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How to cite:

Hankinson, E. (1972) *Some ultrastructural and physiological studies of Phycomyces Sporangiophores*, Durham theses, Durham University. Available at Durham E-Theses Online: http://etheses.dur.ac.uk/10414/

Some Ultrastructural and Physiological
Studies of <u>Phycomyces</u> Sporangiophores.

A thesis submitted by

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for the degree of Master of Science
in the University of Durham.

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University of Durham.

June 1972.

C O N T E N T S

A B S T R A C T

Although the Phycomyces sporangiophore has been the
object of numerous physiological investigations little
is known of its ultrastructure or of the changes that
occur within it during growth and development. This is
partly due to the failure of some workers to obtain
satisfactory fixation of the sporangiophore for
ultrastructural investigations.

Investigations were made for suitable fixation
procedures which might help to elucidate the fine structure
of the sporangiophore during growth and development.
Using the fixation procedures developed, long microfilaments
lying almost parallel to the long axis of the sporangiophore
and closely associated with mitochondria were revealed.
These microfilaments probably determine the path along
which the mitochondria move and may account for the
multistriate streaming of organelles as seen in the phase
contrast microscope. Investigations for a discrete gravity
receptor proved negative but did reveal an upward
displacement of the large central vacuole concurrent with
the development of a geotropic curvature. I was not able
to find any organelle which might function as a
photoreceptor.

The ultrastructural changes occurring during the
development of the sporangium and germination of the spores
are described.

Section I

Aim of Study.

Although _Phycomyces blakesleeanus_ has been the object of numerous investigations and much is known about its physiology, biochemistry, genetics and habits, there remains several phenomena still unexplained, an explanation of which might be assisted if a more detailed knowledge of the fine structure were available.

It was the aim of this study:-

1. To investigate the possibilities of examining the vegetative structures of _Phycomyces_ under the electron microscope. (Several attempts have been made but in all cases considerable difficulty has been experienced in the preparation of the material prior to examination.)

2. To determine if such phenomena as protoplasmic streaming, geoperception and photoperception of the sporangiophore had any basis in recognisable ultrastructural features associated with, and perhaps responsible for, the observed behaviour.

3. To investigate the development and maturation of the sporangia and the germination of the spores.

The investigation was, therefore, essentially a study by electron microscopy and much of the evidence appears in the electron micrographs.

Section II

Introduction.

IIa Phycomyces - A Survey

Phycomyces blakesleeanus belongs to the fungal
class Zygomycetes, members of which are characterised
by the production of non-motile vegetative spores lacking
flagella on a mycelium that is almost completely without
septa

The genus Phycomyces was first created in 1823 by
Kunze who distinguished one species only - Phycomyces
nitens. At the present time there are said to be
three species (Benjamin & Hesseltine 1959) recognised
within the genus although only two are known to be
available. These are Phycomyces blakesleeanus
(Burgeff 1925) and Phycomyces nitens. Burgeff separated
the species on the basis of incompatibility. Although
the two interacted sexually and formed zygospores which
germinated to produce sporangia the spores were sterile.

The organism has been of biological interest
almost since its discovery. Interest was first aroused
by the large, erect, cylindrical, coenacytic
sporangiophores which may grow to a height of 10 cm or
more, and their striking phototropism, and later by the
discovery of self incompatibility by Blakeslee (1904)
and the requirement of Phycomyces for vitamin B_I by
Schopfer (1934) and Burgeff (1934).

Each sporangiophore bears a single spherical
sporangium which may be up to five times the diameter
of the supporting sporangiophore.

Many investigations have been made into the
mechanism of sporangiophore growth of this fungus
(Castle 1942, 1958, 1959) and particularly into the
spiral growth so characteristic of the Stage IV
sporangiophores. About 2-3 hours after the completion
of sporangium development,growth in height of the
sporangiophore, which ceases during sporangium
formation, is resumed and within a few hours a steady
growth of about 3 mm per hour is attained and may
continue for several hours. Growth of the
sporangiophore is limited to a growth zone which
occupies the first 2-3 mm of the sporangiophore below

the base of the sporangium. Growth is not uniform throughout the zone, but reaches a maximum at about 0.3 mm below the sporangium swelling and declines to zero at the base, about 2 mm below the sporangium base (Castle 1958). During the growth of a mature sporangiophore - Stage IV (Castle 1942) the sporangium and each section of the growth zone rotates around the vertical axis, the rotational velocity being fastest for the sporangium and decreasing to zero at the base of the growing region. This rotation is at first anticlockwise (Z helix, Raelofsen 1959) but later changes to a clockwise direction (S helix, Raelofsen 1959)(Castle 1942). Growth within the zone can be considered to be a measure of the rate of synthesis of wall material and its intersusception between the previously existing microfibrils. It is believed by some workers that the spiral growth of the sporangiophore reflects the submicroscopic fibrillar anatomy of the sporangiophore wall (Hawker 1965)

The sporangiophore is sensitive to light - a response first discovered and extensively studied by Blaauw in 1909, 1914, 1915 and 1918 (Bergman et al 1969) and to gravity, the most detailed study of which has been made by Dennison (1959). It has more recently been shown to respond to humidity changes and to an imposed stretching force. The sporangiophore also shows an avoidance response first reported by Elfving in 1881 and recently used in investigations on the habituation of the light growth response by Ortega and Gamow (1970). These various stimuli affect the rate of growth of the sporangiophore by producing either a tropic response or a temporary change in the rate of growth in the growth zone.

Below the growth zone of the sporangiophore and in the mycelial hyphae protoplasmic streaming can be readily observed by phase contrast or interference microscopy. In thin or old sporangiophores particles can be seen moving in opposite directions along a seemingly single strand of cytoplasm. The axes of these strands lie parallel to each other but often deviate by several degrees from the axis of the sporangiophore. This streaming was first described

by Oort and Raelofsen (1932) who believed it played some role in determining the spiral growth of the sporangiophore. Later studies by Pop (1938) produced conflicting evidence which led him to believe that there was no relationship between the two phenomena of streaming and spiral growth.

IIb. Auxins and Tropic Responses.

The sporangiophores of Phycomyces show three pronounced responses to external stimuli viz: a light growth response, a phototropic response and a gravitational response. So far as higher plants are concerned it is generally agreed that the growth responses to light are the result of the regulation of growth rates by auxins and similar regulators are considered to be the controlling influence of growth responses to gravity.

Banbury (1952) was unable to show any growth response to externally applied indolylacetic acid in concentrates of between 10 - 10,000 ug/ml. in the sporangiophores of Phycomyces, despite the evidence for the occurrence of this substance in fungi. He was, however, able to produce sharp curvatures in sporangiophores treated with griseoflavin paste containing 100 ug/ml. The response was the result of a change in direction of growth as a result of the application. Similar applications below the growth zone produced no apparent reaction. Despite the fact that griseoflavin is produced by several species of Penicillium and fulfils one of the criteria for a myco-auxin by its affect on the extension of chitin walled hyphae it has not been demonstrated in Phycomyces mycelium and it seemed reasonable to assume that it is not involved in the photo-responses of Phycomyces sporangiophores.

IIc. History of Ultra-Structural Studies.

Few investigators have attempted to elucidate the fine structure of this much studied fungus. The earliest ultrastructural investigation on Phycomyces was probably that of Raelofsen (1951) when he outlined the structure of the cell wall as understood from the results of experiments using double refraction and electron microscopy. This was followed in 1963 by the

results of the work of Preston on wall structure and
cell growth in which he indicated that it seemed most
probable that there exists a relationship between the
arrangement of the chitin fibrils in the secondary
cell wall, the spiral growth and the spiral streaming
of the protoplasm of the Stage IV B sporangiophore.

Peat and Banbury (1967) reported on the fine
structure of the growing region of the Stage I and
Stage IV B sporangiophores. In this work they were
able to show that in many respects the fungal hypha
possessed most organelles in common with higher plants
and animals – nuclei, mitochondria, vacuoles, lipid
bodies, glycogen and some endoplasmic reticulum. In
their micrographs the latter was comparatively
sparse and unlike the condition commonly reported in
higher plant and animal cells showed no obvious
continuations with the membranes of the plasmalemma and
nuclei. The micrographs published by Peat and Banbury
showed some evidence of possible organelle distortion
during preparation – nevertheless their findings
agreed for the most part with those of earlier workers
with fungal material and summarised in "Ultrastructure
of Fungi", Bracker (1967) and Fine Structure of Fungi",
Hawker (1965).

However Peat and Banbury (1967) were not able
to find any ultrastructural basis for the spiral streaming
of the protoplasm or the striking tropic responses
of the sporangiophore to light and gravity.

A new organelle not observed by Peat and Banbury
is the autophagic vesicle (Thornton 1968). Such
vesicles were reported by that author as occurring in
large numbers below the nuclear zone of the Stage I
sporangiophore where they were often seen to contain
cytoplasmic constituents in various stages of
disintegration. It was suggested that these vesicles,
which start out as small cisternae, coalesce around the
portion of the cytoplasm to be disposed of, so engulfing
it, and finally disgorge the degradated contents into
the central vacuole. Having made their first appearance
in the swollen hyphal primordia of very young
sporangiophores, the vesicles were reported as
persisting throughout the development of the
sporangiophore, and to function after sporulation and
maturation of the sporangium. (In view of the immense
difficulty experienced by myself and other workers in
obtaining satisfactory Gluteraldehyde post Osmium

tetroxide fixation of <u>Phycomyces</u> sporangiophores
and my complete failure to observe comparable
structures, although vesicles which coalesce
with the central vacuole have been observed in
material either similarly fixed and embedded in
Methacrylate (Mohr & Cocking 1968), or fixed in
potassium permanganate and embedded in Araldite, I
remain somewhat unconvinced of the existence of
autophagic vesicles).

Investigations of the fine structure of
<u>Phycomyces</u> have been slow because of the inherent
properties of the cuticle and cell wall which make
preparation of the material for electron microscopical
examination difficult; yet a knowledge of the ultra-
structural features, particularly of the sporangiophore,
might well assist in explaining some of the phenomena
which are so characteristic of this fungus and
which have so far remained inexplicable in terms
of light microscope observations and sensory
physiology.

When beginning investigations on the
ultrastructure of <u>Phycomyces blakesleeanus</u> it was
first essential to develop a technique of material
preparation which would result in the minimum
disruption in shape and distribution and structure
of the constituent organelles of this extremely
large cell.

SECTION III

MATERIALS AND METHODS.

Source of Strain

Phycomyces blakesleeanus plus and minus strains
were obtained from C.B.S. Baarn and were the same
as those used by Peat and Banbury (1967)

IIIa. Media and Growth Vessels

Culture medium. The fungus was grown on plates
or solid slopes of 2% malt agar throughout the
investigation.

The medium was made up as follows:-

Malt-Agar Medium

Malt extract	20.0 grammes
Distilled water	1000.0 cm^3
Agar	20.0 grammes

Two other media were tried at the beginning of the
work but as the growth on these substrates was not
as good as that on the 2% malt agar, they were
abandoned. These media were a malt extract-
glucose agar and a malt extract-glucose-peptone
agar.

Malt extract-glucose agar

Malt extract	20.0 grammes
Glucose	20.0 grammes
Agar	20.0 grammes
Distilled water	1000.0 cm^3

Malt extract-glucose-peptone agar

Malt extract	20.0 grammes
Glucose	20.0 grammes
Peptone	0.5 grammes
Agar	20.0 grammes
Distilled Water	1000.0 cm^3

The growth was particularly poor on the malt extract-glucose-peptone medium, the fungus producing a sparse mycelium and a poor crop of sporangiophores.

Culture vessels.

(a) Petri dish cultures. For routine work the fungus was grown in sterile plastic petri-dishes, diameter 8·8 cm. These were kept in sterile glass petri dish tops,diameter 10·1 cm.,and covered with sterile crystallizing dishes,diamter 9·0 cm. and height 4·6 cm.,(fig 1).

(b) Oxoid cap cultures were used when cultures were to be centrifuged. Small oxoid caps,diameter 2.5 cm. height 2.5 cm,held about 12.5 ml. of culture medium. Eight culture vessels could be placed on a sterile glass petri-dish top,diamter 10·1 cm.,and covered with a sterile crystallizing dish,diameter 9··0 cm. and height 4·6 cm.,until growth of the sporangiophores was sufficient for use in the centrifuge. (figs. 2a and b)

(c) Protective caps. Before use, the oxoid cap cultures were covered with Morton Closure Caps, diameter 2.7 cm. and height 3.8 cm.,(fig 3).

IIIb.1) Turntable for Geotropic Experiments

A turntable of tufnol,38 cm. diameter and 3 cm. deep,was made to fit a slow speed M.S.E. centrifuge.
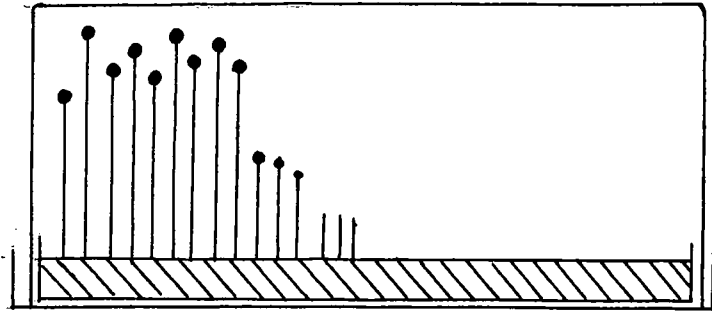
Fig 1. Culture vessel in which material used to investigate protoplasmic streaming and sporangium development was grown.
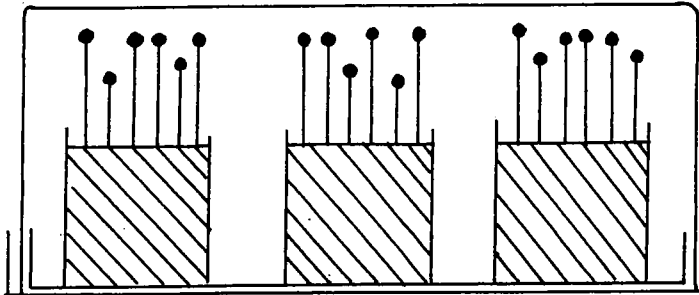

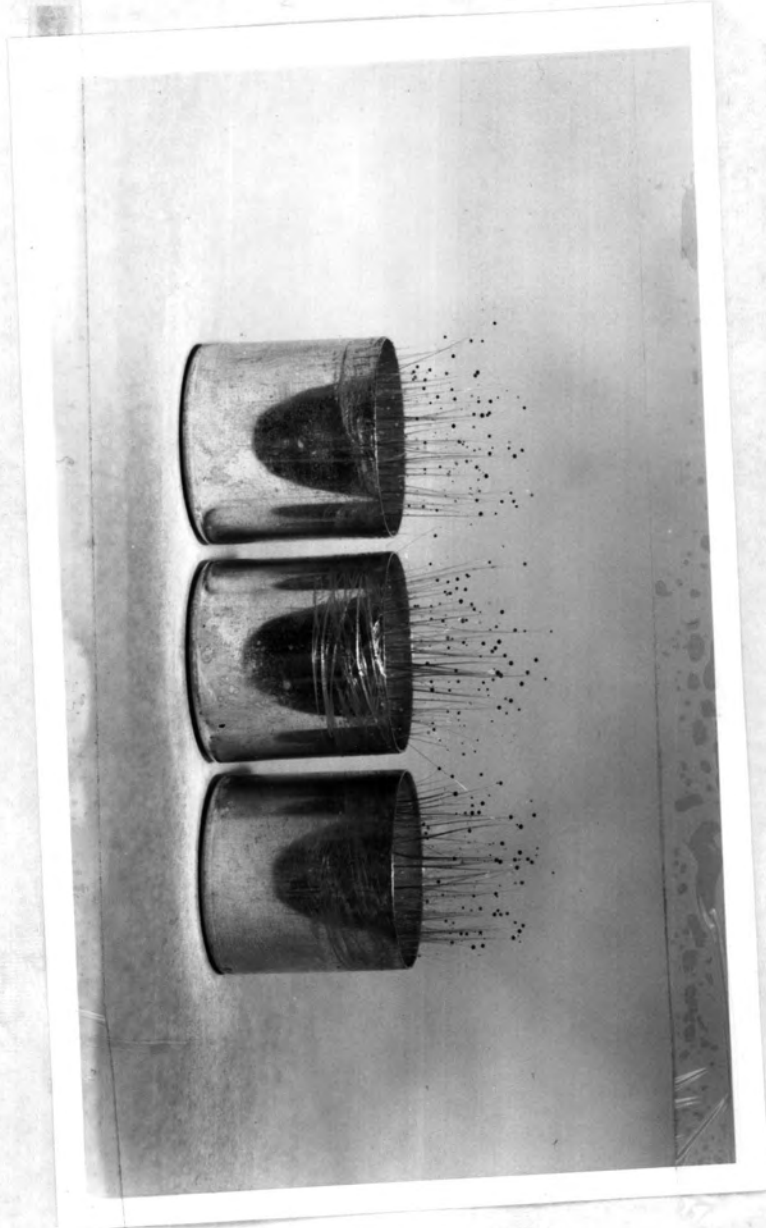Fig 2a. Vessels in which material was grown for use in geotropic experiment.

Fig 2b   Culture vessels used on M.S.E. Centrifuge Turntable
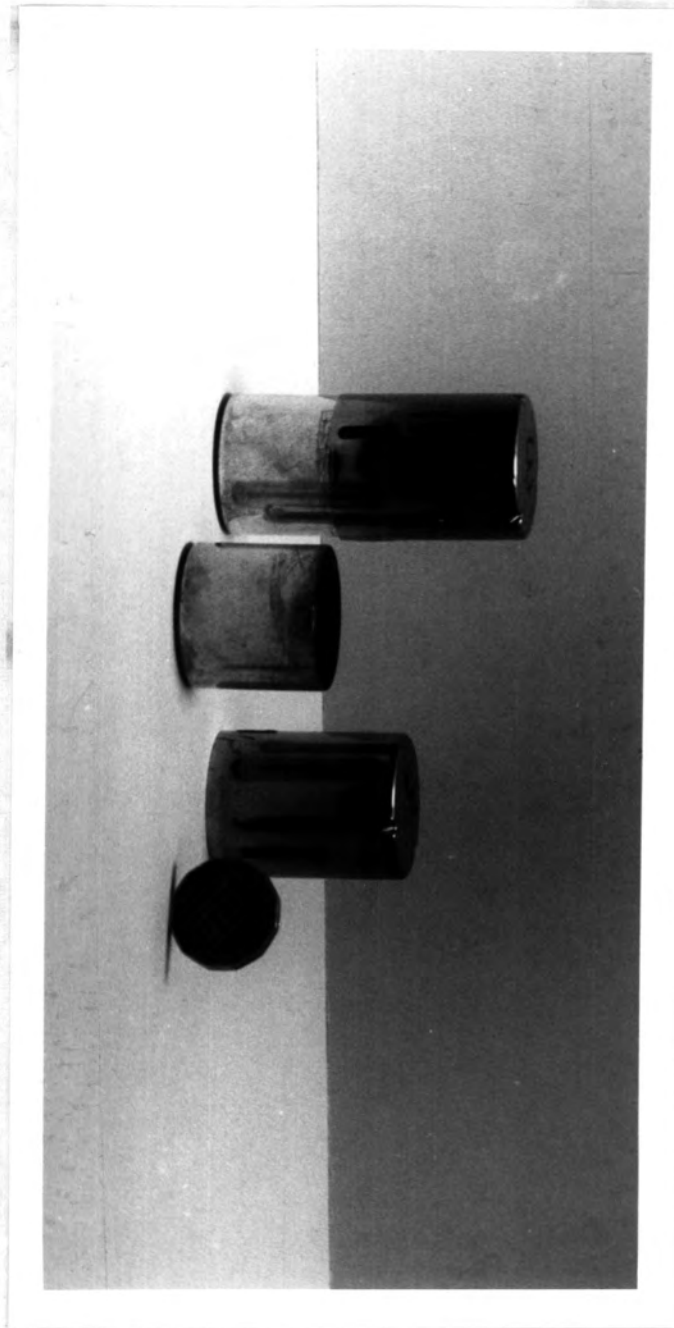
Fig 3 Culture vessel and Morton Closure Cap

This was bored with holes,2.8 cm. in diameter and
2.2 cm. deep,into which the oxoid cap cultures
could be firmly fitted. The holes were
with their centres 3.81 cm., 5.08 cm., 7.62 cm.,
10.16 cm., 11.43 cm., and 15.24 cm. from the centre
of rotation of the turntable (Fig. 4)

2) Growth Chamber for Phototropic Experiment

A box 51cm X 55cm X 56cm was made light proof by
coating both the inside and outside with black
paint. It was fitted with two 3 watt bulbs so
that two opposite light beams each made an angle
of approximately 45° with the centre of the chamber
floor. The two beams were of closely matched
intensity and when necessary the right beam could
be cut off to produce asymmetrical illumination
of vertical sporangiophores placed in the centre
of the chamber floor. The front of the box was
covered with black polythene sheeting.

