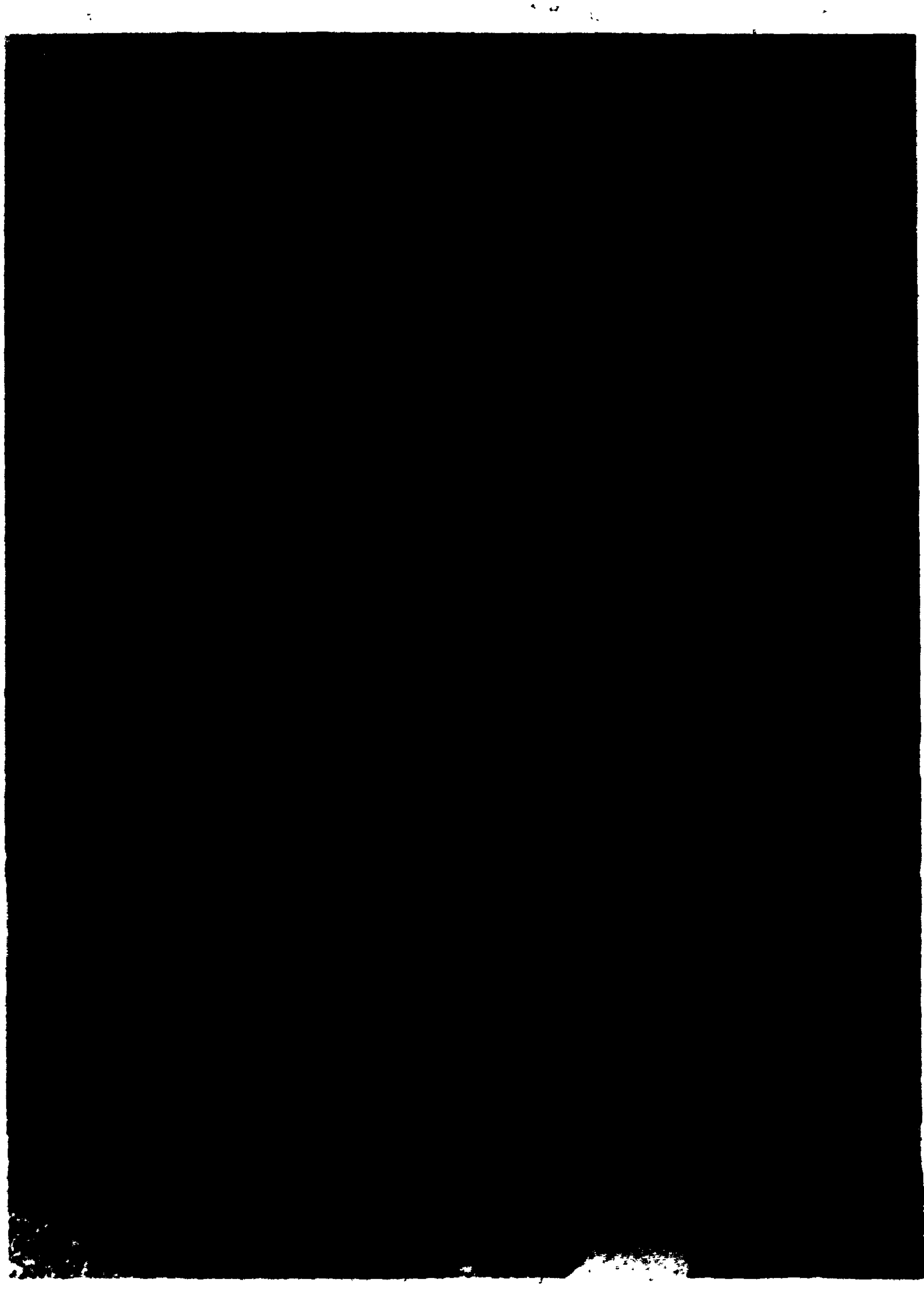
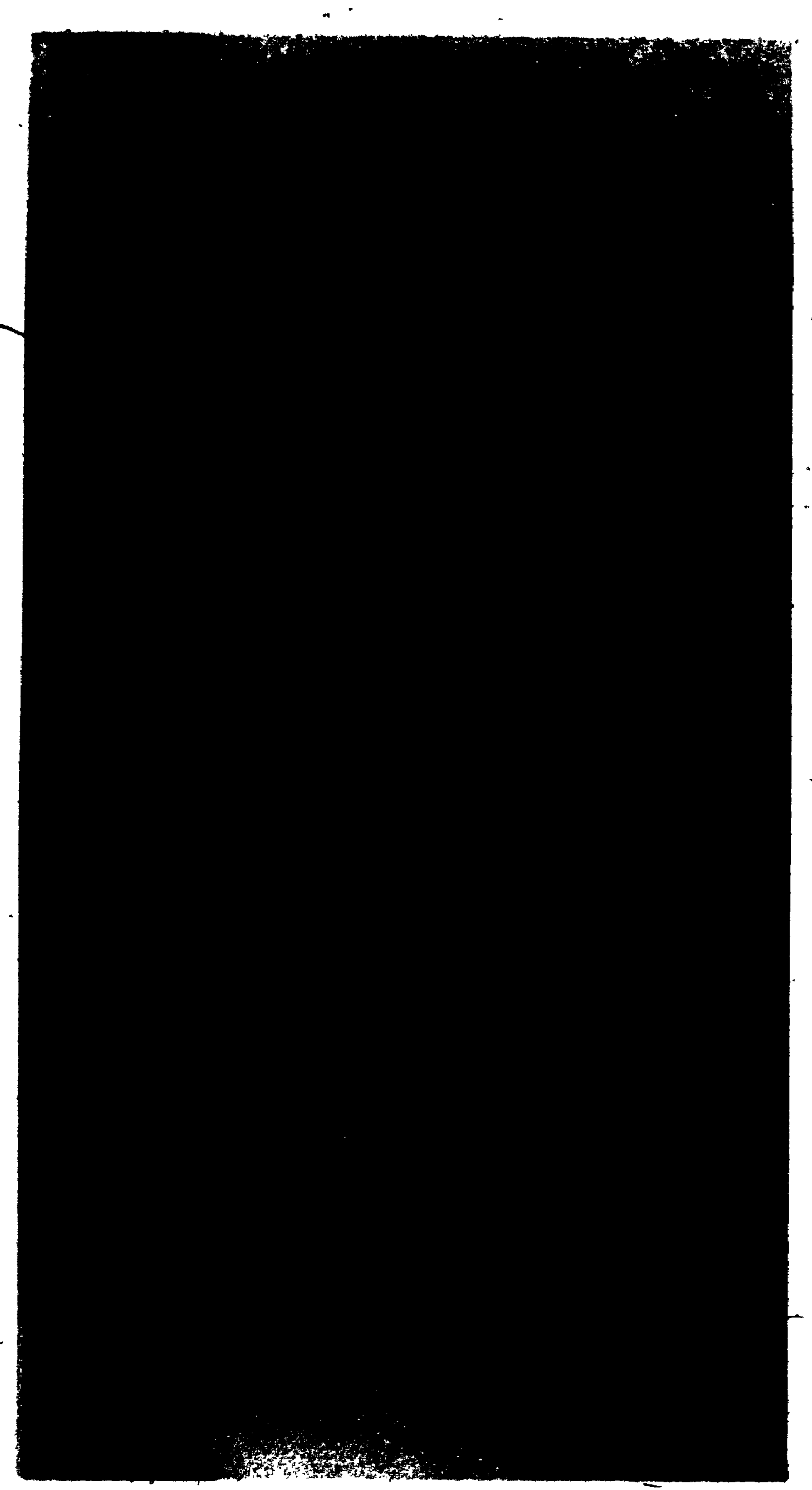