IIIc. Sterilization and Inoculating procedure

The culture medium and vessels were sterilized
by autoclaving at a pressure of 15 lb./sq.in. for
15 min. The culture medium was inoculated with
mycelium from a 6-7 day old culture transferred by
means of a sterile platinum loop. This produced
an excellent crop of sporangia about four days
after inoculation.

Growth conditions.

All cultures were kept at ambient room
temperature - approximately 20°C. Cultures used
in geotropic experiments were transferred to the
dark at the appearance of the Stage I sporangiophores
and kept there until several of the sporangiophores
bearing mature sporangia were 2 cm. to 2.5 cm. tall.

Fig. 4   MSE centrifuge with turntable made to take
oxoid cap culture vessels.

Cultures used in phototropic experiments were subjected to continual illumination from a diffuse light at 60 cm. distance until several sporangiophores bearing mature sporangia were 2 cm. to 2.5 cm. tall.

IIId. Collection of Spores

Spores were collected from 8-9 day old plate cultures as these produced the greatest number of viable spores. The plates were flooded with 3 cm^3 of sterile distilled water drained and rinsed with another 3 cm^3 of sterile distilled water. The concentrated spore suspension thus obtained was diluted to 10 cm^3 final volume.

Medium for Germination of Spores.

The spores were germinated on a potato extract. 30 gm. potato were macerated in 5 cm^3 of sterile distilled water. This was then made up to 25 cm^3 by the addition of a further 20 cm^3 of sterile distilled water. The pulp was shaken vigorously for several minutes to bring as much soluble material as possible into solution. The liquid was then decanted and finally filtered through normal grade Whatman filter paper to remove the insoluble starch grains. Finally 10 cm^3 of this soluble extract were added to the spore suspension in a petri dish.

Culturing of Spores.

The spores were cultured in a thin film of potato extract at the bottom of a petri dish. This was incubated at $40^{\circ}C$ for 15 minutes and

then kept at ambient room temperature. Spores taken from an 8-9 day old culture germinated in 5-6 hours.

IIIe. Electron Microscopy

1. Fixatives

(a) The most satisfactory general fixative was 2% $KMnO_4$ w/v (not buffered)

(b) A fixative composed of equal volumes of 4% Gluteraldehyde and 2% OSO_4 in 0.1 M cacodylate buffer gave the most satisfactory fixation of microfilaments.

Other fixatives were investigated and these are described in a section dealing with fixation

2. Resin

The only one which gave repeated satisfactory embediment of <u>Phycomyces</u> was TAAB Embedding Resin

This was prepared as follows:-

    Resin   50 cm^3

    Dodecenylsuccinic anhydride (D.D.S.A.) 50 cm^3

    Tridimethylaminomethyl phenol

    (D.M.P./30) 0.01 cm^3 per 5 cm^3 of resin

3. Staining

The two stains most frequently used were

(a) Uranyl acetate - a 1% solution w/v made up in absolute alcohol

(b) Lead citrate - Reholds

    1.33 g Pb $(NO_3)_2$; 1.76 g Sodium citrate

    $(Na_3 (C_6H_5O_7)2H_2O)$ in 30 ml. water

    This was shaken vigorously for 1 minute to complete conversion to lead citrate. After 3o min.

8.0 ml. of IN. NaOH were added and the suspension
diluted to 50 ml.

The sections were stained by floating them on
drops of the above solution placed on wax surfaces.
Potassium permanganate was used to stain some of the
sections prepared from simultaneous gluteraldehyde/
osmium tetroxide fixation and gave satisfactory
results.

4. <u>Vessels for fixation</u>.

Rectangular troughs 3 in. by 1 in. by 1 in.
made from microscope slides were used instead of
vials for the fixation of the Stage IV B (fig. 5.)
sporangiophores. In these troughs the sporangiophores
could be drawn down below the surface of the fixative
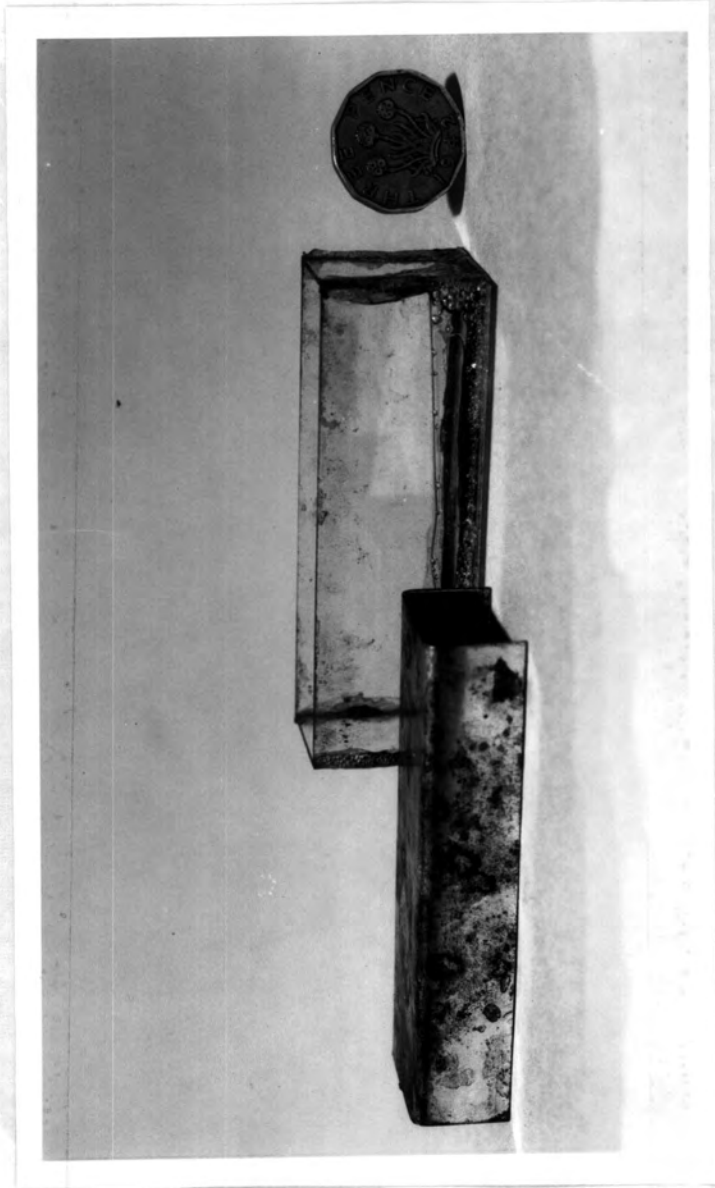by their base without causing deformation of the
cell structure.

Fig 5 Trough used to prevent damage of the-long
Sporangiophores during fixing

SECTION IV

INVESTIGATIONS INTO THE PREPARATION OF PHYCOMYCES FOR ELECTRON MICROSCOPY

IVa. Problems pertaining to Phycomyces.

The presence of a chitinous wall surrounded by a waxy cuticle made the penetration of fixatives and embedding resin extremely difficult in all parts of the sporangiophore except the growing region and sporangiophore base. Fixatives penetrated these latter two regions fairly rapidly, but even so electron microscope examinations of these sections suggested that considerable distortion of the cell contents had occurred.

Fixation.

The following fixatives were tested on the Stage IV B sporangiophore and on sporangia (see Table I) at different stages of development. Only two fixatives were found to be satisfactory.

All fixations in gluteraldehyde were followed by post fixation in 2% aqueous OsO_4 w/v for a period of from 30 min to 2 hours at either $4^{o}C$ or room temperature.

As a fixative for the sporangia, the 2% $KMnO_4$ w/v proved to be the most satisfactory and it was therefore decided to continue to make further studies of the development of the sporangia and germination of the spores using 2% $KMnO_4$ as the fixative. The periods of fixation however varied from 20 mins to 12 hours depending on the stage of development. It is recognised that using only one fixative, $KMnO_4$, for work on sporangia places one

TABLE I

Fixative	Temperature	pH	Fixation period
4% Gluteraldehyde in 0.1M phosphate buffer	4°C	7.1	16 hours
"	"	6.2	"
"	"	4.5	"
" containing 0.007M Calcium chloride	Room temp.	7.1	"
" in 0.1M phosphate buffer	"	"	24 hours
" plus 1% OsO_4 v/v in 0.1 M phosphate buffer	"	"	12 hours
" "	"	"	40 mins.
" in 0.5M sucrose	4°C	6.2	16 hours
2% " in 0.1M phosphate buffer	"	"	"
3% "	"	7.1	"
5% "	"	"	20 hours
6% "	"	"	16 hours
6% "	"	"	20 mins.
25% "	"	"	30 mins.
5% Acrolein in 0.1M phosphate buffer followed by 1% OsO_4v/v in 0.1M phosphate buffer	Room temp.	7.1	1 hour
1.25% Acrolein in 0.1M phosphate buffer	Room temp.	7.1	2 hours
50% aqueous dimethylsulphoxide (DMSO) containing 2% gluteraldehyde buffered with 0.02M S-collidine followed by	"	7.4	1 hour
3% OsO_4 in veronal acetate buffer	"	4.0	2 hours
25% Dimethylsulphoxide plus 3.15% gluteraldehyde in 0.1M cacodylate buffer	"	7.0	"
2% OsO_4w/v	"	"	"
2% $KMnO_4$w/v plus one part of detergent per 1000 parts of fixative	"	"	1 hour
3.1% Gluteraldehyde plus 1% OsO_4 in 0.1M phosphate buffer	0°C	7.0	16 hours
1% OsO_4	0°C	"	2½hours

at a specific disadvantage. Extra information may in general be obtained by comparing micrographs prepared from corresponding sections of material fixed by different procedures, and clear indication may be obtained concerning the destruction, or serious loss, or modification of particular components by one fixative, the same component being well preserved by another fixative. In this case however, potassium permanganate seemed to be the only fixative penetrating sufficiently to give acceptable preservation of a good proportion of the cytoplasmic components. Recognising the characteristic limitations of potassium permanganate preservation, one may still claim that some reliable information may nevertheless be obtained from a study restricted to this fixative.

Protoplasmic Streaming.

After preliminary trials it was decided to use the simultaneous gluteraldehyde, osmium tetroxide fixation with postosmication devised by Franks, Krien and Brown (1969) in further investigation of the fine structure of the mature regions of the sporangiophores.

Equal parts of ice cold 4% gluteraldehyde and 2% osmium tetroxide, both buffered with 0.1 M cacodylate to a pH 7.0, were mixed and used at once for fixation. The fixative vessel was kept in an ice bath throughout the fixation. One part of detergent per 1000 parts of fixative was added to the fixative to ensure the complete wetting of the cell walls and the complete submergence of the sporangiophores during the fixation period. The sporangiophores were fixed for one hour and then washed three times in 0.1 M cacodylate buffer for 30 minutes each, as suggested by Frank, Krien and Brown.

Washing was followed by postosmication in 1% OsO_4
in 0.1 M cacodylate buffer for 3 hours and further
washing in 0.1 M cacodylate buffer for 30 minutes.

IVb. Dehydration.

During the earlier part of this study the
following dehydration procedures were carried out
at room temperature

Either

(1) 25% 50% 75% and absolute alcohol   10 min.each

    Absolute alcohol, 2 changes   15 min.each

    Propylene oxide   15 min

or

(2) 25% 50% 75% 80% 90% alcohol   10 min.each

    Absolute alcohol 2 changes   15 min.each

    Propylene oxide   15 min

These sequences caused considerable shrinkage
of the highly vacuolated sporangiophores. The
shrinkage was accompanied by apparent distortion
of the cytoplasmic contents as seen under the light
microscope. These effects were particularly
noticeable at the 75% alcohol and propylene oxide
stages of the dehydration procedure.

To minimise this distortion associated with
long diffusion paths, the sporangiophores were cut
into approximately 5 mm. lengths at the 25% alcohol
stage. This procedure left an open ended unit into
which substances could move freely. The 5 mm. lengths
of sporangiophore were then passed through 25%,
50% 75% Absolute alcohol for 15 min. followed by a
second period of 15 min. in Absolute alcohol.

Dehydration using the technique of Sitte (1962) (fig. 6) was attempted. In this technique the specimens lying in a small volume of water in a watch glass are placed on a perforated shelf in a small desiccator attached to a vacuum pump. Some alcohol and calcium chloride are placed at the bottom of the desiccator, and above the specimens is a small dish of calcium chloride.

The desiccator is evacuated until the alcohol starts to bubble vigorously and then the tap is closed. The water from the specimens slowly evaporates into the alcohol saturated atmosphere and is absorbed by the calcium chloride.It is replaced by alcohol from the atmosphere.

In my attempts the water evaporated from the specimens but its equivalent replacement by alcohol was not achieved, and the specimens showed signs of shrinkage and distortion.

IVc   <u>Embedding procedures attempted.</u>

(1) <u>Araldite</u>. This resin proved entirely unsatisfactory with <u>Phycomyces</u>. Three embedding procedures being attempted.

(a) <u>Conventional Method</u> (Kay 1961, Mercer & Birbeck 1961). The material was transferred to propylene oxide for 15 minutes and then a 50/50 v/v mixture of propylene oxide and araldite in an open vessel. This was left overnight to allow the propylene oxide to evaporate so that the material would finally contain 100% araldite. Following penetration the sporangiophore lengths were then transferred to fresh 100% araldite plus 0·01 ml of accelerator per 5 ml of resin in the polymerization dishes. Polymerization was brought about by placing the resin containing the material

in a 40°C oven for 2 days and then a 60°C oven for
a further 2 days.

Polymerized blocks were left to harden for a
week before sectioning.

In all attempts the resin within the sporangiophore
failed to harden suggesting lack of penetration
of the 100% araldite plus accelerator.

(b) <u>The method devised by Peat and Whitton - 1966</u>.

This method attempted to achieve good penetration
of the resin into difficult material over a short
period of time.

Following dehydration the material was transferred
into a series of mixtures of propylene oxide and
araldite for a period of 1 hour in each case. The
vessels were left open and the propylene oxide
allowed to evaporate. The mixtures used were:

(a) 20% araldite in propylene oxide at 60°C for 1 hour
(b) 50%   "   "   "   "   "   "   "   "   "
(c) 70%   "   "   "   "   "   "   "   "   "
(d)100%   "   "   "   "   " room temperature for
                                     24 hours

This was followed by polymerization and hardening as
before

Again the procedure failed to give fully impregnated
and hardened blocks. Although the method gave good
results with <u>Chlorogloea</u> it has the disadvantage of
producing a fluctuating concentration of araldite
during impregnation.

(c) <u>A long 'embedding procedure.</u> This was attempted in
the hope of achieving thorough penetration of the
araldite plus accelerator. After dehydration the
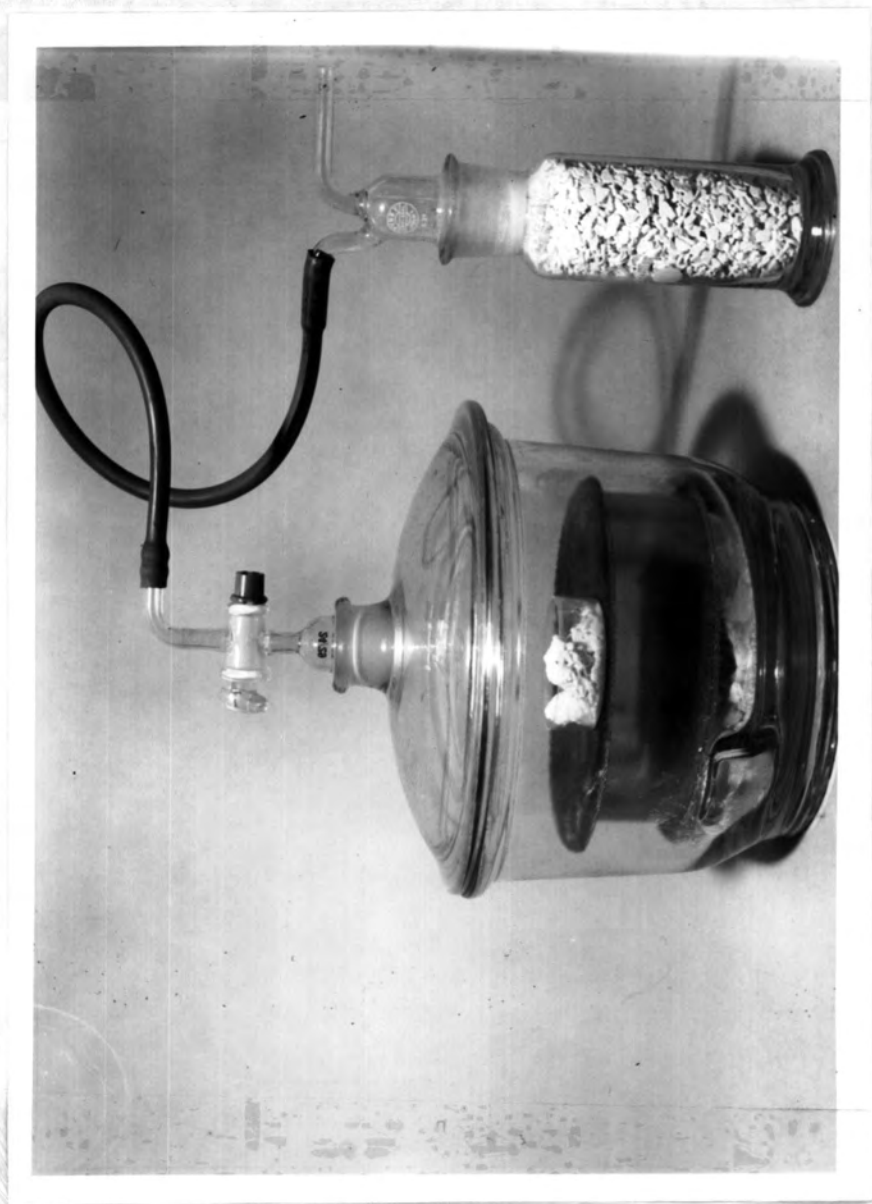sporangiophore lengths were placed in a 50/50 v/v

Fig 6   Dehydration Apparatus   (Sifte 1962)

mixture of resin and propylene oxide for 16 hours. The caps were removed from the vials and the propylene oxide left to evaporate overnight. The material was then transferred to 100% araldite plus accelerator and left at room temperature for 2-3 days. The material was then transferred to fresh araldite plus accelerator and polymerized in a 40°C oven for 2 days followed by 2 days in a 60°C oven. The polymerized blocks were left to harden for a week before sectioning.

In almost all attempts the resin within the sporangiophores failed to harden indicating lack of penetration of the araldite plus accelerator (fig.7).

(2) Methacrylate Embedment. The methacrylate embedding procedure, for highly vacuolated and senescent cells, used by Mohr and Cocking (1968) was tried. This eliminated the need to use propylene oxide and the low viscosity of the methacrylate facilitated easy penetration.

Preparation of the Resin.

The resin was prepared by adding 3 parts styrene to 7 parts n butyl methacrylate v/v. The stabiliser of the n butyl methacrylate was removed with KOH. An equal volume of 5% KOH was added to the mixture in a separating funnel and the two thoroughly mixed and then left to separate. The brown KOH, containing the stabilizer from the n butyl methacrylate, was then run off. This procedure was repeated until the KOH layer remained clear indicating that the removal of the stabilizer was complete. The resin was then washed twice in distilled water to remove any trace of KOH. 2% benzoyl peroxide was added, together with an excess of $CaCl_2$, as a catalyst, to the resin. The resin was finally filtered to remove the $CaCl_2$. Mixtures prepared in this way could be kept for several weeks at 4°C.
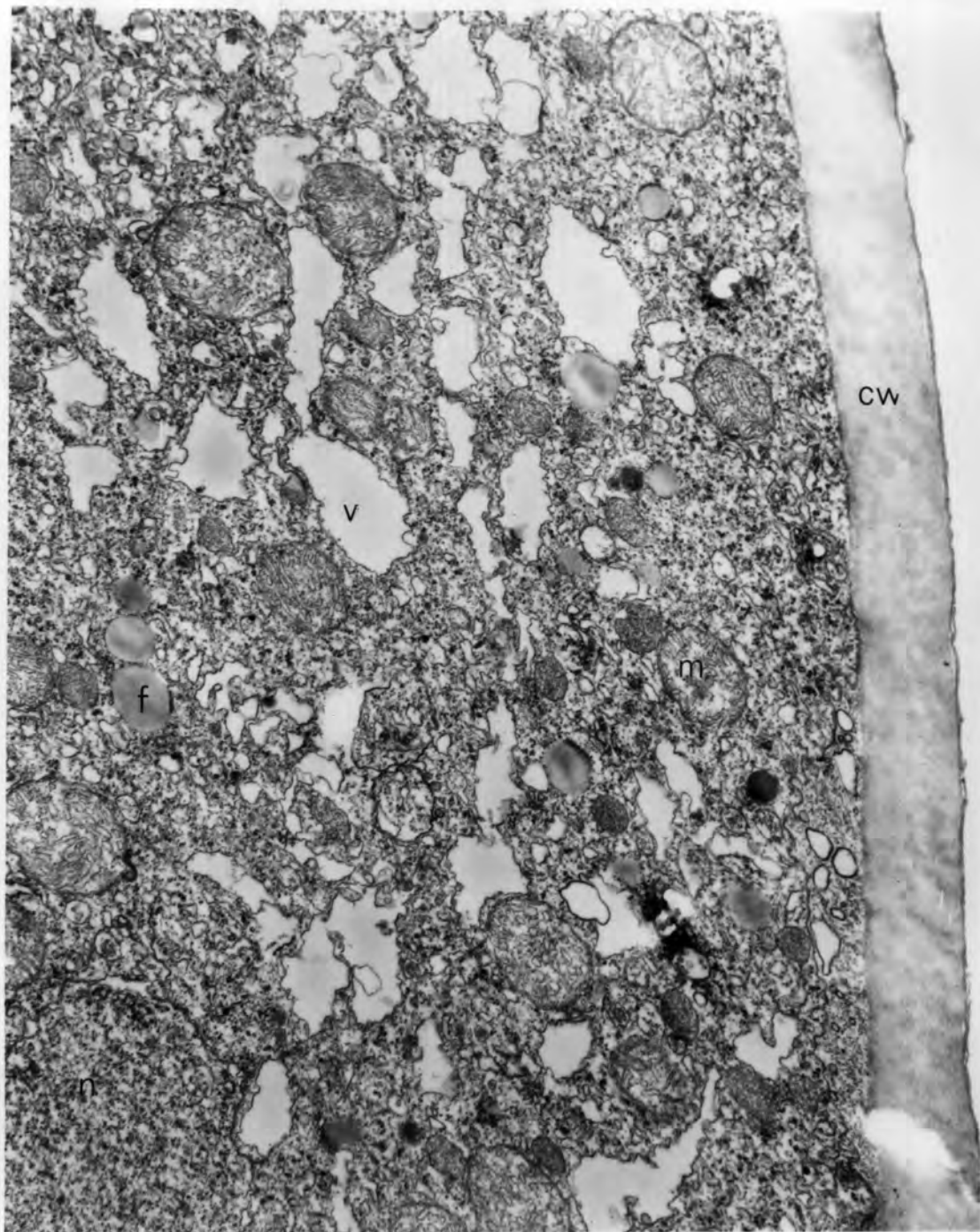
Fig. 7 . Transverse section through growing zone of
Stage IV B sporangiophore. Gluteraldehyde fixation -
Araldite Embedment . Results of this procedure were
usually poor.

Embedment.

The material which had previously been fixed and dehydrated in a graded series of alcohol was transferred to fresh resin and left at room temperature for 15-16 hours. It was then transferred to fresh resin for an hour before a final transfer to the resin in LKB BEEM plastic capsules. Polymerization was carried out at $40^{o}C$ for 24 hours followed by 24 hours at $60^{o}C$.

Good penetration of the material was achieved and although the blocks were often soft, the material cut well. Electron microscope examination however indicated considerable disruption of the fine structure during polymerization. (fig 8b) The sections were not stable under the electron beam.

(3) Water soluble Durcupon

After fixation the material was washed for 15 minutes in two changes of distilled water. It was then passe d through the following series of Durcupon concentrations:

25%	aqueous Durcupon		30	mins
50%	"	"	30	"
70%	"	"	30	"
90%	"	"	30	"

This was followed by two changes of absolute Durcupon for 1 hour each. Finally the material was transferred to fresh Durcupon plus accelerator for 24 hours before polymerization at $40^{o}C$.

(4) Glycol Methacrylate (G.M.A.)

After fixation the material was washed in either distilled water or buffer solution. It was then passed through the following concentrations of
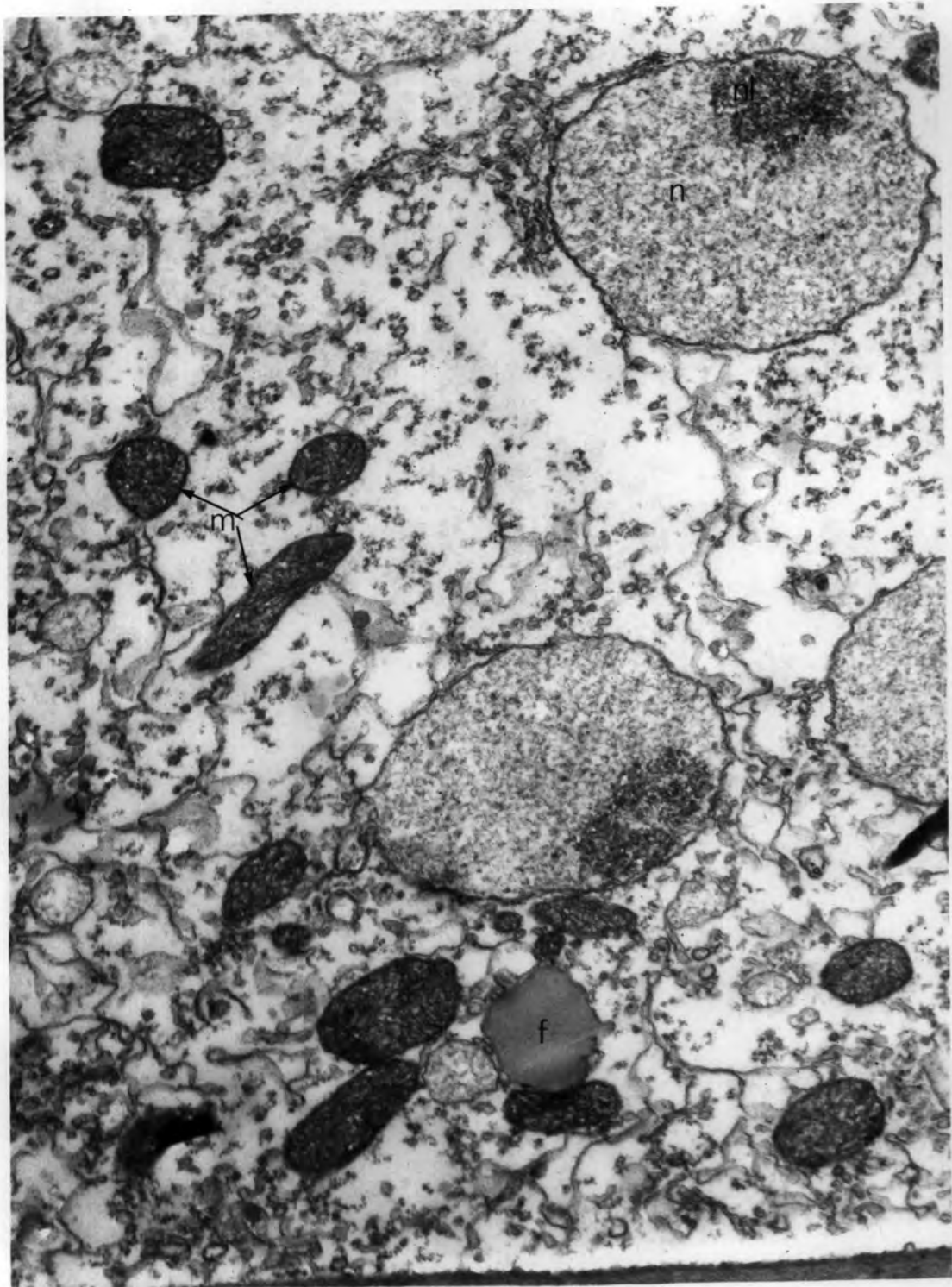
Fig. 8. Transverse section through growing zone of
Stage IV B sporangiophore - Gluteraldehyde fixation,
Methacrylate Embedment .

glycol methacrylate

30% aqueous glycol methacrylate		30 mins
50% " " "		30 mins
70% " " "		30 mins
80% " " "		30 mins
90% " " "		30 mins
Glycol methacrylate		2 hours

The material was then finally transferred to fresh glycol methacrylate and after 24 hours at room temperature polymerized at 40°C.

Material embedded in these two water soluble resins according to the procedure of Glauert and Glauert "Techniques in Electron Microscopy" (1961) proved impossible to section.

(5) TAAB Embedding Resin.

This proved to be the most satisfactory resin for the embedment of Phycomyces.

Preparation of the Resin.

Equal quantities of TAAB embedding resin and DDSA. v/v were thoroughly mixed together. $0 \cdot 1$ cm^3 of the accelerator DMP per 5 cm^3 of the resin were added to the embedding resin just before use. It was found that the best cutting blocks were obtained when the mixture had been made up at least one week before being used.

Embedding procedure.

Material that had been fixed and dehydrated in a graded series of alcohol was left overnight in a 50:50 resin/alcohol mixture. It was then transferred to freshly made up resin minus accelerator and left at 60°C for

2-3 days. This facilitated the penetration of the resin. The material was then transferred to fresh resin plus accelerator and left for 2 days at room temperature. It was then transferred to fresh resin plus accelerator in polymerization dishes and polymerized at 40°C for 2 days followed by 2 days at 60°C. This procedure gave good penetration and an even hardness of resin throughout the blocks. The blocks cut well and the sections were extremely stable under the electron beam. There was little evidence of polymerization damage.

IVd Sectioning.

Sections were cut on an L.K.B. ultramicrotome III using glass knives with a cutting angle of 45°. The sections were collected on T.A.A.B. 300 mesh grids coated with 2% formvar and stained with uranyl acetate for 20 minutes followed by a thorough washing in distilled water and a post staining with lead citrate (Renolds) for 5 minutes. They were then given a final wash in distilled water before examination.

Electron Microscopy.

The sections were examined on an A.E.I 6B Transmission electron microscope at 60 kv magnification 1k to 60k and occasionally at higher magnifications.

Photography.

Photographs were taken on Ilford Special lantern Contrast plates which were developed in Autophen developer. The prints were made on Ilford Photographic Paper.

Section V.

PROTOPLASMIC   STREAMING.

Va. Historical Background.

Protoplasmic streaming is a phenomenon that has been recorded for many organisms,both plant and animal. In plant material it was observed for the first time by Corti in 1774 (Kamiya 1959). According to Kamiya's review (1959) many later workers (Frank 1872, Dehnecke 1886, Keller 1890, Kretzshmar 1904) observing the streaming regarded it as an abnormal phenomenon occurring only when the cell was injured or subjected to environmental changes.

Hauptfleisch (1892) distinguished two classes of protoplasmic streaming:- a primary streaming which occurs in cells under normal and natural conditions and a secondary streaming which is established in cells the protoplasm of which has been in a quiescent state as a result of some physical or chemical stimulus. Velten (1872) thought that protoplasmic streaming was a common phenomenon appearing at a certain stage in the life of the protoplasm of the cell. Kamiya's review (1959) (Schorr 1935, Linderheim 1937, Pop 1938) suggests that it was not until the beginning of the 1930's that protoplasmic streaming was generally accepted as a naturally occurring process in intact cells

with an active metabolism.

The numerous modes of protoplasmic streaming and the vast literature of work dealing with this streaming were well summarised by Kamiya in his review entitled 'Protoplasmic Streaming' published in 1959. Streaming in most organisms is observed because of the rotation of granules. If it were not for the presence of these granules of varying kinds and size which serve as markers to indicate the flow it would be extremely difficult to recognise protoplasmic streaming within the cell. However one should not assume that the visible movement of these granules indicates precisely the flow of the groundplasm in which they are suspended, nor that it is only the granules themselves that actively move.

It has been suggested by Nagai & Rebhun (1966) after reviewing the work of Hoffman-Berling, Bettex-Galland & Luscher, Nakajima, Vorobyeva and Poglazovr & Schmidt that there is possibly a common macro-molecular entity which may be the universal mechanochemical transducer involved in non muscular motility and that this is probably organised into some macro-molecular complex which is then repeated many times in some three dimensional pattern rather than being randomly dispersed. They suggest that the bundles of 50 $\overset{o}{A}$ microfilaments may be the direct mechanochemical transducer of streaming in the cytoplasm of <u>Nitella</u>.

Kavanau (1963), Kane (1962) working on mitotic
apparatus, Ledbetter & Porter (1963) on meristematic
cells and Roth (1964) on various protozoa have suggested
that microtubes are the most probable elements involved
in primitive mobility.

Wohlfarth-Bottermann (1964) found no microtubes
in Physarum but filaments about 70 Å in diameter
which he believed to be the basis of motility
in this organism. This view has been strengthened
by the findings in several protozoa, of similar
filaments possibly involved in contraction
and the presence in smooth muscle of filaments
generally thought to be contractile.

Jahn & Bovee (1969) in their comprehensive
review on protoplasmic movement within cells are
of the opinion that there is sufficient evidence
to indicate a basic biochemomechanical mechanism
that powers protoplasmic movements in all kinds of cells,
and that this exists within the cells as a sort of
actomyosinoid protein. From this pool labile fibrils
and gels are formed which interact and move in
relation to one another, splitting ATP to produce the
energy for the formation and interaction of
the fibrils and gels and for their subsequent
movement.

In those cells which show streaming along well
defined tracks they postulate the formation of
discrete actin fibrils or tubules along which such
organelles as chloroplasts and mitochondria are
carried by the motion of cytoplasmic microstructures

to which they are fixed. It is suggested that the
organelles which possess both ATP and small
quantities of actomyosin with ATPase activity may
have a subsidiary self active role in their
movements, especially the more abrupt ones.

Vb. Protoplasmic Streaming in Phycomyces Sporangiophores.

Pop (1938) describes the protoplasmic streaming
in the sporangiophores of Phycomyces as occurring in
two concentric tubes. A tubular ascent of the
protoplasm takes place around a central sap
vacuole towards the top of the sporangiophore growth
zone where the protoplasm turns back in a tubular
stream towards the sporangiophore base, flowing
between the ascending stream and the sporangiophore
wall. Pop states that he was unable to observe
spiral streaming within the sporangiophore, it
being nearly always parallel to the long axis of
the cell.

Vc. Observations.

Sporangiophores of both the plus and minus
strains of Phycomyces blakesleeanus were plucked
from the mycelium by means of a pair of fine
forceps applied at their bases. The procedure
produced no damage to the cell and they could be
observed for several hours under the phase contrast
microscope.

The plucked sporangiophores were transferred to
a drop of water on a microscope slide and covered with

a coverslip supported on two pieces of fine drawn-out glass tubing. Although hundreds of sporangiophores were examined in this way the tubular streaming of the protoplasm described by Pop (1938) was never observed.

In the Stage IV B sporangiophores of _Phycomyces blakesleeanus_ the protoplasmic streaming appears as a well orientated and well organised phenomenon (Table II) when viewed under the phase contrast microscope. In these sporangiophores vigorous and characteristic multistriate streaming was always observed (Figs. 9a, b). At temperatures of about 20°C the velocity of movement, calculated by timing the movement of particles between two fixed points in the calibrated eye piece, was estimated as being 2.2u-3.6u/sec. (Table III). These calculations agree with those cited by Kamiya (1962). It was observed that the smaller particles - mitochondria etc., moved at a greater velocity than the larger ones i.e. nuclei. The various particles within any one microstream of cytoplasm could be seen moving in the same or opposite direction along the longitudinal axis of the sporangiophore. The particles were frequently seen to be deflected from their line of movement on meeting what can only be envisaged as an obstacle to progression. This deflection was usually followed by several similar abortive attempts to proceed before finally returning along the original path. Particles moving in the same direction within a cytoplasmic strand were often observed increasing their speed of movement as they moved along the

TABLE II

Table reflecting Spatial Organization of Protoplasmic Streams within the Sporangiophores

Height in cms.	Width in μ	*Distance between protoplasmic streams	Average Distance
1.8 cms	1065 μ	15μ,14,25, 22μ	19μ
1.8 cms	480 μ	42, 45, 50 50μ	46.75 μ
2.2 cms	300 μ	50, 58, 52, 91μ	62.75 μ
3.7 cms	233 μ	85u, 100, 175μ	120μ
6.5 cms	337 μ	59u, 193, 209μ	153.6 μ
7.0 cms	330 μ	88, 150, 170μ	102.6 μ

* Distance measured at various points along the sporangiophore

TABLE III

Table to show Velocity of Organelles moving in a Protoplasmic Stream of Phycomyces blakesleeanus

Sporangiophore Streams	Time in sec. for Organelles to move across a distance of 40μ	Average Time
Minus strain 1a	16,16,15,17,10,16,18,21	16.38
1b	13,10.5,11,13,11.5,15,13	12.42
2a	20.5,20,17.5,18,17,15.5,19,20	18.44
2b	12,12.5,11,11,10.5,11,13,15	12.0
3a	12.5, 12,12,13,14.5,9.5,10,10.5	11.62
Plus strain 1a	11,10,16,12,11,11.5,10,10	11.44
2a	10,9.5,10,5,11,13,5,10	10.75
3a	12.5,12,13.5,13,12,14,12	12.71 Chanel not particularly obvious
3b	11,13,14,15,13,20,14,16	14.5
4a	10,11,12,10,10.5,11,13,12	11.18

a- movement from base to apex of sporangiophore
b- movement from apex to base of sporangiophore

Fig 9a Two sporangiophores showing protoplasmic streams   X.100
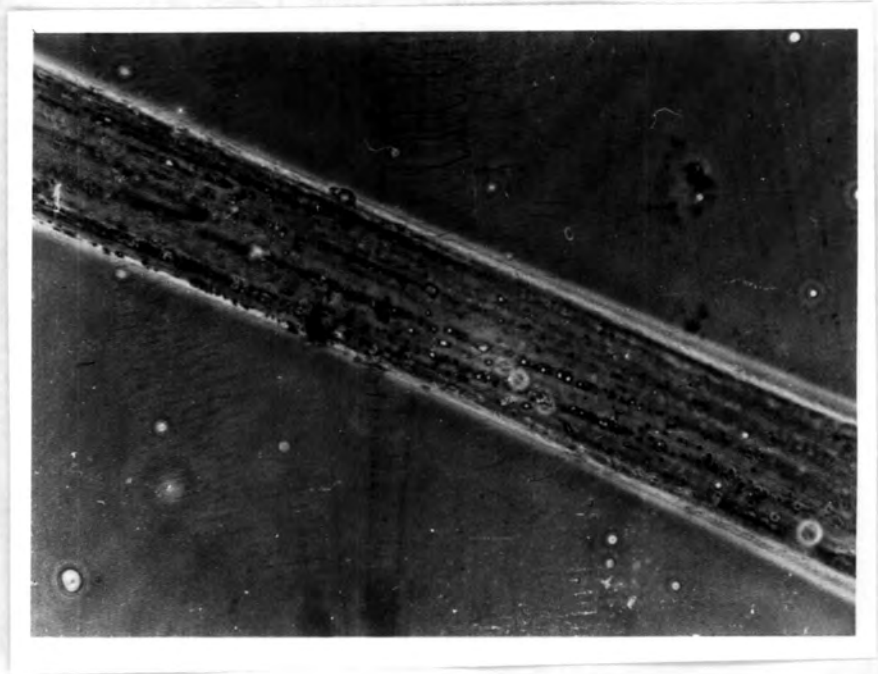
Fig 9b The Protoplasmic streams are almost parallel to the long axis of the sporangiophore X.100

strand so that they frequently passed each other
as they progressed. In order to pass round a
particle moving at a lower velocity a particle
moving at a higher velocity often first collided
with the obstacle particle, retraced its path over
a short distance and then moved forward again on a
slightly deviated path, moving back to its apparent
original course after having negotiated the obstacle.
The movement resembled in many ways the negotiating
of an obstacle by many protozoan organisms.

When observed under a Leitz phase contrast
microscope with an oil immersion lens the cytoplasmic
strands were at times seen to exhibit a pulsating
movement. (Figs. 9c, d).

All these features strongly suggested the existence
within the cytoplasm of a structure,to which the
particles were in some way elastically linked,which
determined the general direction of flow. The
variations in velocity however suggested that the
particles move with their own localised impulse,
although one cannot ignore a possible role of the
ground cytoplasm in assisting movement in one
direction.