PLATE 63 Section of mitochondria from a germinating cyst incubated in succinate/ferricyanide medium to demonstrate succinate dehydrogenase activity. The copper ferrocyanide reaction product is localised in mitochondrial membranes.

Fixed with osmium tetroxide. Stained with uranyl acetate and lead citrate. x 40,000.

PLATE 64 Section of a mitochondrion from a cyst incubated in ferricyanide medium in the absence of succinate. There is no deposition of copper ferrocyanide reaction product in the membranes (compare with Plate 63).

Fixed with osmium tetroxide. Stained with uranyl acetate and lead citrate. x 72,000.



B CHANGES IN METABOLIC RESERVES.

Electron microscopic observations have revealed the presence of large stores of lipids and proteins in zoospores and cysts of P. palmivora. The lipids are present in amorphous "vesicles" and proteins in granular vesicles, both of which undergo disintegration during germination. Although carbohydrates were not recognised in electron micrographs, the cytoplasm of zoospores and cysts treated with α-amylase was less electron-dense. This was an indication of the probable presence of carbohydrates in the cytoplasm.

Hickman (49) suggested that vesicle contents may serve as energy sources for sustaining long periods of zoospore motility. However, no visible changes in vesicle contents are observed after 6 h motility in zoospores of P. palmivora.

The probable quantitative changes in endogenous metabolic products during motility and germination was investigated. Samples were taken from freshly liberated zoospores (designated T1), after incubating zoospores at 17°C for 6 h (T2) and, after a further 2 h incubation (encystment followed by germination) following the 6 h motility period (T3).

I CARBOHYDRATES

The amount of carbohydrates in 0.5 ml of sonicated suspensions (designated Total), 22,000 x g supernatant (cell-free extract - CFE) and the 22,000 x g pellet (Pellet) were determined for the three stages described above.

The phenol-sulphuric acid method (27) was used for the

quantitative micro-determination of sugars and their methyl derivatives, oligosaccharides and polysaccharides. The resulting yellow-orange colour was measured at 488 nm, the wavelength at which absorbance was highest.

The values obtained for 0.5 ml of sample were used to calculate carbohydrate concentration of the total volume of samples. The data thus obtained were related to unit (mg) dry weight of spores.

The results are presented in Table VII. The level of significance of differences is P = 0.01, unless otherwise stated.

After 6 h motility, there was a significant* decrease in total carbohydrate concentration of zoospores but it increased during germination. This increase was significant, although the quantity, 101.45 ± 1.06 μg/mg, was a little less than that present in freshly liberated zoospores, 118.80 ± 1.70 μg/mg dry wt.

The changes in carbohydrate concentration of the CFE fractions were not significantly different.

The pellet fractions contained unbroken spores, cell wall fragments and other unidentified particulate matter. The general trend of changes in carbohydrate concentration in these fractions was similar to that of the total fractions. There was a decrease in carbohydrates during the motile period, from 41.46 ± 2.46 at T1 to 28.76 ± 6.0 μg/mg

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* Analysis of variance and the Student Newman - Keul's test were used.

dry wt. at T2. Carbohydrates increased during germination; this increase resulted in significantly higher values than was originally present in freshly liberated zoospores (58.02 ± 2.23 at T3 and 41.46 ± 2.46 μg/mg dry wt. at T1).

TABLE VII

CHANGES IN CARBOHYDRATE CONCENTRATION AT DIFFERENT STAGES IN ZOOSPORES AND CYSTS OF _P. palmivora_

STAGE	MEAN+ CARBOHYDRATE CONCENTRATION OF FRACTIONS (µg/mg DRY WT.)		
	TOTAL	CFE*	PELLET**
T1	118.80 ± 1.70	14.20 ± 5.52 [a]	41.46 ± 2.46
T2	89.75 ± 3.04	14.55 ± 5.02 [a]	28.76 ± 6.00
T3	101.45 ± 1.06	16.95 ± 3.61 [a]	58.02 ± 2.23

+ Data representative of two experiments ± standard deviation.

* Supernatant after centrifugation at 22,000 x g.

** Pellet after centrifugation at 22,000 x g, resuspended in 2.0 ml buffer.

a No significant difference, P = 0.05.

II LIPIDS

From electron microscopic observations, lipids constitute the major source of storage material in zoospores and cysts of P. palmivora. The entire contents of the amorphous "vesicles" are lipid. Lipids also form part of the contents of crystalline vesicles. Electron microscopic observations further showed that there is little change in lipid content at the end of the 6 h motile period but that they are broken down during germination. Since binding of saturated lipids by OsO_4 is limited, it is possible that the lipids present in and utilised by motile zoospores, would not be shown solely by ultrastructural observations alone. Quantitative analyses of lipids were therefore undertaken to clarify this point, and also to obtain comparative data for lipids in motile zoospores and germinating cysts.

Lipid analyses were carried out on sonicated cell-free extracts without centrifugation; i.e. on total fractions. Values obtained for each analysis were based on unit (mg) dry weight of spores used in the experiment.

Acyl ester linkages. The method of Stern and Shapiro (91) measured monoglycerides, diglycerides and triglycerides in 1.0 ml of chloroform extract (Folch's extraction procedure) of each sample.

Phosphorus. The amount of lipid phosphorus in 1.0 ml of chloroform extract (Folch's extraction procedure) was analysed by a method adapted from Rouser et al. (84; Chapter 2, p. 27).

<u>Free fatty acids</u> in aqueous samples were analysed using the same batch of suspensions as for carbohydrate and protein analyses. This was because the volume of chloroform extracts obtained with the Folch's extraction procedure, was insufficient for analysing acyl esters, lipid phosphorus as well as free fatty acids. An alternative method of analysing the latter was therefore necessary. Chloroform extracts were prepared from 1.0 ml samples in phosphate buffer (0.05 M, pH 6 to 7) as described in Chapter 2, page 28. The resultant yellowish-brown colour (copper complex of free fatty acids) developed immediately was measured at 440 nm.

Confirmatory tests on chloroform extracts (Folch's extraction procedure) showed a trend of free fatty acid changes at different stages generally similar to those obtained with the aqueous chloroform extract method of Itaya and Ui (56).

The results in Table VIII show the concentration of glycerides (acyl esters) and lipid phosphorus in 5.0 ml of chloroform extract and free fatty acids in 5.0 ml of aqueous samples. The level of significance is P = 0.01 unless otherwise stated.

There was a sharp decrease in acyl esters and free fatty acids from stage T1 to T2.

The decrease in glyceride (acyl ester) concentration over the entire experimental period (T1 - T3, i.e. 6 h motility followed by 2 h encystment/germination) represen-

ted a highly significant total loss of 73.80 μmole/mg dry wt. Of the initial amount present in freshly liberated zoospores (T1), 45.47 μmole was utilised during the 6 h motile period; and of the remainder, 28.33 μmole was used up during the 2 h encystment/germination period.

Of the total lipid content the greatest decrease occurred in free fatty acids. About 88 μmole of the original (T1) content was utilised during motility and 43 μmole of the remainder (T2) during the 2 h encystment/germination period. In total, this amounted to approximately 132 μmole decrease of free fatty acids during the entire experimental period (T1 - T3).

These results indicate that the energy requirements of motile zoospores were supplied primarily by free fatty acids and to a lesser, but still significant extent, by glycerides (acyl esters). The energy requirements during germination were also satisfied more by free fatty acids (74%) than by glycerides (48%).

The lipid phosphorus content of freshly liberated zoospores (T1) and zoospores after 6 h motility (T2) did not alter significantly. However, there was a significant increase in lipid phosphorus during the 2 h encystment/germination period (T3).

TABLE VIII

CHANGES IN TOTAL LIPID CONTENT AT DIFFERENT STAGES IN ZOOSPORES AND CYSTS OF *P. palmivora*

STAGE	MEAN[+] CONCENTRATIONS OF VARIOUS LIPIDS (μmole/mg DRY WT.)		
	ACYL ESTERS	LIPID PHOSPHORUS	FREE FATTY ACIDS
T1	102.40 ± 5.02	0.011 ± 0 [a]	145.78 ± 1.80
T2	56.93 ± 3.71	0.013 ± 0 [a]	57.88 ± 2.93
T3	28.60 ± 0.99	0.022 ± 0	14.85 ± 0.35

+ Data representative of two experiments ± standard deviation.

[a] At P = 0.05 the change is not significant.

III PROTEINS

Ultrastructural observations of zoospores of P. palmivora have shown that no recognisable changes occur in the protein contents of granular vesicles during the 6 h motile period. These studies have further shown that the protein in granular vesicles are broken down and completely disappear after 2 h encystment/germination. The proteins broken down during germination could serve as energy sources or they could be reconstituted as other forms of protein. The following chemical analyses were made to assess the use to which these proteins were put in motile zoospores and germinating cysts.

Proteins in different fractions of sonicated spore suspensions (as outlined on pages 28 and 29) at different stages were analysed by the filter paper-dye technique of Bramhall et al. (14). The total amount of proteins, precipitated by trichloroacetic acid, was measured to give quantitative estimates of proteins in 5.0 ml of sonicated samples.

The results are presented in Table IX. The level of significance used is P = 0.01 unless stated otherwise.

Although no changes in granular (protein) vesicles were observed in electron micrographs after 6 h motile period, the results (T2) in Table IX show a decrease in total proteins after 6 h motility. This decrease represented about 88 μg loss of the proteins in zoospores at stage T1; however, this decrease was statistically not signifi-

cant at P = 0.05 level of significance. After 2 h encystment/germination, the protein concentration of cysts increased substantially, exceeding the level of proteins initially present in freshly liberated zoospores (T1).

Protein concentration in the CFE fraction (which was used as enzyme source in later experiments, pages 160 to 166) showed a slight but insignificant increase after 6 h motility. After zoospores had encysted and germinated (T3), proteins increased significantly.

Changes in protein concentration of the pellet fractions followed the general trend of changes in the total fractions. That is, there was a decrease in proteins during zoospore motility (T2) but then protein concentration increased again during the 2 h germination period.

From ultrastructural observations and the results in Table IX above, it appears that proteins lost during motility (Total fraction) were not from the granular vesicles but possibly could have been from the cytoplasm.

TABLE IX

CHANGES IN CONCENTRATION OF PROTEINS AT DIFFERENT STAGES IN ZOOSPORES AND CYSTS OF P. palmivora

STAGE	MEAN[+] PROTEIN CONCENTRATION IN FRACTIONS (µg/mg DRY WT.)		
	TOTAL	CFE*	PELLETS**
T1	430.75 ± 14.49 [a]	213.00 ± 15.56 [a]	93.40 ± 3.11
T2	343.25 ± 2.47 [a]	226.50 ± 30.41 [a]	58.90 ± 4.10
T3	567.50 ± 79.90	331.50 ± 4.95 [a]	111.30 ± 6.08

\+ Data representative of two experiments ± standard deviation.

* Supernatant after centrifugation at 22,000 x g.

** Pellet after centrifugation at 22,000 x g, resuspended in 2.0 ml buffer.

a Indicates lack of significance at P = 0.05.

C CHANGES IN WEIGHT OF SPORES

The long motile periods in the absence of external nutrients of zoospores of P. palmivora and other species, has led to the suggestion that internal reserves supply the energy requirements for motility.

From the results on metabolic reserves presented in Tables XII to IX, it is clear that zoospores use primarily lipids and some carbohydrates for motility, and that during germination lipids are utilised and carbohydrates and proteins are synthesized.

The effects on the weight of zoospores and cysts of utilisation of lipids and the synthesis of proteins and carbohydrates, without exogenous nutrients were investigated.

Zoospores and cysts at different stages (T1, T2 and T3) were weighed as previously described on page 45 .

The results are given in Table X.

Results of changes in weight showed significant ($P = 0.01$) decreases throughout the duration of the experiments (stages T1 - T3). Zoospores lost about 81% of their weight during the 6 h motile period. A further decrease of about 6% was recorded during the period of encystment/germination.

TABLE X

CHANGES IN DRY WEIGHT OF ZOOSPORES AND CYSTS OF P. palmivora DURING MOTILITY AND GERMINATION

STAGE	MEAN* DRY WEIGHT (mg) OF 1.0×10^4 SPORES
T1	0.0070 ± 0.0003
T2	0.0013 ± 0.0001
T3	0.0009 ± 0

* Data representative of two experiments ± standard deviation.