These findings with the light microscope led
me to believe that there might possibly be a system
of microtubules or fibrils within the cytoplasm along
which the organelles moved as a consequence of lasting
or transient attachment. Movement of a structural
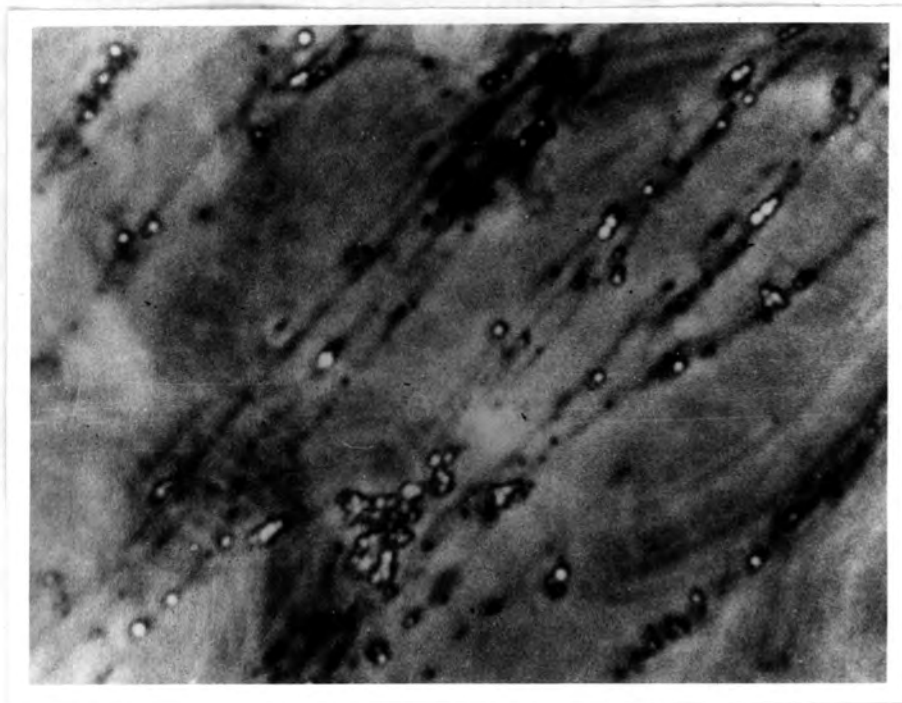system has been envisaged in terms of jet propulsion

Fig 9c A 'vesicle' can be observed in two of the
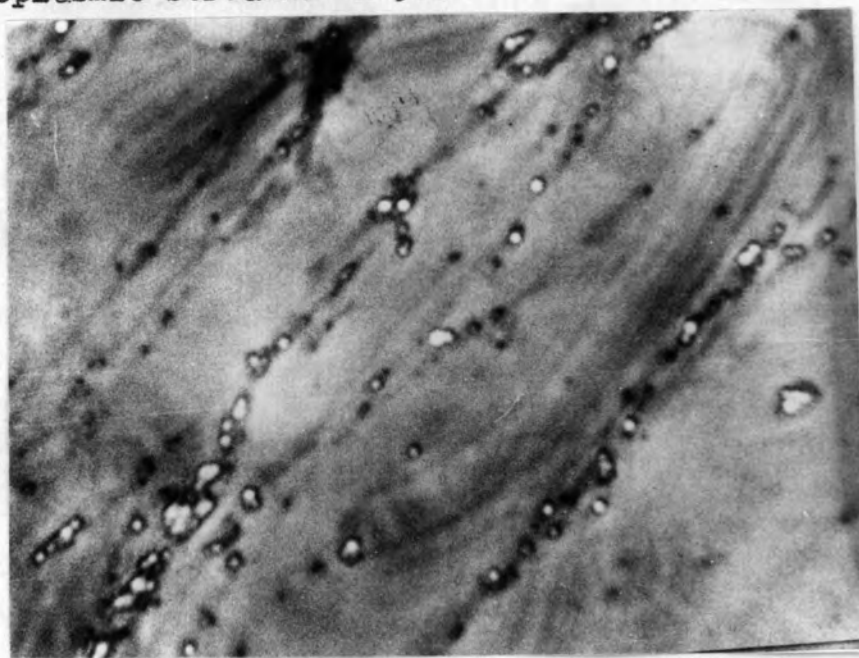Protoplasmic streams. X 900

Fig 9d less than one second later the vesicles are no
longer visible - such vesicles appeared to pass in wave
like motion along the streams   X.900

of fluid from microtubules (Kavanau 1963) and as previously stated as cycles of contraction and relaxation of fibrils.

Vd. <u>Organelle Distribution in Relation to Protoplasmic Streaming.</u>

An attempt was made using various staining techniques for the light microscope, to determine the nature of the organelles moving along the fine strands of protoplasm. Since most staining techniques involve pretreatment with fixatives that caused considerable distortion of the cytoplasmic contents of <u>Phycomyces</u> they did not always provide the desired information.

<u>Materials and Method.</u>

1. <u>Nitroblue tetrazolium - Mitochondria</u>

Living sporangiophores were placed in equal volumes of nitroblue tetrazolium (1 mg 1 mol.) and 0.05 M sodium succinate buffered to pH 7.1 with 0.05 M phosphate buffer. The sporangiophores were then kept at a temperature of $35^{o}C$ for 20 mins. This was followed by thorough washing in distilled water and mounting in Zeiss water mountant W 15 n_D 1.515. The sporangiophores were examined immediately.

2. <u>Janus Green - Supravital stain for Mitochondria</u>

Living hyphae were placed in a solution of Janus Green 1/1,000 in distilled water on a microscope slide. They were then covered and examined immediately. This is a supravital stain for mitchondria and their

position in the living hyphae could thus be
readily determined.

3.   Feulgens stain (Jones 1947) - Nuclei

Sporangiophores were fixed for 2 hours in
Carnoy's B fixative (6 parts ethyl alcohol, 1 part
glacial acetic acid and 3 parts chloroform). They
were then rinsed for 20-30 mins in distilled water.
The fixed material was then hydrolysed in
N. hydrochloric acid for 5 mins at room temperature,
followed by 5-7 mins at $70^{\circ}C$ and a further 5 mins
at room temperature. The sporangiophores were then
removed from the acid, washed thoroughly, placed in
distilled water for 5 mins and finally stained in
Feulgens reagent for 5-10 hours at room temperature.
After staining the material was bleached in water
containing sulphur dioxide for 30 secs to 5 mins.
This was followed by rinsing in distilled water,
dehydration, clearing and mounting in Euparal.
Some sporangiophores were mounted immediately after
rinsing in Zeiss water mountant.

Dehydration was carried out by placing the
material for 5 mins in each of 20%, 30%, 50% and
70% alcohol and 10 mins in each of 85%, 95% and
absolute alcohol. The sporangiophores were cleared
by leaving them for 5 mins in 75/25%, 50/50% and
25/75% alcohol-xylene mixture and 100% xylene.

A few sporangiophores were counter stained with
10% Orange G in 70% alcohol. The counter staining
was carried out at the 70% alcohol stage of dehydration

when they were transferred to the stain for 5 mins.
Excess stain was removed in 70% alcohol before the
material was dehydrated, cleared and mounted as
above.

4.   Sudan III - Fats

Living hyphae were placed on a microscope slide
and flooded with Sudan III (0.1 gms Sudan III in 50 mls
of 95% ethyl alcohol and 50 mls of glycerol). After
5 mins the Sudan III was drained away and the
sporangiophores were mounted in equal parts of
95% ethyl alcohol and glycerol, covered and
examined immediately.

5.   Korson's Stain (Korson 1951)

Sporangiophores were fixed for 30 mins in a
modified Carnoy's B fixative (2 parts ethyl alcohol,
3 parts chloroform and 1 part glacial acetic acid).
They were then washed for 30 mins each in two
changes of 85% alcohol and then brought down to
water through 75%, 50% and 25% alcohol. The
fixed material was then stained as follows:-

It was first placed in Korson's solution A
(4% Orange G in distilled water) for 3-5 mins, washed
in distilled water containing a drop of Korson's
solution A. The material was then transferred to
Korson's solution B (0.15% methyl green in distilled
water) for 15 mins, drained and then transferred to
Korson's solution C (one part 0.15% methyl green in
distilled water and one part 0.1% toluidine blue
in distilled water) for 5 mins. After staining the

material was taken through a graded series of
n butyl alcohol and finally left overnight in fresh
n butyl alcohol to complete destaining. This long
period of destaining is essential for delicate and
precise staining. Following destaining the material
was then washed in xylol and mounted in Zeiss mountant
or Euparal.

Results.
1.   Nitroblue tetrazolium.
Aggregates of mitochondria appeared in the following
locations:- (Fig.10, a-f)
(a) From 0-1 mm below the sporangium of the Stage I
and IV B sporangiophores.
(b) From $0-\frac{1}{2}$ or 1 mm up from the base of the Stage I
and IV B sporangiophores.
(c) In the storage vesicles of the mycelium.
(d) At the points of branching of the mycelium.
(e) In the columella.

2.   Janus Green - Supravital stain. (Figs. g&h)
The results of this staining procedure again showed
the presence of large numbers of mitochondria:-
(a) below the sporangium of the Stage IV B sporangiophore.
(b) at the base of the sporangiophore
(c) in the storage vesicles of the mycelium and, in
addition to the above locations, in the fine
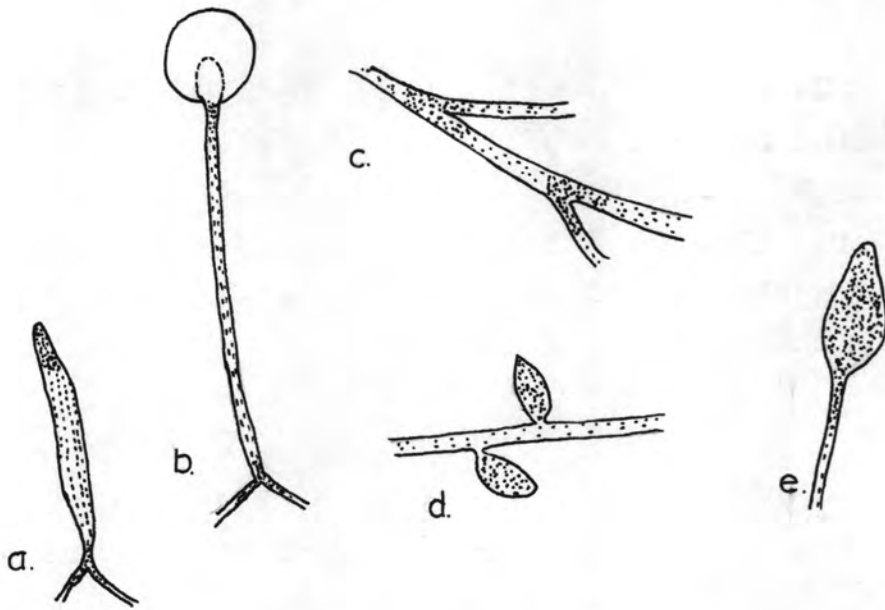protoplasmic streams running along the long axis of
each sporangiophore.

Fig.10a Results of staining with nitroblue - tetrazolium
a)stage I sporangiophore, b)stage IVB sporangiophore
c)branching of mycelium, d) reserve vesicles, e)columella
after dehiscence of sporangium

Fig.10b Growth zone of sporangiophore stained with
nitroblue - tetrazolium   X 100

Fig 10c Sporangiophore base containing numerous
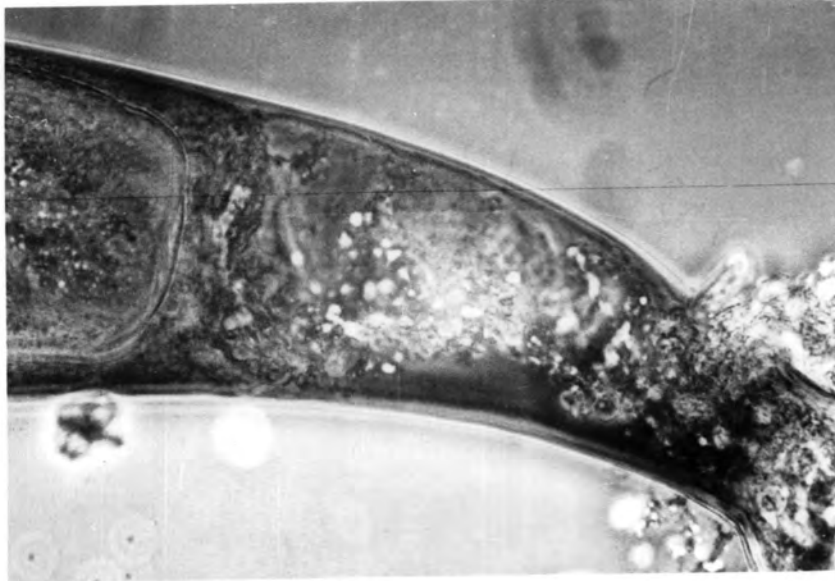mitochondria - nitroblue-tetrazolium stain X.200
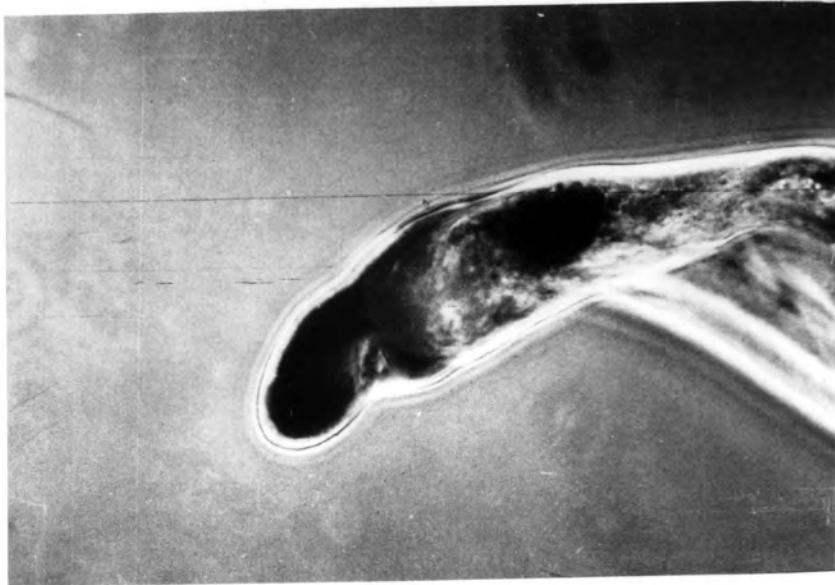
Fig 10d Apex stage I sporangiophore showing dense
aggregation of mitochondria - nitroblue-tetrazolium
stain X.100

Fig 10e Columella and growth zone of Sporangiophore
-nitroblue-tetrazolium stain   X.100

Fig 10f Part of Mature sporangiophore -nitroblue
-tetrazolium stain. Note absence of protoplasmic
streams. X.200

Fig 10g Part of sporangiophore showing protoplasmic
streams – Supravital Janus Green stain   X.200

Fig 10h As Fig 10g – Note the dark staining indicating
the presence of mitochondria in the protoplasmic
streams. X.200

3. Feulgen's Stain.

Nuclei, located by means of the D.N.A. staining with Feulgen's stain, were present:-
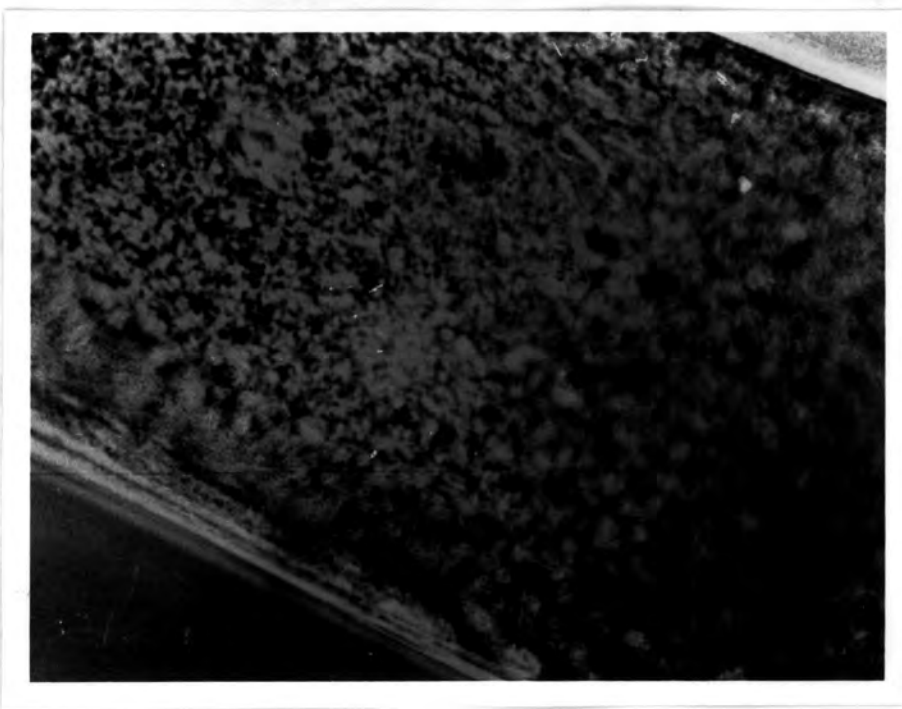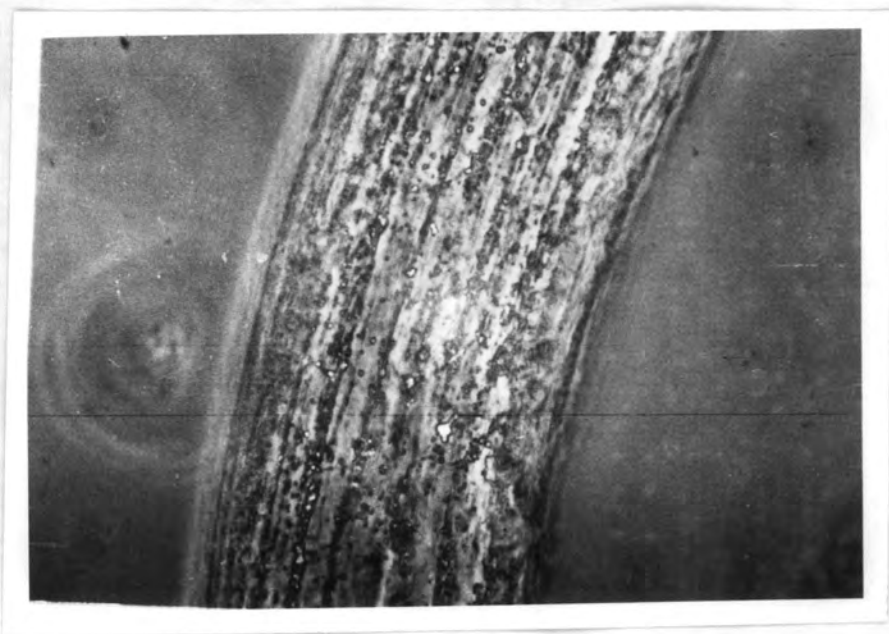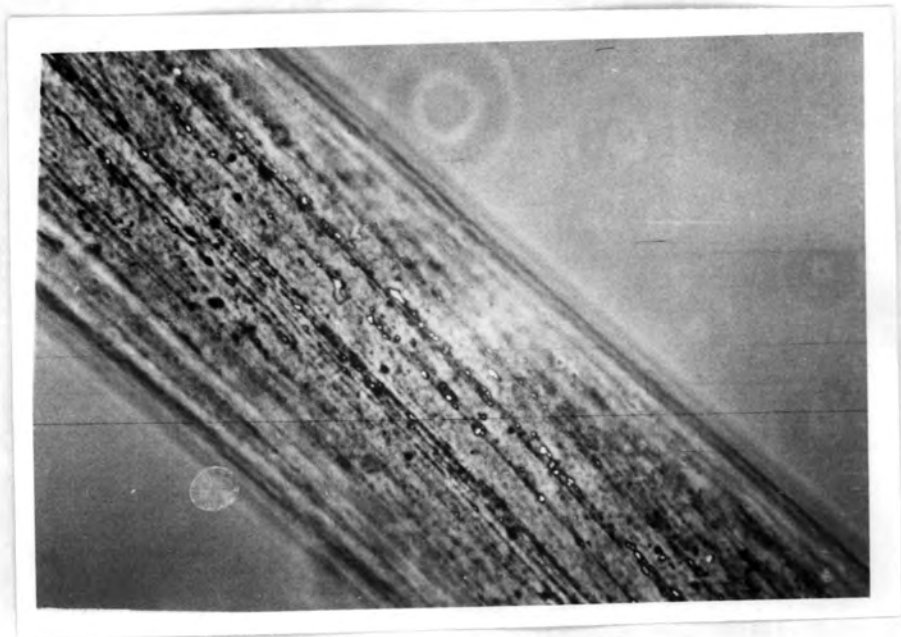
(a) In large numbers in the Stage IV B sporangia. Squashed sporangia showed these nuclei to be located in the spores which had stained deeply.

(b) In the sporangiophore where there was a fairly even distribution of nuclei along the whole length. Although the fixation procedure used prior to staining brought about some distortion of the linear arrangement of organelles and protoplasmic strands it was evident that the nuclei had a linear orientation together with the mitochondria along the length of the sporangiophore.

4. Sudan III.

Fats were apparent along the whole length of the sporangiophore. They appeared as droplets of varying size. An intense orange stain appeared in the Stage IV B sporangium and a deep yellow stain in the columella. In the latter there appeared a well defined peripheral region without stain. There was no evidence of a linear arrangement of the fat droplets.

5. Korson's Stain.

(a) In the terminal 1 mm of the sporangiophore there was an intense green staining indicating the presence of D.N.A. in large quantities.

(b) The sporangiophore itself was stained blue

indicating the presence of ribosomal R.N.A., but
scattered throughout this were areas of green
staining indicating the presence of D.N.A. alongside
the ribosomes.