D CHANGES IN ENZYME ACTIVITIES

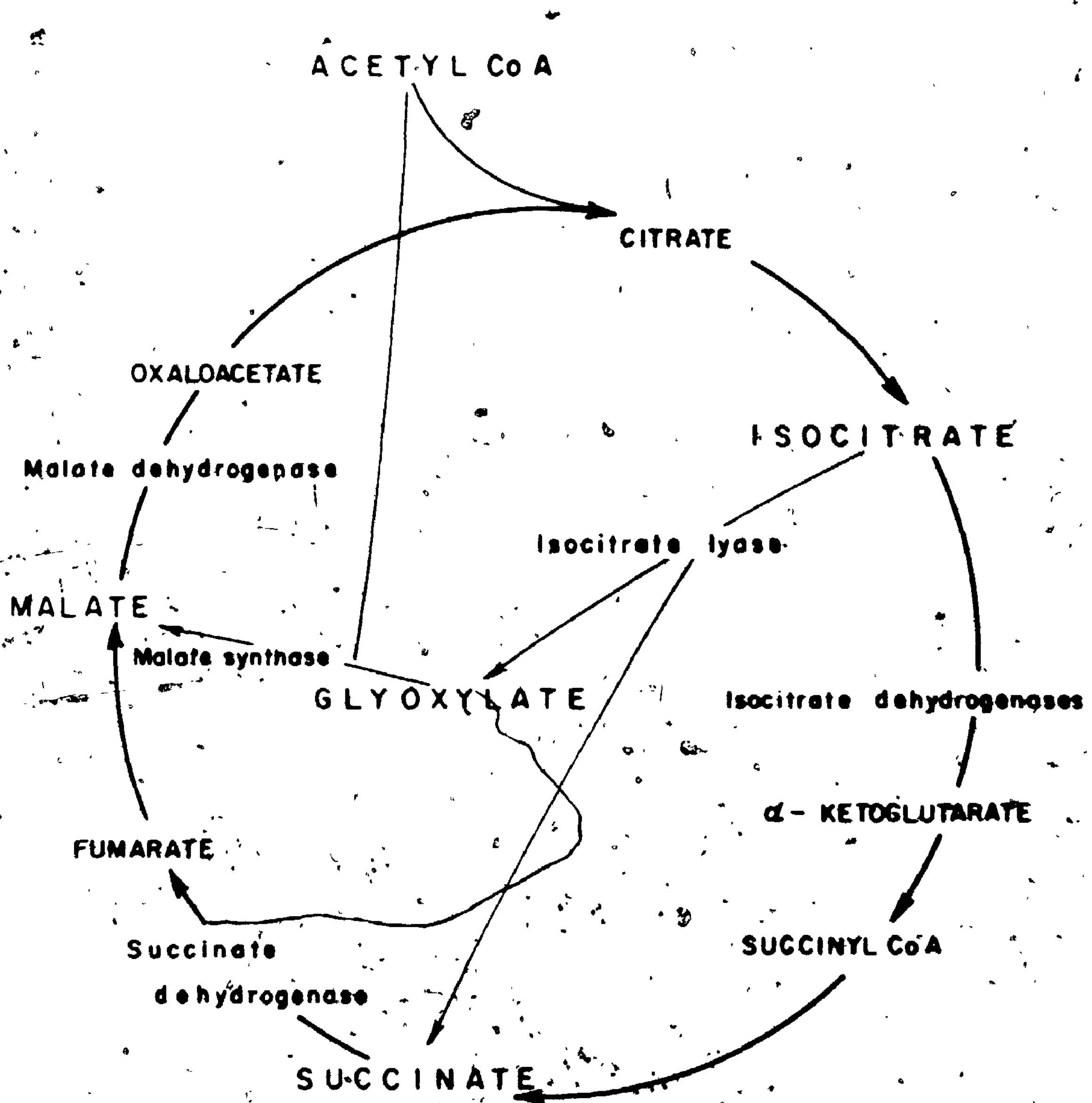
Fine structure observations and data on metabolic reserves in zoospores and germinating cysts of P. palmivora have so far revealed some of the changes that occur during these developmental stages. After 6 h of motility, there is a drastic decrease in lipids and a slight decrease in carbohydrates but no significant change in protein content of zoospores. Germinating cysts utilised lipids to synthesise carbohydrates and protein. These degradative and synthetic processes are mediated by enzymes and as these alter, so must the enzymes and metabolic pathways associated with them.

Since earlier results showed the importance of lipid degradation during zoospore motility and cyst germination, the key enzymes of the glyoxylate cycle (Fig. 8) were investigated. This pathway supplies carbon atoms, from lipid degradation, for the operation of the tricarboxylic acid cycle in lipid storing organisms.

Four enzymes of the tricarboxylic acid cycle were also investigated. Isocitrate dehydrogenase - NAD and NADP forms were studied. Both enzyme forms mediate the conversion of isocitrate to α-ketoglutarate. The $NADP^+$ enzyme was of particular interest, since $NADP^+$ is directly involved in biosynthesis of lipid products. The activities of malate dehydrogenase (the only enzyme investigated which was common to both the glyoxylate and tricarboxylic acid cycles) and succinate dehydrogenase were also measured.

FIGURE 8

ENZYMES OF THE GLYOXYLATE AND TRICARBOXYLIC ACID CYCLES MEASURED IN ZOOSPORES AND CYSTS OF *Phytophthora palmivora*



The aim of these investigations was to ascertain which cycle or cycles were in operation and/or dominant during zoospore motility and cyst germination in P. palmivora.

The specific activities of the following enzymes are tabulated in Table XI and Appendix (p. 203):

Isocitrate lyase	-	ICL
Isocitrate dehydrogenase : NAD	-	d-IDH
Isocitrate dehydrogenase : NADP	-	t-IDH
Malate dehydrogenase	-	MDH
Succinate dehydrogenase	-	SDH

The methods of assay and calculations to derive specific activities are described in detail in Chapter 2, pages 30 to 41 .

Each value in Table XI represents the mean specific activity from three experiments, with the standard deviations. Unless otherwise stated, the level of significance used is $P = 0.01$.

TABLE XI

CHANGES IN SPECIFIC ACTIVITY[+] OF ENZYMES AT DIFFERENT STAGES IN ZOOSPORES AND CYSTS OF *P. palmivora*

STAGE	MEAN SPECIFIC ACTIVITY[+] OF VARIOUS ENZYMES PER mg DRY WT. OF SPORES				
	ICL	d-IDH	t-IDH	MDH	SDH
T1	19.91 ± 2.64	*33.56 ± 1.11	15.71 ± 0.52	*247.32 ± 0.68	0.079 ± 0.019
T2	*9.71 ± 1.12	*36.47 ± 1.23	6.53 ± 0.55	*320.52 ± 57.09	0.033 ± 0.004
T3	*6.39 ± 0.90	45.20 ± 2.94	23.08 ± 0.70	436.85 ± 30.32	0.109 ± 0.001

+ Specific activity expressed as units per mg protein.

Data representative of three experiments, ± standard deviations.

* At P = 0.05 the change is not significant.