(c) Considerable green staining also appeared in
the sporangium.

(d) The peripheral region of the terminal 2 mm of
the sporangiophore and also the region of the mycelium
from which the sporangiophore originates stained a
deep violet.

(e) The columella showed a blue staining together
with considerable deep violet and a green staining
at the base indicating the location of nuclei.
It was however impossible to detect any precise
arrangement of organelles within the sporangiophore.

Ve.   Electron Microscope Results.

In transverse and longitudinal sections of
regions of the Stage IV B sporangiophore between
1 cm and 4 cm below the sporangium the cytoplasm
forms a lining to the cell wall and surrounds an
extensive vacuole. As the distance from the
sporangium base increases the cytoplasmic layer
becomes progressively thinner. At a distance of
4 cms or more as seen in Transverse section (Fig 11)
the cytoplasmic layer is extremely variable in
depth consisting of deep regions in which are embedded
organelles such as nuclei and mitochondria separated
by thin regions often devoid of organelles. This
arrangements of the cytoplasm probably explains the
appearance of the longitudinal cytoplasmic strands
apparent in entire sporangiophores when viewed under

cytoplasm

cell wall

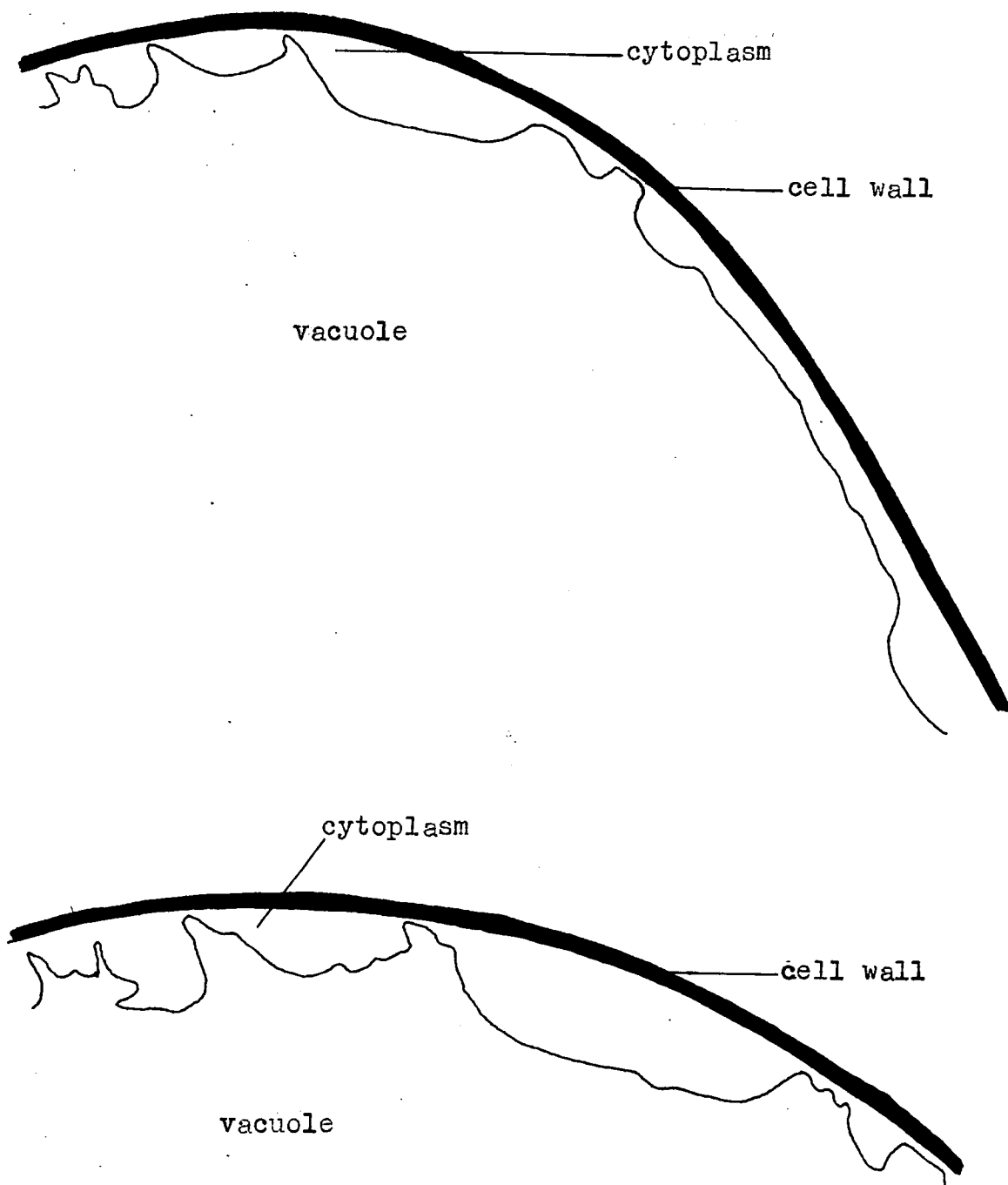vacuole

cytoplasm

cell wall

vacuole

Fig.11 Transverse section of parts of stage IVB sporangio-
phores of Phycomyces approximately 4 cm below the
sporangium to show the variation in depth of cytoplasm -
tracings from an electron microscope plate.

the light microscope.

In preliminary experiments I was unable to find any conclusive evidence of either microtubules or fibrils within the cytoplasm. Although several methods of fixation were used (see Table I, section IV a) nothing was observed which gave any evidence at all for the presence of microtubules. With the Gluteraldehyde-Osmium tetroxide fixative (Franke et al 1969)however many, and in some instances extremely long, microfilaments were observed (Figs. 12 and 13). In some instances mitochondria appeared to be attached to these microfilaments (Fig. 14). These microfilaments were 160 $\overset{o}{A}$ in diameter and were only observed in longitudinal sections suggesting an orientation parallel, or approximately so, to the long axis of the sporangiophore. Microfilaments of similar dimensions were occasionally observed in material prepared by using gluteraldehyde fixative with post osmication (Fig. 15). They were not however so common or so long in this material.

The microfilaments observed in Phycomyces are very similar in diameter to those occurring in Physarum (Crawley 1966). Both are considerably larger in diameter than the microfilaments occurring in most other organisms. No structures corresponding to the 210 $\overset{o}{A}$ microtubules described by Sabnis and Jacobs (1967) in Caulerpa were observed.

From the observations it seems reasonable to suppose that the orientation of the microfilaments
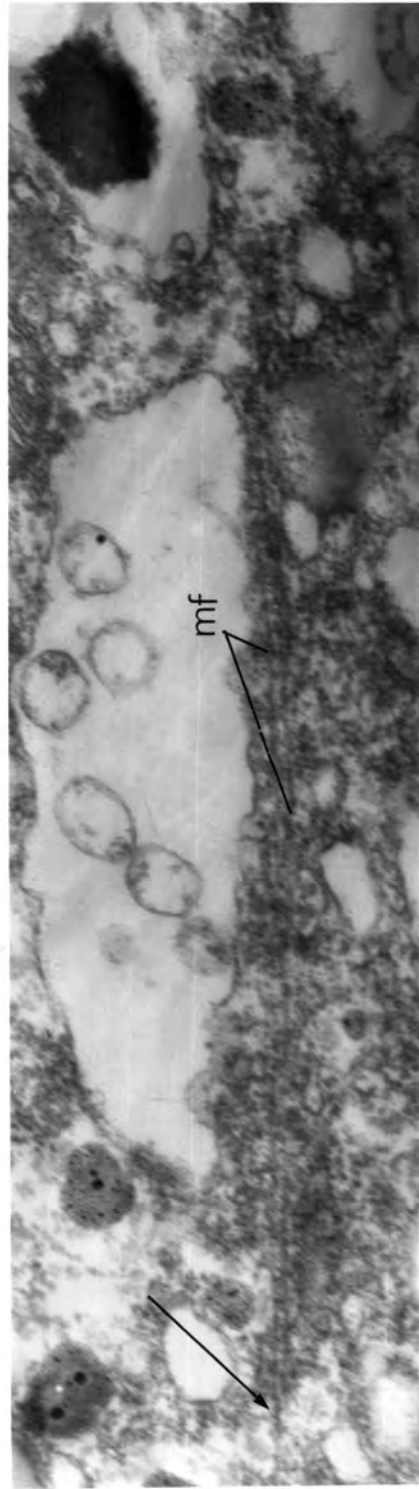
Fig 12
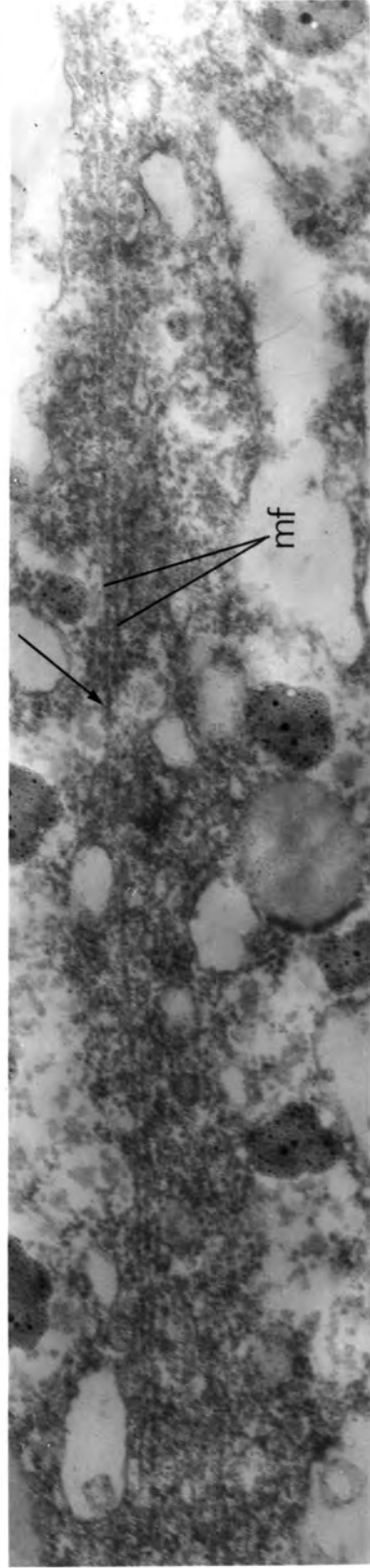
Fig 13.

Fig.12 and Fig.13. Long microfilaments running approximately parallel to the long axis of the sporangiophore. These filaments were over 8.5 μ in length. Arrows indicate point of overlap of two micrographs. X 30,000 Simultaneous gluteraldehyde – osmium tetroxide fixation.

Fig. 14. Microfilaments with closely associated mitochondria. Arrow indicates an apparent link between a filament and a mitochondrion. Simultaneous Gluteraldehyde – Osmium tetroxide fixation.   X 30,000

cw

mf

mf
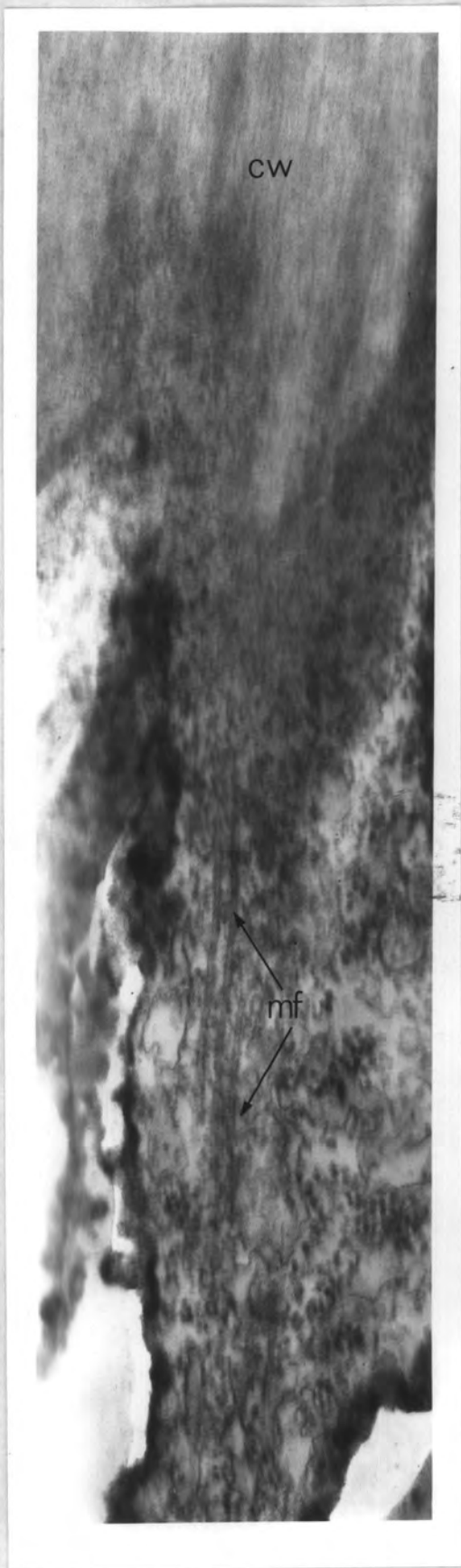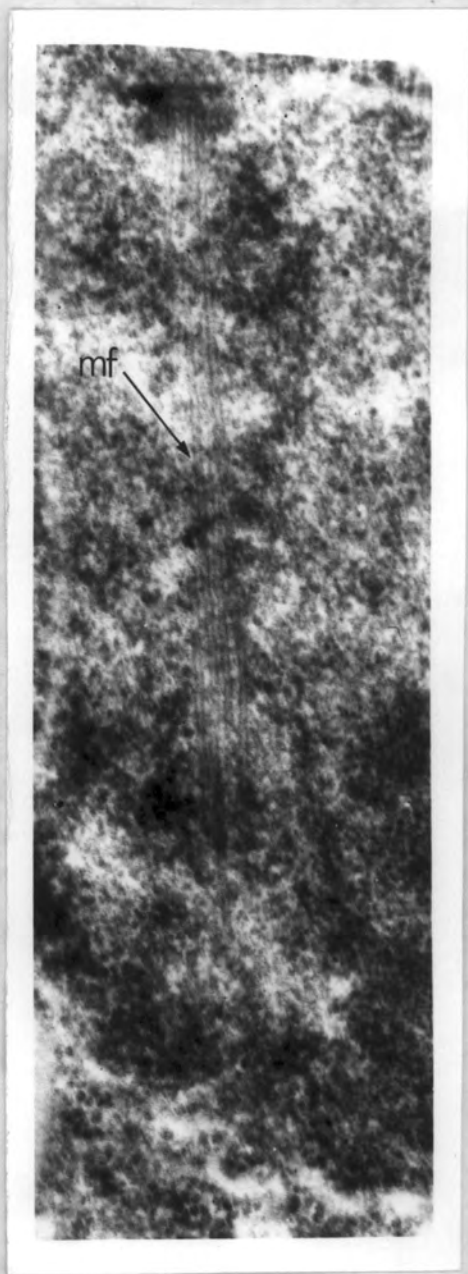
Fig 15.

Fig 15 Microfilament passing across
a nucleus - the double lines
suggest microtubules -
Gluteraldehyde fixation X 63,750

Fig 16 Microfilaments passing from
the cytoplasm into the cell wall
Their orientation is very close
to that of the chitin fibrils

X 60,000

Fig 16.

parallel to the long axis of the sporangiophore determines the orientation of protoplasmic streaming. It is possible to envisage a system similar to that suggested by Jahn and Bovee (1969) existing within the sporangiophores of Phycomyces. The mitochondria, which are generally accepted as possessing ATP and ATPase activity, could provide some of the motive force required for their movement and be linked to the cytoplasmic microfilaments by temporary bonds. Such a system would explain why it is possible for moving organelles within a strand of cytoplasm to be able to pass each other and then to continue their forward movement on the same path but moving at different velocities.

This does not however explain the mechanism which determines when the organelles will move from base to apex or from apex to base of the sporangiophore. From the observed rates of movements of the organelles in the two opposing directions (see Table III) it seems unlikely, as was once suggested, (see Kamiya 1959) that the primary force develops from a turgor pressure at the base of the sporangiophore.

Vf. Protoplasmic Streaming in Relation to Secondary Wall Development.

It is believed by some investigators that the spiral flow of the cytoplasm might play some part in the deposition of the secondary chitin molecules into the primary wall structure laid down in the growing region of the sporangiophore. In oblique sections of

the Stage IV B sporangiophore taken from more than
2 cm below the sporangium the internal boundary of the
wall was seen to be extremely irregular in form
possessing numerous sharp inward projections.
Passing from the cytoplasm into these projections of
wall materials microfilaments could often be observed
(Fig. 16). From the nature of the wall as seen in
transverse, longitudinal and oblique sections a three
dimensional model of the structure of the wall can
be built up.(Fig. 17). It will possess numerous
raised areas orientated with their long axes obliquely,
at an angle in the region of 45° to the long axis of
the sporangiophore. The position of the microfilaments
seen passing into these raised areas indicates that
they enter at an angle to the long axis of the
sporangiophore closely approximating that of the
secondary wall fibres, thus suggesting an orientation
in a loose spiral within the cytoplasm. Such an
orientation would also account for the appearance
of the fibrils in the wall as seen in longitudinal
sections.

'lomasome like'
bodies

plasmalemma

a

Chitin fibrils

b

cytoplasmic microfilament

plasmalemma

wall
protrusions

c

d
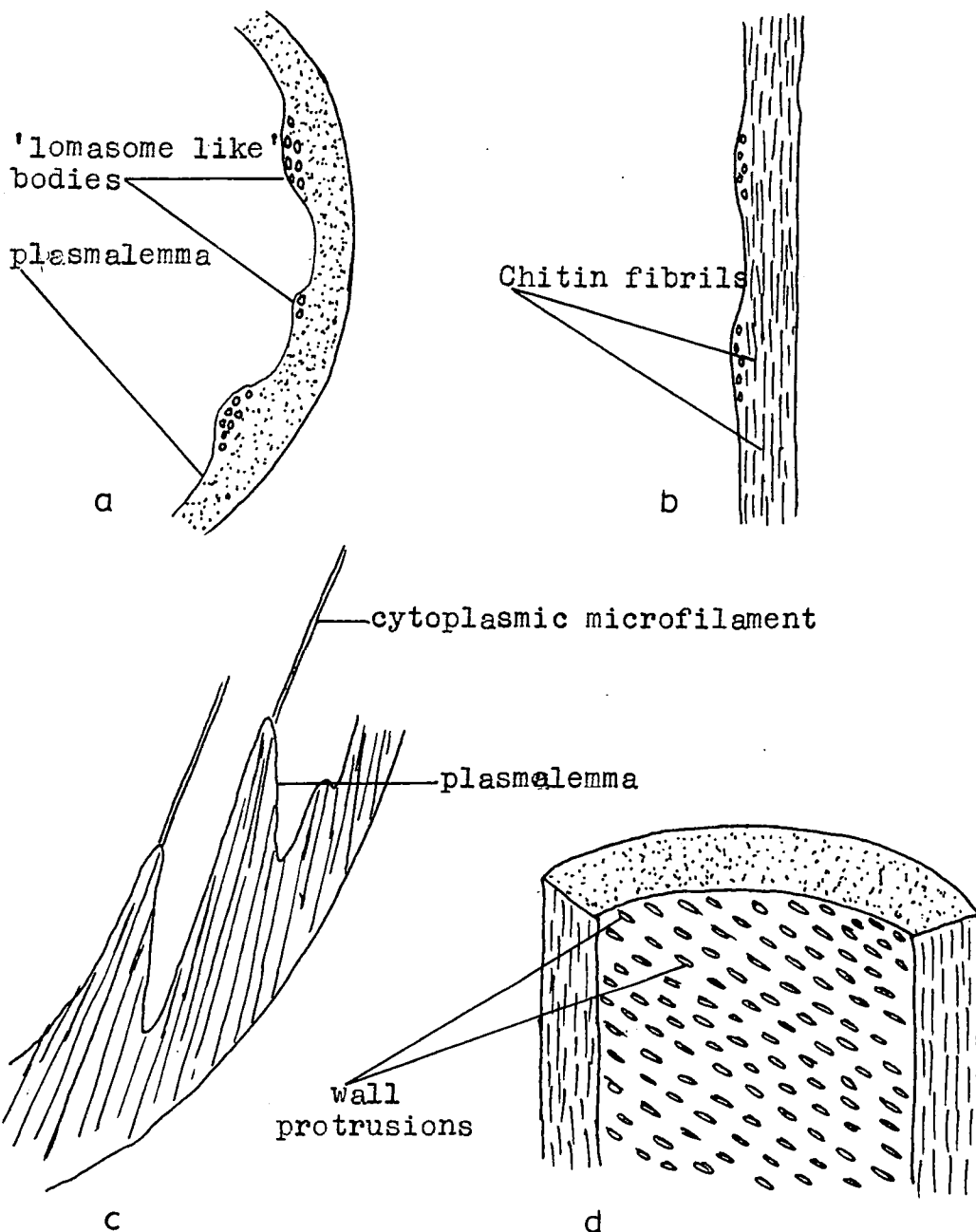
Fig 17 Diagrams to illustrate the appearance of the chitin
fibrils in the wall of the growth zone _Phycomyces_
sporangiophore a) transverse section-no fibriller structure
apparent, b) longitudinal section - short fibrils present
c) oblique longitudinal section - long fibrils present-
microfilament pass into wall from cytoplasm d) three
dimensional plan of sporangiophore wall

Section VI

Geoperception in Phycomyces Sporangiophores

VIa Historical

When a sporangiophore of Phycomyces blakesleeanus is placed with its long axis in a horizontal plane, the growing region curves upwards until its direction of growth is vertical. It thus exhibits a negative geotropic response. The response begins after a variable latency of 30-180 mins in mature stage IV B sporangiophores. The bending rates for wild types may be as high as 0.3°/min initially when the angle between gravity and the growth zone is 90°, but drops to about 0.17°/min. when the angle is 45° and about 0.08°/min when the angle is 20°. Thus, starting from the horizontal, about 12 hours is required for the sporangiophore to reach the vertical (Dennison 1961).

Dennison also found that there is considerable variability in the latent period and in the initial bending speed. He suggested that this might reflect a variation in the growth rate or in the sensitivity to gravity, due to such factors as sporangiophore maturity, culture age, and possibly, humidity. However, one cannot overlook the possibility that two or more of these factors may be involved.

Further results of Dennison showed that when vertical sporangiophores are centrifuged about a

vertical axis at a Resultant Gravitational Force
greater than 1.47 g the geotropic response is more
rapid than with natural gravity, and continues until
the direction of growth is parallel to the resultant
of the gravitational and centrifugal vectors.
Dennison found that at any specific angle the
geotropic bending speed increased with increased
centrifugal force in the range of 1-4.3g.

In addition to the response to steady gravitational
force a transient response, occurring only after a
change in centrifugal stimulus, also occurs in
Phycomyces sporangiophores. This response has two
phases. The first, occurring immediately upon
changing the relative centrifugal force, is in the
direction of the change of force and appears to be
an elastic flexing under the changing imposed force.
The second or active phase begins within two minutes
after the beginning of the change in relative centrifugal
force and lasts for about 5 mins. It represents an
active growth response, and occurs in a sense opposite
to the effect of the change in the flexing force.
This transient response is superimposed on the
relatively slow geotropic response elicited by steady
gravitational stimulus.

Dennison was able to show that the transient
response is due to the change in the external forces
acting on the sporangiophore, and the resulting
deformation of the growth zone, whereas the long term

response is not due to the deformation of the growing
zone under external forces. When the sporangiophores
were totally immersed in FC-43 (a fluorine-substituted
hydrocarbon fluid of high relative density- 1·87) during
the centrifugation, the transient geotropic response
was reversed, but the long term geotropic response was
substantially unaffected, despite the fact that
the greater density of FC-43 would result in the
externally acting forces being the reverse of those
on sporangiophores centrifuged in air. He concluded
that the long term response to a steady gravitational
stimulus must be due to the actions of the stimulus
on an internal sensory apparatus.

Investigation for Sensory Apparatus.

Sporangiophores of Phycomyces blakesleeanus grown
on 2% malt agar under normal daily light variations
were plucked from the mycelium when they were
approximately 2 cm tall and prepared for electron
microscopy. Transverse sections at 0·5 mm, 1 mm,
1·5 mm and 2 mm below the sporangium were examined
under the electron microscope.

(1) Ultrastructure of the growth zone of the sporangiophore.

The ultrastructural features of the cytoplasm are the
same at all levels in the growth zone. The sporangiophore
is bounded by a cuticle which sometimes during the
preparation of the material may become separated from
the chitinous (Roelofsen 1951) cell wall which in
the growth zone is approximately 2μm thick. From
immediately below the sporangial swelling
the columella wall extends downwards as an inner

lining within the sporangiophore wall for a distance of about 0.05 mm (Fig.18). In the terminal 0.5 mm of the growth zone small vacuoles coalesce (Figs.19 and 20) to form the large central vacuole which extends down to the sporangiophore base. Within this vacuole, and occasionally within the cytoplasm, crystals of various sizes and forms are found; they have been identified (Wolken 1969) as probably being a riboflavin protein complex (Fig.21).

These crystals bear a close resemblance to the intra mitochondrial crystals found in the epitheliomuscular cells of sexual and regenerating Hydra (Davis 1967) and to some of the macrocrystals found in the seeds of Fraxinus excelsior and Fraxinus americana (Villiers 1968). At high magnification crystal lattices could often be resolved. These appeared either as a regular system of parallel electron dense lines 130 $\overset{\circ}{A}$ apart with a repeating pattern of electron dense short side branches also of approximately 130 $\overset{\circ}{A}$ apart. In other sections, presumably cut in other planes, the lattice appeared more nearly as two sets of parallel lines intersecting at angles of 105° and 75°. Villiers suggests that the intranuclear crystals of Fraxinus represent storage protein for use in germination, and Davis also indicates that the intra mitochondrial crystals of Hydra may also be a storage product, although similar crystals were found by Oschman in Hydra viridis which had been starved for five days.

columella
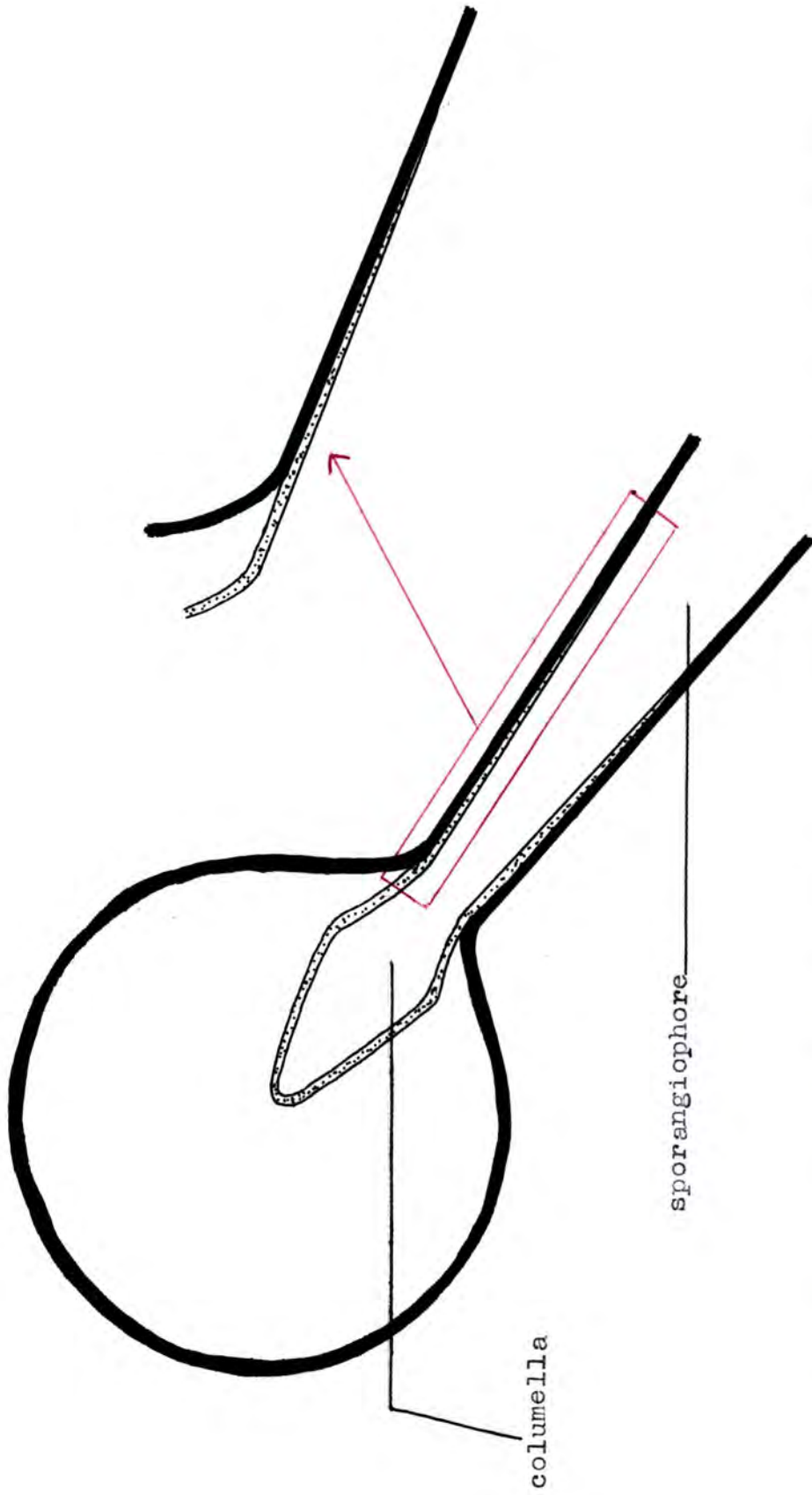
sporangiophore.

Fig.18. Longitudinal section of a mature sporangium to show the union of the columella wall with the wall of the sporangiophore
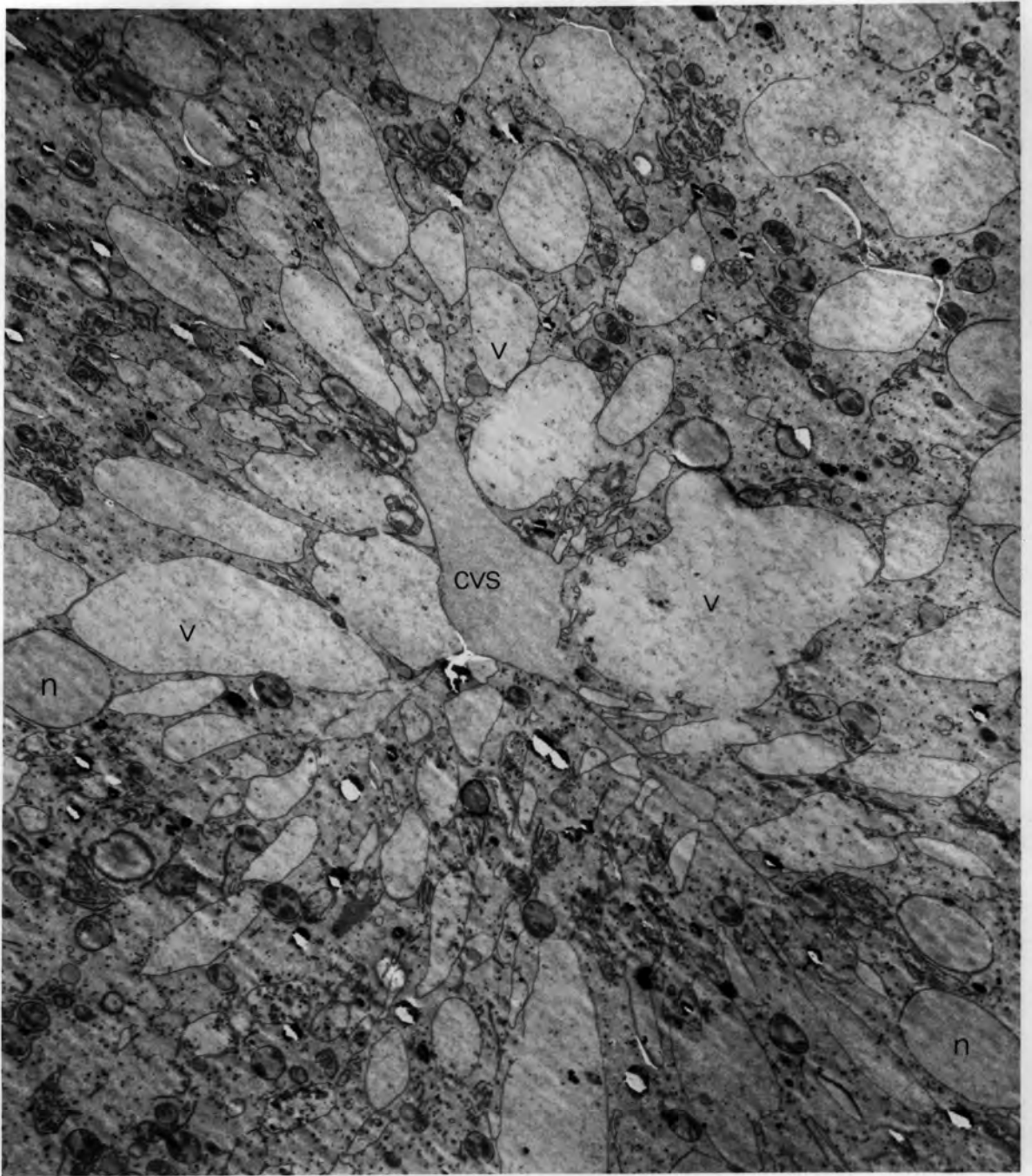
Fig. 19. Transverse section of the growth zone 0·5 mm below
the sporangium of a Stage IV B sporangiophore showing
numerous small vacuole and the central vacuole   X 5,600

Fig. 20. Central vacuole of sporangiophore 1 mm below the
sporangium. The cytoplasm vacuoles are less numerous
than in Fig. 19   X 6,800
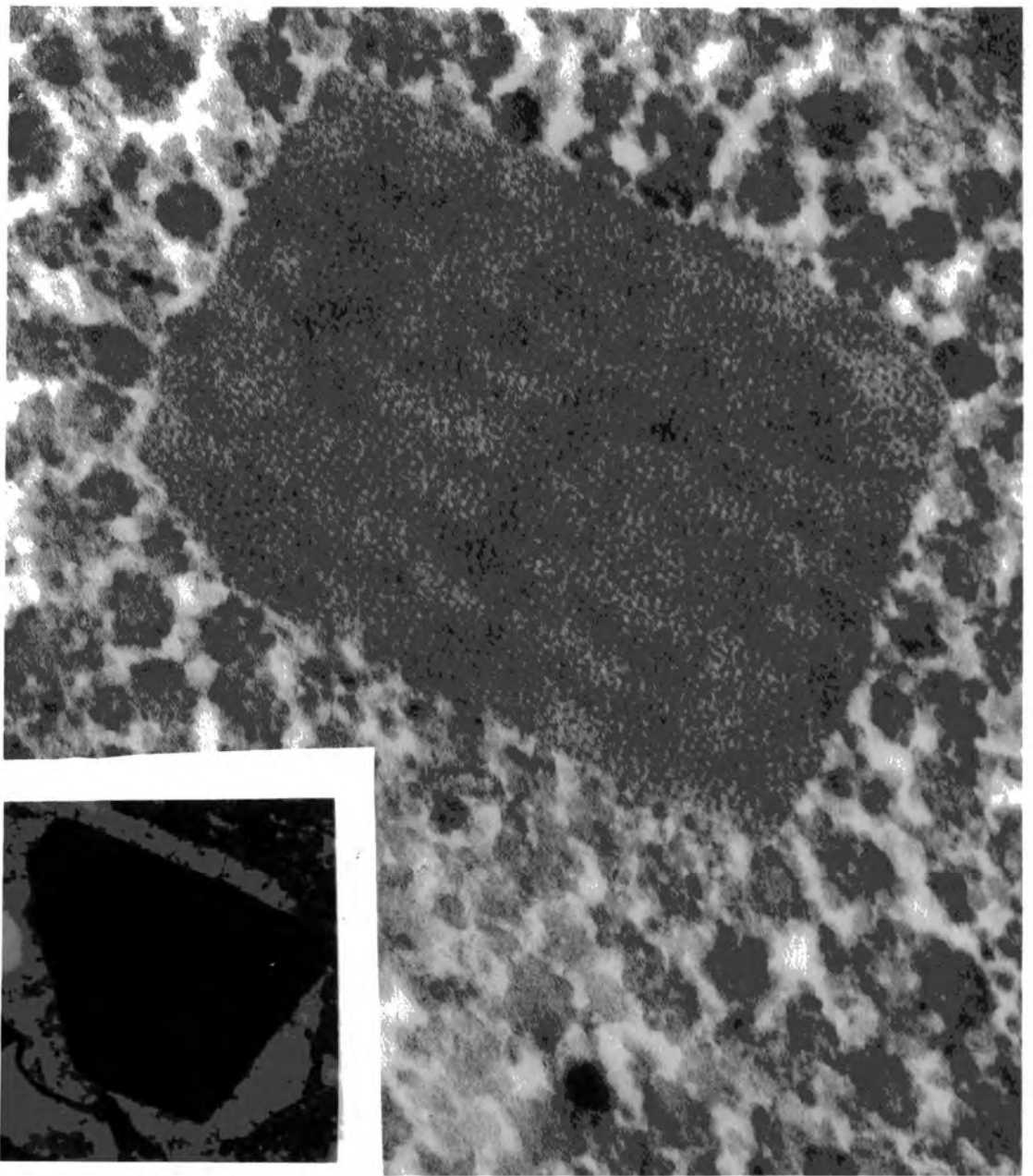
Fig. 21 Rhomoidal crystal in the cytoplasm. The crystal
is approximately 1.07 μ between the more widely separated
vertices and 0.95 μ between the other vertices. The
crystal lattice is seen as intersecting electron dense
lines.   X 115,000
Inset A vacuole crystal. Note the different profile.
Gluteraldehyde fixation.   X 21,560

Their presence in relatively large numbers in the vacuole of the older parts of <u>Phycomyces</u> sporangiophores, which in the Stage IV B development have little need of storage material, might perhaps more satisfactorily be interpreted as a means by which waste materials are removed from the cytoplasm and deposited as harmless insoluble substances.

There is no organised distribution of cytoplasmic organelles within the growth zone. Mitochondria nuclei,glycogen granules, fat droplets bound with a crystalline layer of ferritin molecules forming a two dimensional pattern over the surface (Peat and Banbury 1968) (Fig.22), vesicles of several forms and layers of membrane have a random distribution (Fig.23).

Pockets of vesicles causing numerous invaginations of the plasmalemma are of frequent occurrence. These very much resemble the lomasomes originally described by Mohr and McAlear (1961)(Plate 24).

VIb (ii) Primary Gravity Receptor

The possibilities for a primary gravity receptor for the long term geotropic response were:-

1. The sedimentation of particles - this was not likely because of the rapid streaming of protoplasm within the sporangiophore which would tend to disturb any concentration gradient induced by gravity.