I KEY ENZYMES OF THE GLYOXYLATE CYCLE

Isocitrate lyase activity decreased during motility and continued to decrease during germination (Table XI). Although the decrease during the 2 h encystment/germination period (T3) represented about 35% loss over the enzyme activity at T2, this decrease was statistically not significant.

The activity of malate synthase was too low to record with the samples used in these experiments. However, this enzyme was present in zoospores and cysts of P. palmivora (as shown in experiments in section V below, pages 166 to 168).

II ISOCITRATE DEHYDROGENASE

Zoospores and cysts of P. palmivora contained both NAD- and NADP-isocitrate dehydrogenases (Table XI). In all stages, the NAD enzyme had higher specific activities than the NADP enzyme.

There was a slight but non-significant increase in isocitrate dehydrogenase:NAD activity during the 6 h motile period. During the 2 h encystment and germination period, the enzyme activity increased significantly.

Isocitrate dehydrogenase:NADP decreased significantly

after 6 h motility, but then increased after the 2 h encystment/germination period, resulting in higher values than were initially present, i.e. 15.71 ± 0.52 at T1 compared to 23.08 ± 0.70 units/mg protein/mg dry wt. at T3.

III MALATE DEHYDROGENASE

Malate dehydrogenase activity was the highest of all the values observed (Table XI).

After 6 h motility (T2), the specific activity had increased about 23% over the enzyme activity at T1. However, this increase was not significant, possibly a result of the variability in replicates at the T2 stage.

After 2 h encystment/germination period (T3), malate dehydrogenase activity had increased significantly from 320.52 ± 57.09 at T2 to 436.85 ± 30.32 units/mg protein/mg dry wt. (Table XI).

IV SUCCINATE DEHYDROGENASE

Specific activity of succinate dehydrogenase was generally very low in all the stages, compared to the other enzymes assayed.

During 6 h motility, the specific activity decreased significantly from 0.079 ± 0.019 at T1 to 0.033 ± 0.004

units/mg protein/mg dry wt. at T2. After 2 h encystment and germination (T3), succinate dehydrogenase activity increased to 0.109 ± 0.001 units/mg protein/mg dry wt. (Table XI).

V DISTRIBUTION OF ENZYMES IN CELL FRACTIONS

The distribution of five enzymes in differentially centrifuged fractions of zoospores as described in detail in Chapter 2, pages 42 to 44 was studied. These enzymes were isocitrate lyase, malate synthase, NAD-isocitrate dehydrogenase, NADP-isocitrate dehydrogenase and malate dehydrogenase.

The crude enzyme preparations were obtained by ultrasonic disruption of spores (about 30 mg wet wt.) for 5 min. Cell-free extracts were centrifuged (as described in Chapter 2, pp. 42 to 44) to obtain fractions P1, P2, P3 and S4.

The results are presented in Table XII:

Isocitrate lyase - ICL
Malate synthase - MS
Isocitrate dehydrogenase : NAD - d-IDH
Isocitrate dehydrogenase : NADP - t-IDH
Malate dehydrogenase - MDH

Specific activities of the enzymes are expressed as units/mg protein.

The results in Table XII show that most of the isocitrate lyase activity and all the activity of malate syn-

thase were in fraction S4. The isocitrate lyase activities recorded in fractions P2 and P3 were relatively low. The presence of this enzyme in these two fractions could be attributed to insufficient rinsing of the pellet fractions preceding them in the centrifugation procedure, i.e. P1 and P2, respectively (as described on p. 42 and Fig. 2). The overall activity of isocitrate lyase was higher than that of malate synthase (about four times as great). The relatively low activity of the latter enzyme could explain the inability to detect malate synthase activity in earlier experiments (p. 164) when smaller quantities of spores were used.

Malate dehydrogenase activity was also mostly in the S4 fraction, although lower activities were also measured in fractions P1 and P3. The activity of the enzyme in fraction P3 could be part of the mitochondrial MDH. The enzyme activity recorded in fraction P1 was most probably contributed by unbroken spores in that pellet.

Similarly, NAD-isocitrate dehydrogenase activity in P1 was possibly from unbroken spores. However, the bulk of this enzyme was in fraction S4. Isocitrate dehydrogenase : NADP enzyme was only present in fraction S4.

TABLE XII

DISTRIBUTION OF ENZYMES IN DIFFERENTIALLY CENTRIFUGED FRACTIONS OF SPORES OF P. palmivora

FRACTION	SPECIFIC ACTIVITY OF VARIOUS ENZYMES, IN UNITS/mg PROTEIN				
	ICL	MS	d-IDH	t-IDH	MDH
P1	0	0	185.19	0	216.67
P2	47.62	0	0	0	0
P3	386.91	0	0	0	291.67
S4	1317.60	337.83	833.33	147.73	5378.79

CHAPTER 4

DISCUSSION AND CONCLUSIONS

In those pathogenic fungi in which they occur, zoospores have several important functions in the life cycle. One of the most important is their role in disease dissemination and infection. Consequently they have drawn intense attention from several investigators who have provided a lot of information on zoospore biology. From these studies one fact has been established over and over again. This is the ability of zoospores to remain motile for surprisingly long periods in the absence of any external energy supplies.

Most of the studies on longevity of zoospore motility have been with root-infecting pathogens (38, 51). However, zoospores of species attacking aerial parts of plants including Phytophthora palmivora share this capacity for long motile periods (10, 51).

Previous studies on the relationship between external energy sources and motility of zoospores indicate that very little of the exogenous sugars, amino acids, organic acids and fatty acids tested was metabolised (3, 92). In P. drechsleri (3) the evolution of $^{14}CO_2$ from labelled D-

glucose, acetate and glutamic acid by germinating cysts was over twice the rate of $^{14}CO_2$ evolution by zoospores, and this increase correlated with the progress of germination. This led Barash and his co-workers (3) to suggest that while zoospores could utilise some exogenous nutrients, they were less dependent on external energy sources than germinating cysts.

These results, together with observations on longevity of the motile period in the absence of external nutrients, have inevitably drawn attention to the nature of the substances that support these long motile periods, but in spite of the vast amount of knowledge on zoospores, information on this particular point is practically non-existent.

The present investigation with P. palmivora was an attempt to establish precisely any links between changes in ultrastructure and changes in endogenous nutrients and enzymes during zoospore motility and the early stages of cyst germination.

The electron microscopic observations described earlier in Chapter 3 (pp. 47 to 130) have confirmed the existence of a variety of "vesicles" which have been reported in zoospores and cysts of other species (34, 37, 41, 47, 56, 57, 80, 101) as well as identifying inclusions whose function at the present time is unknown. With the exception of these latter structures, the contents of the major "vesicles" in zoospores and cysts of P. palmivora have been positively identified as metabolic reserves.