2. The shifting of the large central vacuole.

3. The redistribution of the vesicle system.

VIb (iii) Examination for the Primary Gravity Receptor

Petri dish and oxoid cap cultures of <u>Phycomyces</u> <u>blakesleeanus</u> grown on 2% malt agar bearing Stage IV B
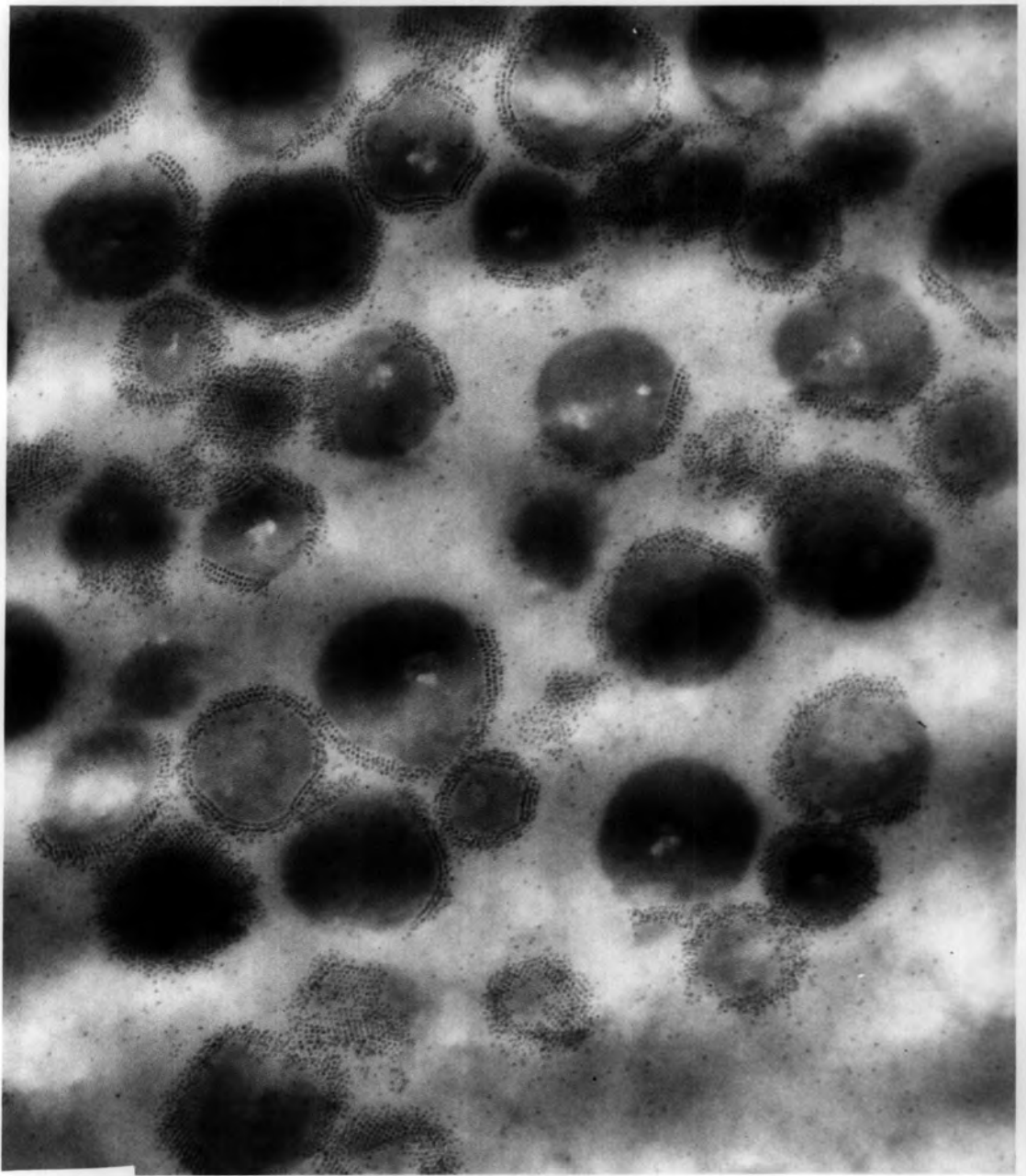
Fig. 22.   Ferritin bound to lipid droplets in the cytoplasm
of the growth zone of Stage IV B sporangiophore.
Note the two dimensional crystalline pattern
Gluteraldehyde fixation.   X 82,500
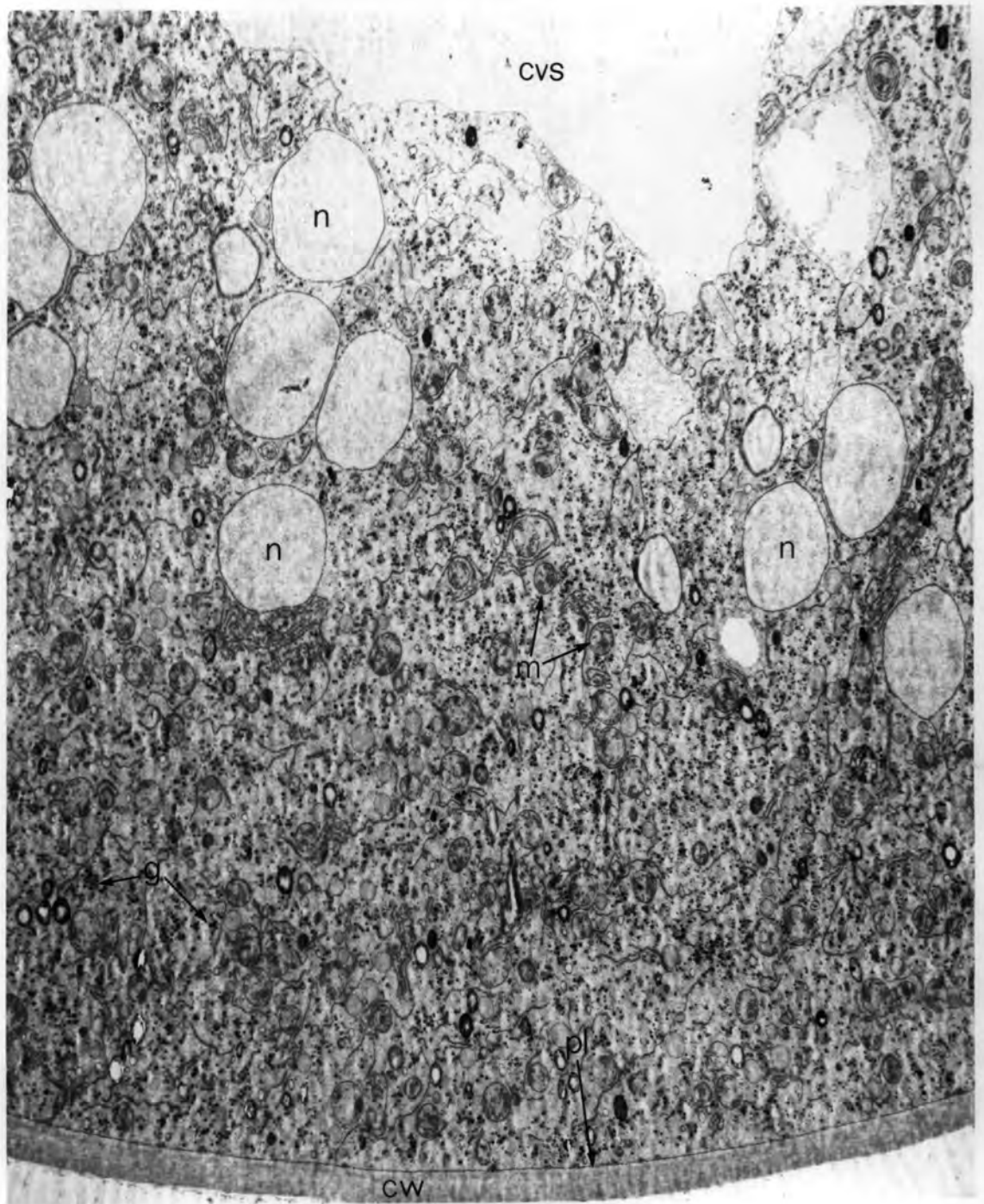
Fig. 23. Transverse section of growth zone of Stage IV B sporangiophore. KMnO$_4$ fixation.   X 5,780
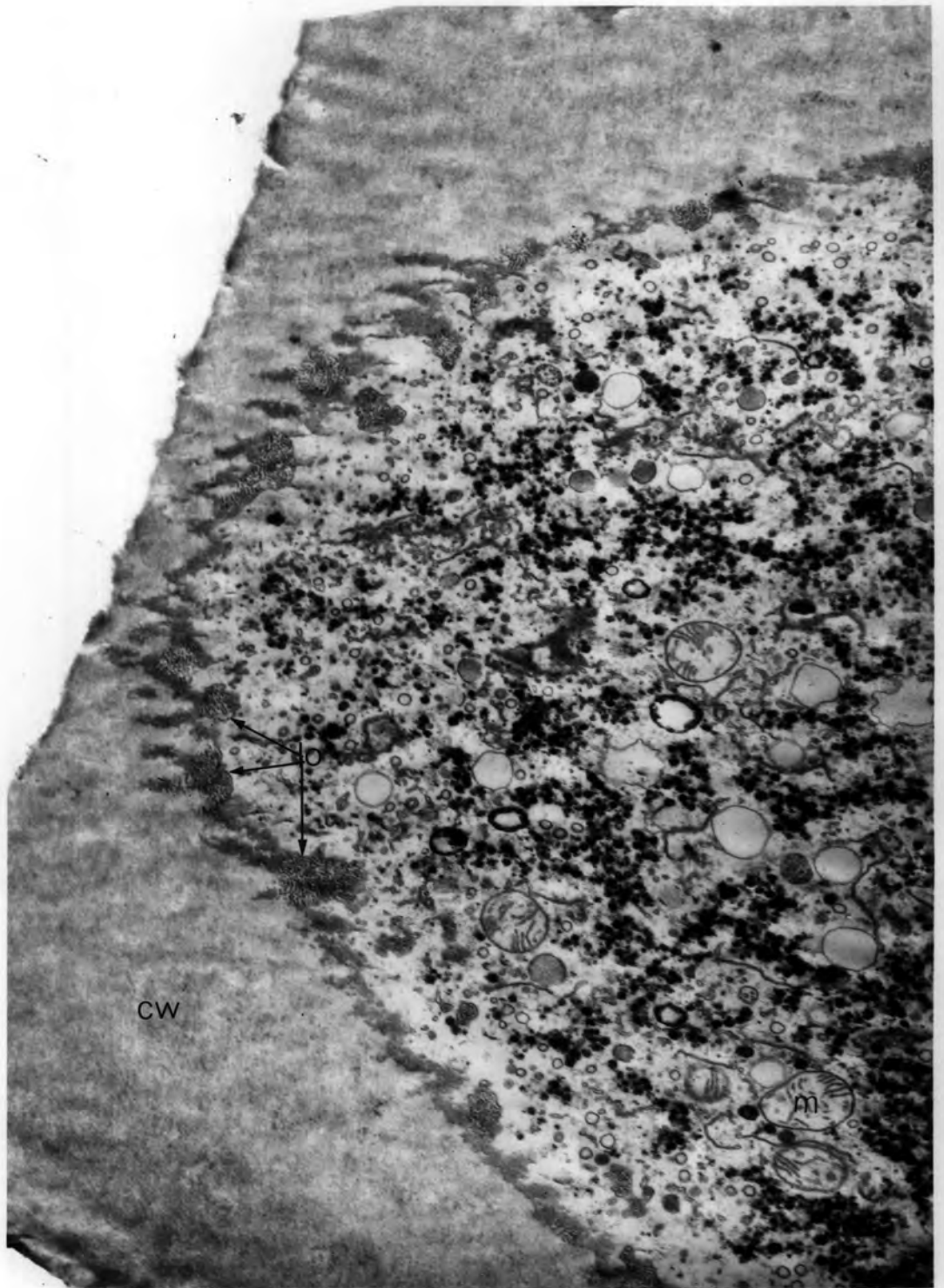
Fig. 24. Oblique section of growth zone of Stage IV B
sporangiophore 0.5 mm below sporangium. Note the large
number of 'lomasome-like' bodies associated with the
plasmalemma and cell wall. $KMnO_4$ fixation.   X 13,400

sporangiophores between 20-25 mm in height and standing vertically were treated as follows:-

All sporangiophores markedly less than, or greater than 20-25 mm in height were plucked from the cultures Some petri-dish cultures to be used as controls were placed in the dark and left to continue vertical growth for a period of 2 hours.

Other petri dish cultures were placed in the dark and turned so that the sporangiophores were lying with their along the axes making an angle of approximately 90° to the force of gravity.

The oxoid cap cultures were placed in the dark for 2 hours and then fitted with Morton closure caps to protect them from air currents during centrifugation. They were then placed on the centrifuge table in supporting cavities position 2 (Fig.25) i.e. 5.08 cm from centre of force and position 3 (Fig.25) i.e. 7.62 cm from centre of centrifugal force. The relative centrifugal forces at these positions were calculated as follows:-

$$\text{Centrifugal force} = \frac{M \times 4 \pi^2 \times N^2 \times R}{M \times g}$$

where M = mass; N = revolutions per second; R = radius and g = acceleration due to gravity = 980.6 cm/sec/sec and where:-

Position 2 (Fig. 25)

(a) When the disc was centrifuged at 200 R.P.M. the relative centrifugal force = 2.29 g.
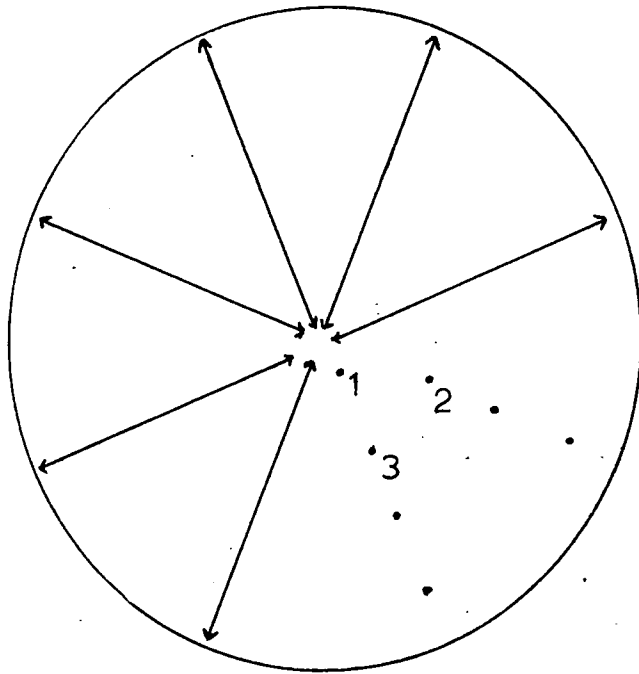
Fig. 25a. Surface view of centrifuge table to
show relative positions of holes drilled to take
the 'oxoid cap' cultures (shown on two radii only)
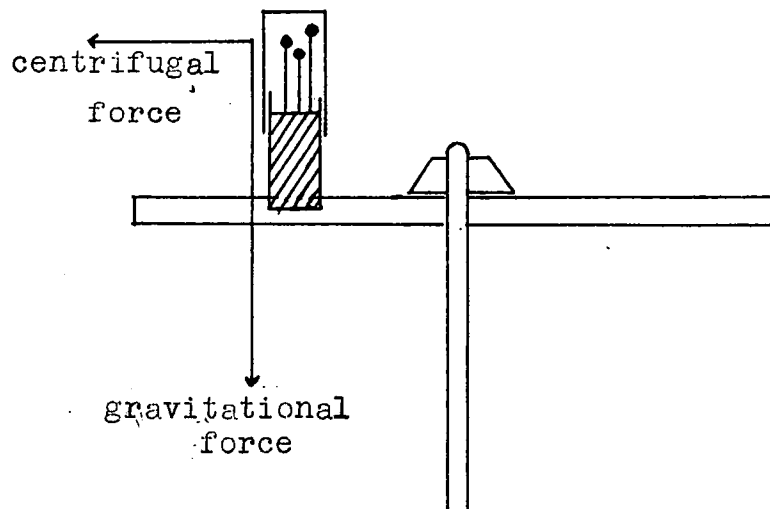
Fig. 25b. Side view of centrifugal table - only
one culture tube is shown

(b) When the disc was centrifuged at 300 R.P.M. the relative centrifugal force = 5·13g.

Position 3 (Fig. 25)

When the disc was centrifuged at 200 R.P.M. the relative centrifugal force = 3·43g.

The sporangiophores were centrifuged at approximately

    (a) 2·3 g for 30 mins

    (b) 5·1 g for ·5 mins

    (c) 5·1 g for 30 mins

An absolute positioning of the sporangiophores relative to the axis of rotation was not possible because of the need to obtain sufficient material for electron microscopy. However all the sporangiophores used were positioned within a radius of 1 cm from the midpoint of the culture discs.

Preparation of Material for Electron Microscope examination.

The curvatures produced in the sporangiophores in response to gravitational and centrifugal stimulation remained throughout the subsequent preparation of the material for electron microscopy, and it was thus possible to make accurate orientation of the specimens to ensure that true transverse sections across the curvature were obtained during sectioning (fig. 26). The block was marked on the outer side of the curvature to enable immediate identification when the sections were examined under the microscope (Fig. 27).

Preparation of Thick Sections.

Sections 1 μ thick were transferred to microscope slides by means of film loops, dried onto the slides and
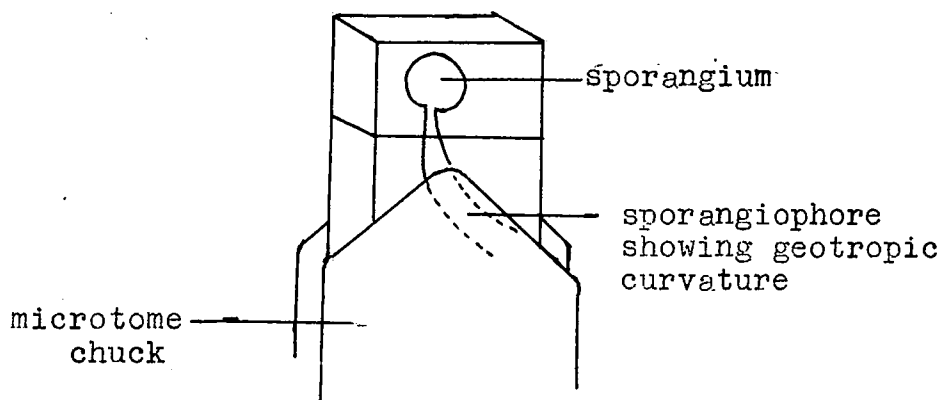
Fig. 26. Resin block in position in L.K.B. chuck ready for trimming
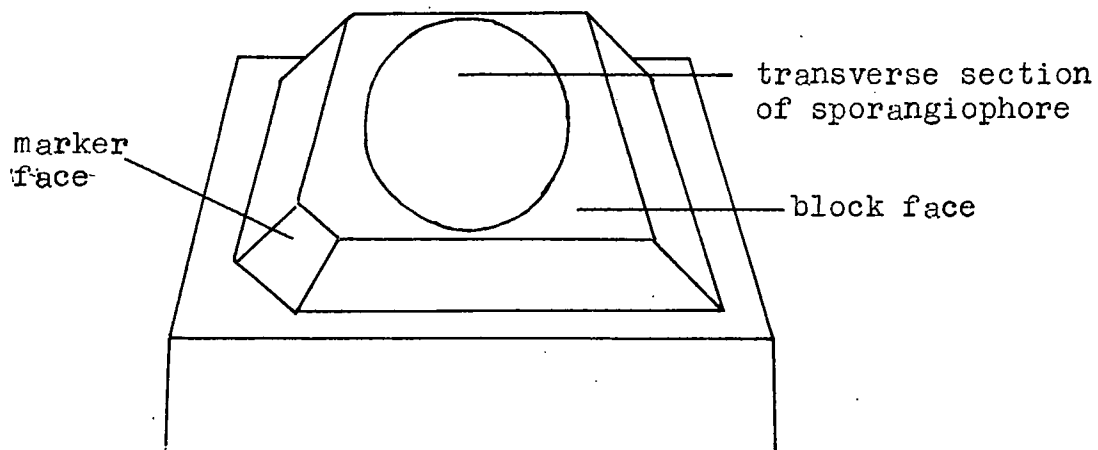
Fig. 27. Trimmed block ready for sectioning showing marker for outer edge of geotropic curvature in sporangiophore

then stained by warming in 1% Toluidine blue in borax
for several minutes. Following staining, the sections
were then rinsed under running water, air dried and
mounted in Euparal

VIc Results.

After reaching the required centrifugal force
by gentle acceleration for about 2 mins. the cultures
experienced a steady force that was both centrifugal
and gravitational in origin, resulting from the
vectorial addition of a component originating from
centrifugal acceleration and another from the
gravitational pull.

N.B. The magnitudes of all gravitational force
expressed as multiples of the acceleration due to
gravity (980 cm/sec ^{2}) to which the sporangiophores
were subjected, were selected on the basis of
Dennison's work (1961).

In the sporangiophores centrifuged at 5 g for 5 mins
inward curvatures (Fig.28) in the growth zone of 35°
to 37° from the vertical were measured and because
of selection showed a remarkably uniform response.
These were in agreement with Dennison's calculations
of "transient" tropic bending speed of 5°/min for
the active phase of the transient growth response
exhibited when the sporangiophores are subjected to
a change in the centrifugal force. The direction of
the curvature was related to the direction of change
of the centrifugal force in that it was always
opposite to it. Thus the direction of curvature was
inward as the centrifugal force was raised from
1 g to 5 g.