Comparison of the fine structure of freshly liberated zoospores with that of zoospores after 6 h motility, showed that no substantial changes occurred in the contents of vesicles or inclusions. However, quantitative chemical analyses over the same period demonstrated considerable decrease in lipids and a slight decrease in carbohydrates (Tables VII and VIII). These substrates could have occurred in the freshly liberated zoospores in a form or forms which did not react with the fixatives used for electron microscopy. This would mean that the lipids and carbohydrates that were utilised during motility (between T1 and T2) failed to form stable or recognisable reaction products - hence the absence of any noticeable changes in contents of vesicles or inclusions.

Bearing out the suggestions outlined above, treatment of zoospores and cysts of P. palmivora with α-amylase generally led to a reduction in electron density of the cytoplasm. The carbohydrates in zoospores could be present in the cytoplasm in some unrecognisable form, unlike glycogen, which is fixed and easily identified in osmium tetroxide-treated materials. The fixation of zoospores in glutaraldehyde and osmium tetroxide has been reported to cause the removal of different amounts of carbohydrates. Thus, Desjardins and his co-workers (24) reported a loss of 31% total carbohydrates when zoospores of P. palmivora were fixed in 4% glutaraldehyde compared to a 0.5% carbohydrate loss in 1% osmium tetroxide.

Additional information on fixation of carbohydrates (and lipids) is also provided by Millonig and Marinozzi (69) who report that although hexoses, pentoses or most of their polymers react with osmium tetroxide, this may not lead to a reduction of the fixative and therefore no stable reaction products are formed. If no stable reaction products are formed, the unfixed carbohydrates could be removed through dehydration and embedding procedures.

The level of binding of osmium tetroxide with lipids depends on the fatty acid composition (69). The hydrolysis of lipids and hence electron-density, increases with the amount of unsaturated fatty acid components in the lipid. Therefore, if the lipids which were utilised during the motile period were saturated, this could account for the absence of recognisable changes in lipid content of zoospores after 6 h motility. A possible location of such saturated lipids, that are removed during dehydration or embedding, could be the areas of crystalline vesicles which appear electron-transparent.

These results emphasise the value of complementing the ultrastructural studies with biochemical studies wherever possible.

The general morphology and ultrastructure of zoospores of *P. palmivora* resembles those of other members of the Peronosporales, e.g. *P. megasperma* var. *sojae* (54), *P. parasitica* (47, 80) and *Pythium aphanidermatum* (41) as well as the secondary zoospores of *Aphanomyces euteiches* (Sapro-

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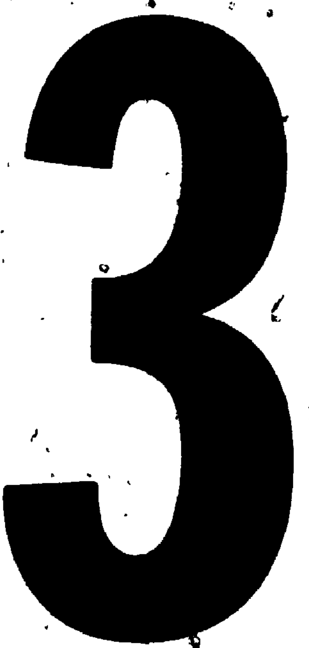

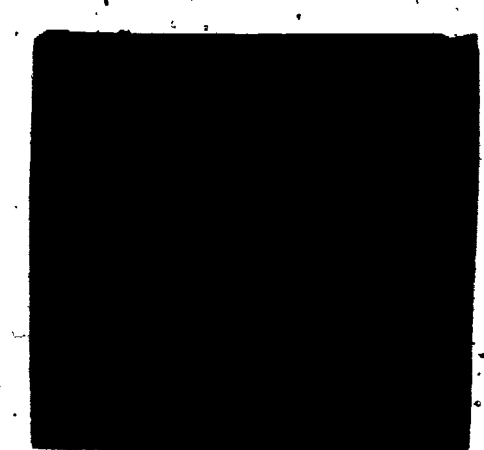

leginales, 56). However, there are minor differences in detailed fine structures.

Thus, although in both Phytophthora palmivora and Aphanomyces euteiches (56) the area around the contractile vacuole is characterised by membranes with rough surfaces and double membrane-bound vesicles; Hoch and Mitchell described these vesicles as a product of dictyosome proliferation. In P. palmivora zoospores, however, no connection was observed between Golgi membranes and the parallel membranes which delimit the vesicles surrounding the contractile vacuole. Moreover, Golgi proliferated vesicles in P. palmivora were bound by single membranes and were much smaller than the double membrane-bound vesicles under discussion. It is concluded that the coalescence of these vesicles with the tonoplast of the contractile vacuole in zoospores of P. palmivora (see page 48) is part of the periodic functioning of the water expulsion vacuolar system, which is also typical of zoospores of other organisms (47, 54, 56, 80).

Spherosome/microbody-like organelles with or without bars were observed in P. megasperma var. sojae (54). In P. parasitica (47, 80), similar organelles were also reported. Microbodies in zoospores of P. palmivora are very similar to the organelles described above; some contain the bar-like material and others have only an amorphous matrix. In germinating cysts, another type of microbody contains a combination of bars and thin striations (Plates 40 and 41).

These microbodies, particularly the form with striations and bars, are similar to structures labelled parastrasomes in precleavage sporangia and zoospores of P. capsici by Williams and Webster (103). The different microbodies in P. palmivora could represent three distinct types possibly with different properties, for example, in the nature of their enzyme systems. On the other hand, they may simply represent variations in the form or stage of development of the same type of organelle, or perhaps variations associated with the plane of section (eg. microbodies with and without bars).

The structure of flagella, kinetosomes and rootlets in zoospores of P. palmivora is very similar to those of P. infestans (61), P. parasitica (47, 80) and Aphanomyces euteiches (56) and Saprolegnia sp (46). Although there is a short rootlet bundle from the anterior kinetosome towards the narrow end of the pyriform nucleus in P. palmivora, there is no contact with the nucleus or nuclear envelope compared to the direct contact reported in developing zoospores of P. infestans (61).

Another structure that has been reported in zoospores of several organisms are those designated as enlarged endoplasmic reticulum containing tubules (ERt) in A. euteiches by Hoch and Mitchell (56). In P. parasitica similar structures described by Reichle (80) as vesicles containing tubules or described as microtubular bundles by Hemmes and Hohl (47) were also observed. In zoospores and cysts of P.

palmivora, similar organelles were observed (Plates 20, 37 and 38). Although the connection with endoplasmic reticulum shown in A. euteiches (56, Figs. 18-20) was not so clearly observed in the present study, it is believed that they too represent an enlargement of endoplasmic reticulum cisternae. The tubules in the enlarged ER cisternae are presumptive mastigonemes (flimmers) as reported in several species including Saprolegnia sp. by Heath et al. (46). In motile and encysted zoospores of P. palmivora, these tubular structures may represent remnants of the presumptive mastigonemes (flimmer hairs), held over from sporangia. These tubules would also provide a source of mastigonemes should the germinating cysts produce zoosporangia at a later stage as occurs in P. parasitica (47) and other species.

The major storage product in zoospores of P. palmivora is lipid. Lipids have also been observed in other zoospores, biflagellate and uniflagellate, including P. parasitica (47, 80), Sclerophthora macrospora (Peronosporales, 34) and Aphanomyces euteiches (55, 87); Allomyces macrogynus (52), Blastocladiella emersonii (81) (Blastocladiales), Monoblepharella sp. (Monoblepharidales, 36), Olpidium brassicae (Chytridiales, 93) and Rhizidiomyces apophysatus (Hyphochytriales, 35). In Phytophthora megasperma var. sojae (54) similar unidentified amorphous material was also observed. In most of these species, no limiting membranes were observed around the amorphous material and therefore it will be more appropriate to label them as lipid bodies

instead of amorphous "vesicles".

Granular vesicles observed in P. palmivora have also been found in some other species including P. megasperma var. sojae (54) and P. parasitica (80). In the latter, the contents of granular vesicles were not always uniformly distributed; this could be a result of fixation procedures used in those studies for at times similar organisation within granular vesicles were noted in the present study, associated with incomplete fixation procedures as judged by other criteria. Zoospores of Aphanomyces euteiches on the other hand, contained vesicles described as granular which possessed distinct cortex and matrix, an organisation quite different from what is generally regarded as characteristic of granular vesicles. Furthermore, Hoch and Mitchell (56) found that during encystment some of these vesicles were extruded from the spore. It seems evident that these structures comprise a distinct type of organelle occurring only in this species. Returning to the granular vesicles in zoospores and cysts of P. palmivora cytochemical tests showed that they contain protein material.

With regard to crystalline vesicles, these have also been reported in zoospores of all the biflagellate species mentioned above (47, 54, 56, 61, 80), and in sporangia of some of them (55, 57, 87, 101, 103). The historical basis for naming the contents of these vesicles as "crystalline" does not appear to have any connection with the presence of crystals as such but rather with the regularity of the stri-

ations of the material constituting the inclusions (101).

The contents of these major "vesicles" have not been identified to date in most cases nor have the changes that they undergo during motility and germination been followed. As a result of the present work with P. palmivora, considerable information is now available on their biochemical composition and fate in zoospores and germinating cysts. The contents of the amorphous, non-membrane-bound bodies are lipid whilst crystalline vesicles contain lipid together with other unknown components, which are neither protein nor carbohydrate in nature.

Several unidentified bodies have been reported in zoospores of some species of the Peronosporales and Saprolegniales; for example in Aphanomyces euteiches, Hoch and Mitchell (56) observed an unknown body containing helical fibres. In P. palmivora zoospores and cysts an unknown body, previously unreported in zoospores or cysts, with ribosome-like particles and amorphous matrix remains unidentified both as to composition and function.

Another unknown body most commonly observed adjacent to, and appearing to arise from dictyosomes in cysts of P. palmivora are those with internal concentric membranes (Plate 23). These bodies have not been reported in zoospores of other species. Several types of membrane structures have been observed in different organisms and various functions have been assigned to them. For example, in Neurospora tetrasperma (64) membrane structures associated

with ER have been related to the synthesis of ER during germination of ascospores. The membrane bodies associated with dictyosomes in P. palmivora may possibly play a role in germination (104) particularly with the initiation of germ tubes or branching (43). This is because these bodies were not observed in motile zoospores or freshly encysted zoospores but they were observed after 30 min encystment and in germinating cysts. These bodies are morphologically different from the vesicles associated with hyphal apex described by Grove et al. (41, 42). Further cytochemical work will be required to identify and assign a role to these membrane structures in P. palmivora.

The general features of encystment and germination of P. palmivora zoospores are similar to those of P. parasitica (47), Pythium aphanidermatum (41) and Aphanomyces euteiches (56). Although similar changes as those described for P. palmivora during cyst germination were observed in electron micrographs of A. euteiches cysts, Hoch and Mitchell (56) made no mention of vesicle contents. During germination, the lipid in lipid bodies and crystalline vesicles of P. palmivora were broken down and the protein contents of the granular vesicles disappeared in the cysts.

Turning now to the biochemical changes, the objective of my investigations was to establish the role of endogenous metabolic reserves and associated enzyme systems during motility of zoospores and cyst germination and to relate these phenomena to any ultrastructural changes.

On the subject of the contents of lipid bodies, crystalline vesicles and granular vesicles and their function, lipids are utilised as the energy source for motility and germination. Associated with the decrease in lipids, there was an increase in proteins and carbohydrates during germination (Tables VII to IX) in P. palmivora. Increase in total protein has also been observed in germinating spores of other, quite unrelated fungal species including Aspergillus niger, Penicillium atrovenetum, P. oxalicum - imperfect states of Eurotium and Carpenteles, respectively (Eurotiales) and Trichoderma viride - imperfect state of Hypocrea (Hypocreales) (73). Ultrastructural observations

showed the disappearance of protein (granular) vesicles at a time when the protein content of the germinating cysts was increasing in Phytophthora palmivora. It appears that the proteins in these vesicles and possibly elsewhere in the cytoplasm, e.g. the multivesicular bodies (Plate 34) frequently observed in germinating cysts, are resorbed and the material possibly rearranged to produce other proteins. Accompanying these processes, the breakdown of storage lipids also contributes to the production of more protein.

The major storage product in zoospores is lipid and this resembles the situation in some other fungal spores as does the decrease in lipid content during cyst germination. Thus, in Rhizopus arrhizus (Mucorales, 44) total lipids decreased for 2 h and then increased again for the next 6 h of germination. During the same period, free fatty acids also decreased. In P. palmivora, lipids as measured by acyl esters and free fatty acids decreased both during zoospore motility and cyst germination.

Ultrastructural observations showed a close association between microbodies, mitochondria and lipid bodies in zoospores and cysts of P. palmivora. Associations such as these have been reported between microbodies (peroxisomes) and lipid bodies in fat storing seeds and endosperm tissue (44, 46, 71, 72, 180). Since the microbodies in P. palmivora contain α-hydroxy acid oxidase and the mitochondria catalase (peroxidatic) activity, their spatial relationship

with lipid bodies would tend to support the theory that in fat storing cells, microbodies are involved in lipid metabolism. This includes the mobilisation of lipids and their subsequent conversion to carbohydrates or proteins through the glyoxylate cycle. If the microbodies in zoospores and cysts possess enzymes of the glyoxylate cycle, in addition to the oxidases, then these organelles should be more specifically termed "glyoxysomes" according to the definition of Breidenbach and Beevers (15). However, use of this terminology must await further enzymatic characterisation of the microbodies involved.

In investigating the distribution of key enzymes of the glyoxylate cycle, both isocitrate lyase and malate synthase, were found in the 46,000 x g supernatant (S4). This fraction could have contained intact microbodies, but it was not possible to determine the exact composition of organelles in the S4 fraction. It is also possible that this fraction contained a mixture of soluble, cytoplasmic enzymes as well as those from organelles. In the latter case, enzymes normally restricted to mitochondria or microbodies may have been released into the cytoplasm if these organelles were broken when spores were sonicated to yield cell-free extracts. This would mean that the S4 fraction is not composed of any one particulate matter. It would be interesting, impossible though it may seem, to produce the vast quantities of zoospores necessary for the identification and analysis of the enzymes in the microbody and

mitochondrial fractions. In the present study, it was extremely difficult to obtain sizable pellets, after differential centrifugation, which could be processed for electron microscopic identification of the organelles in the various fractions, i.e. P1, P2, P3 and S4.

As described for the glyoxylate enzymes, most of the activity of NAD-isocitrate dehydrogenase, which is normally restricted to mitochondria, was also registered in the 46,000 x g supernatant. Other enzymes which are both mitochondrial and cytoplasmic, e.g. NADP-isocitrate dehydrogenase and malate dehydrogenase, were also restricted almost completely to the S4 fraction. For this reason it was concluded that this supernatant fraction contained enzymes from both the soluble, as well as particulate portions of the cytoplasm. Perhaps separation of this fraction by sucrose density gradients (29) could separate the organelles and enzymes further.

The presence of both isocitrate lyase and malate synthase in spores of P. palmivora (Table XII) would indicate that lipid conversion to protein and carbohydrates was through the glyoxylate pathway. The inability to detect malate synthase when smaller quantities of spores were used (Table XI) was most probably a result of the low activity of this enzyme compared to isocitrate lyase (Table XII). Isocitrate lyase activity was highest in freshly liberated zoospores when the lipid content was also at its highest, and then it decreased as the lipid

substrate also decreased during the 6 h of motility and 2 h encystment/germination period. In *Aspergillus niger*, *Penicillium oxalicum* and *Trichoderma viride* (73), isocitrate lyase activity also decreased during spore germination.

The tricarboxylic acid cycle enzymes which were assayed remained unchanged (malate and NAD-isocitrate dehydrogenase) or decreased (NADP-isocitrate and succinate dehydrogenase) during zoospore motility. After 2 h encystment/germination, the activities of all these enzymes increased. Thus, at the time when the tricarboxylic acid cycle intermediates were being converted into carbohydrates and proteins, the specific activities of the enzymes mediating this cycle increased.

This increase in activity of enzymes during germination of cysts of *Phytophthora palmivora* is not a unique process as bourne out by studies on spores of other fungi (17). Thus, the germinating spores of *Ustilago zeae* (Ustilaginales, 39) were found to have synthesised some enzymes of the hexose monophosphate shunt and tricarboxylic acid cycle which were absent from or present at very low levels in resting spores or spores after 6 h incubation. The synthesis and increase of these enzymes occurred after 12 h germination. At the same time, enzymes of the glycolytic pathway which were present at low levels in resting spores increased greatly during germination. In *Aspergillus niger* and *Trichoderma viride* spores (73) succinate dehydrogenase activity increased during protein synthesis and germina-

tion.

The present investigations with P. palmivora show that the glyoxylate cycle is important in lipid conversion and provides the carbon atoms and energy required for motility. However, the tricarboxylic acid cycle also operates during this period. During germination, the tricarboxylic acid cycle assumes a more important role in providing energy for germ tube growth and intermediates for the synthesis of carbohydrates and proteins.

From the results of their work with P. drechsleri, Barash and his co-workers (3) hypothesise that the main energy requirements for motility and initial development of a germ tube appeared to be independent of the availability of external nutrients.

My own investigations with P. palmivora show that the suggestions outlined above are indeed true. Of the total lipids present in freshly liberated zoospores, about 44% of glycerides or 60% free fatty acids were utilised during 6 h motility. Of the remainder about 48% glycerides or 74% free fatty acids were utilised in just 2 h encystment/germination. Carbohydrate content also decreased slightly, about 24%. However, after 2 h germination, carbohydrates and proteins were synthesised resulting in an increase of 13% and 65%, respectively, over the amounts present in encysted zoospores. The results in Table VIII indicate that more lipids are used up per hour during germination than for motility. This could explain why motile zoospores would

be less dependent on external energy sources than germinating cysts, as Barash et al. suggested (3).

The net result of carbohydrate and protein synthesis during germination and lipid utilisation for both motility and germination was the drastic decrease in dry weight of spores. The method adopted for production of zoospores of P. palmivora for these studies made it possible to produce suspensions which were relatively nutrient-free (Table I). Results of nutrient analyses show that the low levels of carbohydrates and protein in the suspensions were not utilised by zoospores during the 6 h of motility and there was no significant difference between the values obtained for suspensions at 0 and 6 h. The zoospores and germinating cysts continued to lose weight with time since lost carbon atoms were not (i.e. zoospores) or could not (i.e. cysts) be replenished from external sources. Thus it appears that the longer the periods of motility and germination, the more endogenous substrates are used up and hence the greater the need for external nutrients for continued growth.

In conclusion it should be emphasised that this study with zoospores and cysts of P. palmivora confirmed the ultrastructural similarities with other Peronosporales and Saprolegniales. But more importantly it has provided a considerable amount of new knowledge on vesicle contents, and inclusions and their relation to metabolism. In the latter connection, it is interesting to note that the biochemical processes in zoospores and germinating cysts are

similar to those in spores of quite unrelated fungi. The presence of hitherto unrecognised organelles have also been demonstrated. And as so often happens in investigations of this kind, this study has also raised various points of great biochemical interest, which could not be pursued within the general framework of this study. These include such questions as what enzymes do the different forms of microbody contain and what is the composition (enzyme or otherwise) of the various unidentified bodies especially the ones with internal concentric membranes and their role in germination. Cytochemical and biochemical solutions to these and related questions would help to elucidate more fully the processes that go on in zoospores and cysts during motility and germination.

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APPENDIX

SPECIFIC ACTIVITIES* OF ENZYMES AT DIFFERENT STAGES IN ZOOSPORES AND CYSTS OF *Phytophthora palmivora*

ENZYME	STAGE AND REPLICATE								
	T1			T2			T3		
	I	II	III	I	II	III	I	II	III
Isocitrate lyase	17.02	22.21	20.49	10.92	8.71	9.49	7.42	6.03	5.74
NAD-isocitrate dehydrogenase	34.83	32.75	33.11	35.65	37.88	35.88	44.64	42.57	48.38
NADP-isocitrate dehydrogenase	15.12	16.08	15.94	7.13	6.39	6.06	22.32	23.70	23.22
Malate dehydrogenase	246.54	247.62	247.80	270.90	382.92	307.73	402.12	450.37	458.06
Succinate dehydrogenase	0.10	0.06	0.07	0.04	0.04	0.03	0.11	0.11	0.11

* Specific activity expressed as units per mg protein.