Direction of curvature of sporangiophore

Direction of centrifugal displacement of cytoplasm
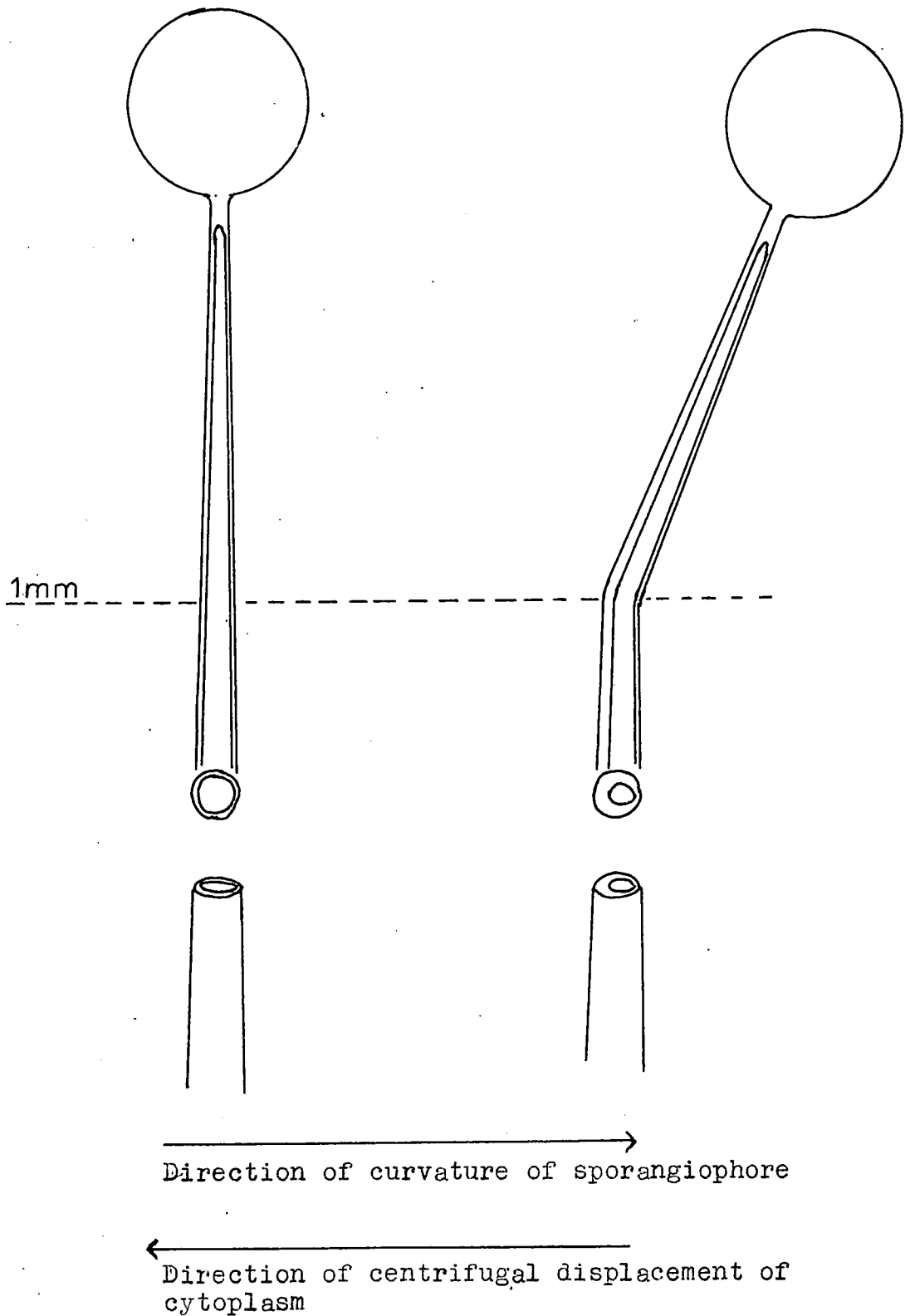
Fig. 28. Sporangiophore centrifuged at 5g for 5 mins produced an inward curvature of 35^0 to 37^0 from the vertical .

<u>Results of Examination of thick sections.</u>

The prepared sections were examined under a
Baker phase contrast microscope at magnification
of X 100 and X 400

<u>Sporangiophores centrifuged for 5 mins at 5.1 g</u>

Examination of thick sections showed a well
pronounced asymmetry in the distribution of the
cytoplasmic mass between 1.5 mm and 2.0 mm below
the sporangium base. In this area there was
approximately three times the depth of cytoplasm on
the outside of the resultant curvature, as compared
with that on the inside. (Fig.29). This asymmetry was
presumably the result of an outward displacement of
the cytoplasm and an opposite and corresponding
inward movement of the less dense vacuole.

The occurrence of this asymmetry between 1.5 mm
and 2.0 mm below the sporangium base closely corresponds
with the region of the maximum curvature of the
active phase of the transient response curve at
1.5 mm below the sporangium base reported by
Dennison (1961) There was no asymmetry of the cytoplasm
in those parts of the sporangiophore lying outside
the growth zone where secondary wall material was
being deposited. (Fig.30).

<u>Results of Examination of ultrastructural features.</u>

Thin sections were cut from the same areas as the
thick sections. These, after staining were
examined under the electron microscope and numerous
micrographs covering the whole area of each section
were taken at 2.5 k. From these micrographs, which
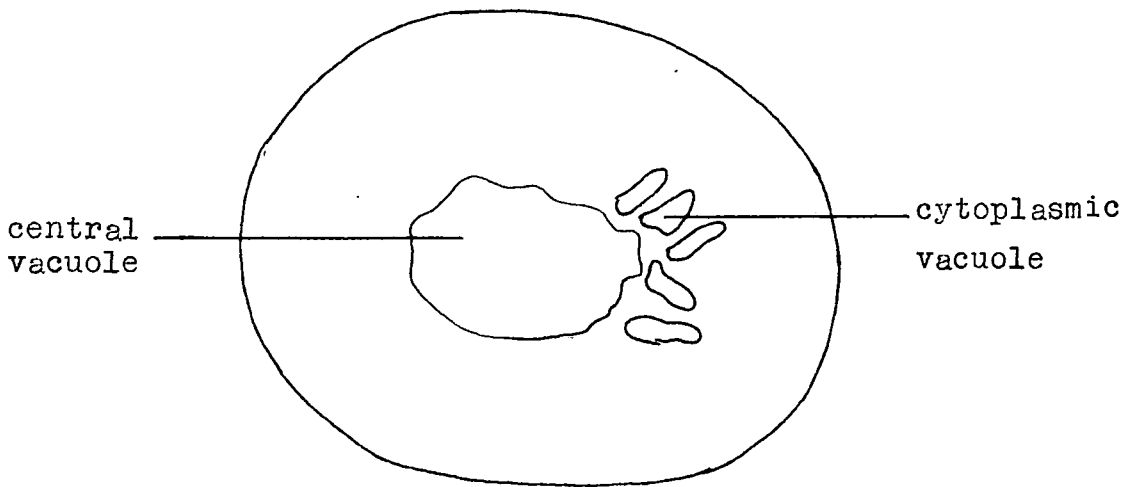
Fig. 29a Transverse section of a sporangiophore
centrifugal at 5 g for 5 min. Section cut 1 mm
below the base of the sporangium.
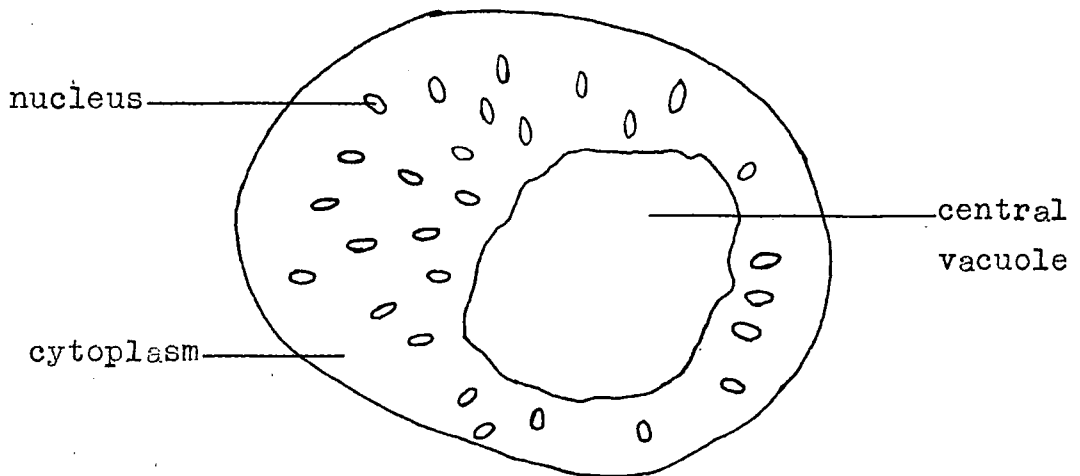
Fig. 29b Transverse section of same sporangiophore
at 2 mm below the base of the sporangium
At this point there is a marked asymmetry of the
cytoplasm. Camera lucida drawings
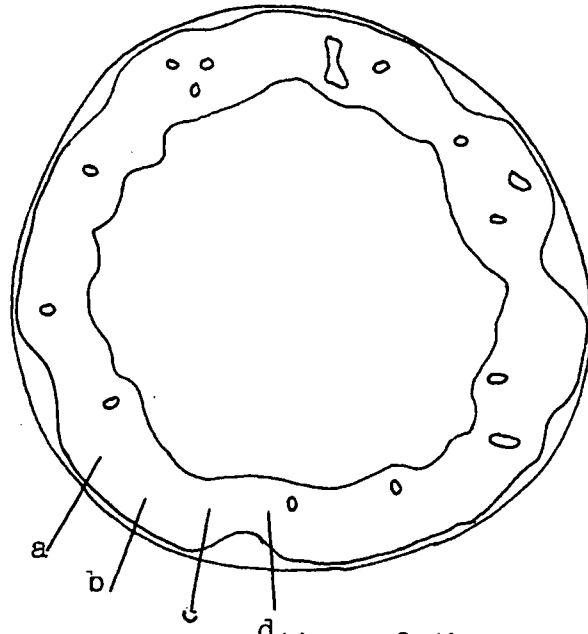
Fig. 30a. Transverse section of the same sporangiophore
as Fig. 29 but taken 4 mm below the base of the
sporangium. Note the absence of asymmetry of the
cytoplasm. Camera lucida drawing.
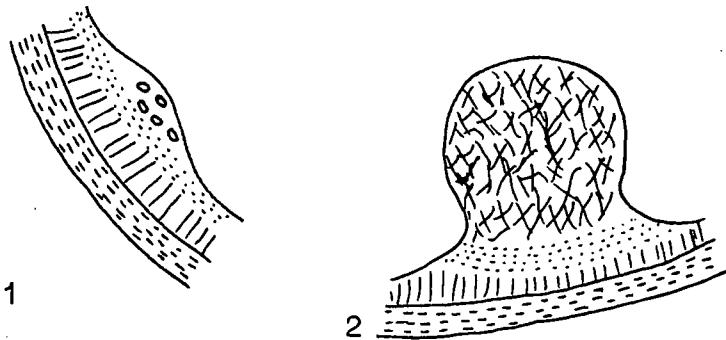
Fig. 30b. I. Part of the sporangiophore similar to
that between A-B, and 2. similar to that between C-D in
Fig. 30a to show the deposition of secondary wall
material between the primary wall and the plasmalemma.
Drawings from electron micrographs such as Plates 34
and 35.

had at least 25% overlap in order to allow for
spherical aberration, montages were prepared.
These were examined carefully for organelle
distribution. Examination of these montages taken
at various levels within the growth zone of both
the centrifuged material and that subjected to gravity,
did not reveal any asymmetry of organelle distribution
or any marked re-organization of organelles or inclusions
See Table IV for results.

VIc (i) Cytoplasmic Asymmetry in Centrifuged Sporangiophores

Dennison attributes the transient response to a
mechanical distortion of the cell wall under the
externally acting resultant force arising from the
action of gravitational and/or centrifugal force on
the relatively massive sporangium. He concludes
that the resulting stretching or compression of the
cell wall would stimulate or retard the rate of cell
wall elongation. The transient response must however
be a significant fraction of the total long term
geotropic response.

It seems probable that the asymmetry of the cytoplasm
that develops during the transient response might well
be the triggering mechanism for the release of
substances affecting the rate of cell wall growth on
opposite sides of the sporangiophore, that results in
the long term geotropic response, although it is not
known whether any ultrastructural change can be
recognized after brief stimulation by flexing, to
produce a minimal transient response.

Distribution of Organelles in Normal and Centrifuged

Sporangiophores

Using a 5 cm square counts were made of the various organelles present in the cytoplasm on the inside and outside of the geotropic curvature.

TABLE IV A

Sporangiophore not subjected to Gravitational and Centrifugal Force. Samples taken at random.

Block 173a Section 5. Magnification 2.5K Diameter 180.8 μ

Number of Organelle in each 5 cm square

Micrograph No —	1	2	3	4	5	6	7	8	9
Organelle									
Mitochondria	3	5	4	6	4	3	2	5	4
Nuclei	1	1	-	1	-	1	2	1	2
Fat droplets	6	4	4	1	3	1	4	4	3
Spherical vesicles	8	3	5	7	8	2	4	4	4
Small vacuoles 2.	-	3	4	1	3	3	4	4	1
Large vacuoles	-	-	-	-	-	-	-	-	-
E R	-	√	√	//	√	//	√	√	//

√ = present;   // abundant

Micrographs taken in sequence round tranverse section

TABLE IV B

Sporangiophore centrifuged for 5 g for 5 mins - Samples taken at random

Block 173d Section 5   Magnification 2.5k   Diameter 608.0μ

Number of Organelles in each 5 cm square

Micrograph No	-1	2a	2b	3	4	5	6a	6b	7a	7b	8	9	10	11a	11b	12a	12b
Organelle																	
Mitochondria	4	4	4	5	4	3	3	6	3	2	5	10	7	6	6	9	8
Nuclei	1	-	1	-	-	-	1	1	-	1	-	-	-	-	-	-	2
Fat droplets	2	4	2	2	6	3	1	3	5	2	4	9	7	3	4	1	7
Spherical Vesicles	3	5	7	5	5	2	2	2	2	2	2	3	7	2	4	2	6
Small Vesicles	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
Large Vesicles	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E R	✓✓	✓	✓✓	✓	✓	✓✓	✓	✓	-	✓✓	✓	✓	✓	✓	✓	✓	✓✓

Micrographs taken on inside of curvature

Micrographs taken on outside of curvature

TABLE IV C

Counts taken from same section as Table B but
from selected areas of the Cytoplasm on the
outside of the Geotropic Curvature.

	Number of Organelles in each 5 cm sq.									
Micrograph No -	8		9		10		11		12	
Outer Middle Mitochondria	5	3	9	4	5	5	5	7	10	6
Nuclei	-	-	-	1	-	-	-	-	-	-
Fat droplets	2	3	2	4	4	9	5	6	2	7
Spherical vesicles	4	1	5	1	3	3	4	2	-	1
Small vesicles	-	-	-	-	-	-	-	-	-	-
Large vesicles	-	-	-	-	-	-	-	-	-	-
E R	-	-	-	-	-	-	-	-	-	-
Inner Middle Mitochondria	1	7	-	2	3	2	5	9	8	4
Nuclei	1	-	-	1	-	1	-	-	-	1
Fat droplets	6	1	5	2	8	4	5	6	3	9
Spherical vesicles	1	2	1	2	4	2	5	1	13	4
Small vesicles	-	-	-	-	-	-	-	-	-	-
Large vesicles	-	-	-	-	-	-	-	-	-	-
Inner Mitochondria	-	3	-	3	1		2	-	3	2
Nuclei	1	-	-	-	-		1	1	-	2
Fat droplets	-	3	-	2	-		5	4	1	-
Spherical vesicles	-	1	-	3	-		-	3	1	1
Small vesicles	-	-	-	-	-		-	-	-	-
Large vesicles	-	-	-	-	-		-	-	2	-

TABLE IV D

Counts taken from same section as Table IV B
but from selected areas of the Cytoplasm on
the Inner side of the Geotropic Curvature.

Number of Organelles in Each 5 cm Square.

Middle layer	Micrograph No.	1	2	3	4
	Mitochondria	2	4	2	4
	Nuclei	1	–	1	1
	Fat droplets	3	5	5	4
		–	1	–	2
	Small vacuole	–	–	–	–
	Large "	–	–	–	–
	E R	√	√√	√	√√
Inner layer					
	Mitochondria	1	1	1	
	Nuclei	–	1	–	
	Fat droplets	–	2	6	
		–	–	–	
	Small	1	–	–	
	Large	–	–	–	
	E R	√√	√	√	

VIc (ii) Absence of Organelle Asymmetry

The absence of asymmetry of organelle distribution
in sporangiophores subjected to gravitational and
centrifugal forces are in agreement with the findings
of Zalokar (as reported by Bergman et al) when he
attempted the intra-cellular centrifugal separation
of the organelles in the Stage I sporangiophore. Zalokar
found that centrifugal forces of about 50,000 g were
necessary to obtain separation of organelles from
cytoplasmic connections.

It is therefore most unlikely that the mechanism
of geoperception is based on differential sedimentation
of organelles. The role of sedimentation, relative
viscosities of organelles and inclusions in geoperception
has been critically discussed by Audus (1962)

VIc (iii) Lomasome Development

In Sporangiophores subjected to a steady force
of either 1 g for 2 hours, 5 g for 30 mins or 2 g for
30 mins or more the asymmetry of the cytoplasm was
not so well defined but did nevertheless exist.

In these sporangiophores however, particularly
those centrifuged at 2 g for 30 mins or more there
was found to be an extensive increase in the lomasome-
like bodies on the outside of the resulting curvature
(Fig.31) when compared with :-
(a) the lomasome-like bodies of the vertically grown
sporangiophore
(b) the lomasome-like bodies on the inside of the
curvature (Fig.32). Lomasome-like bodes were also
present between the plasmalemma and sporangiophore

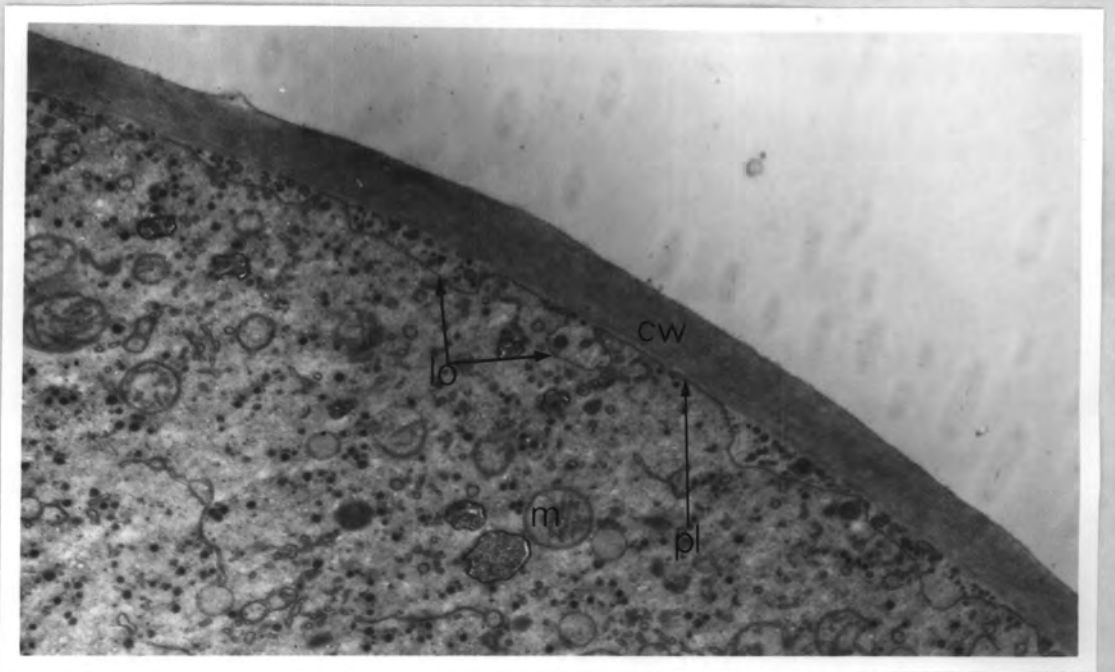Fig. 31 Extensive development of lomasome like
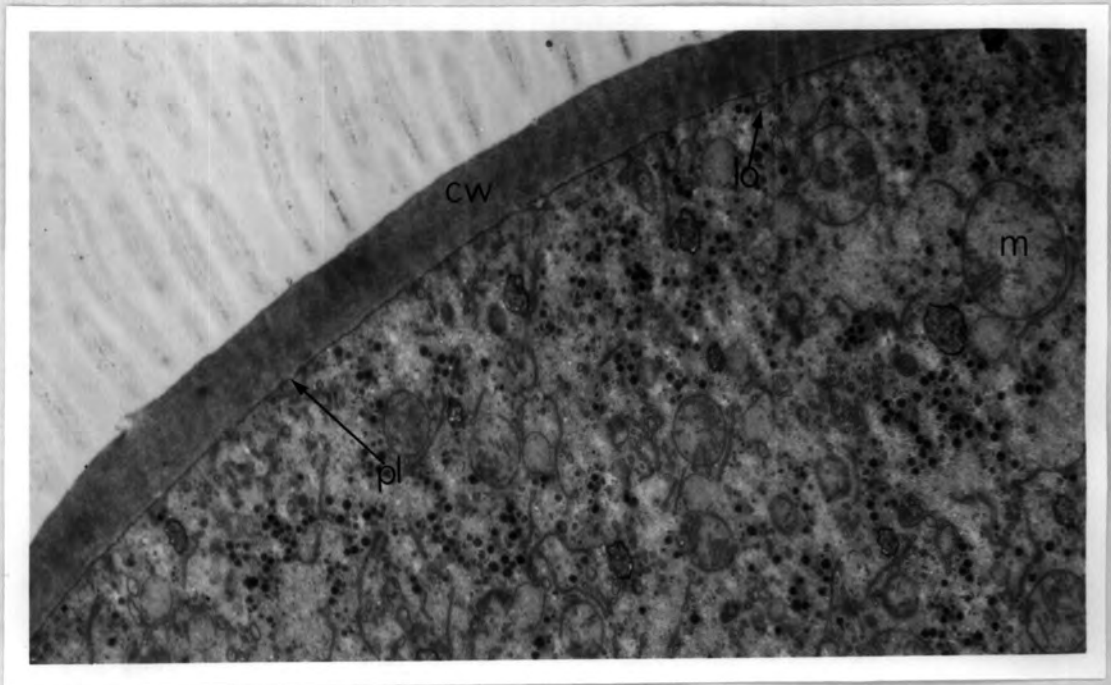bodies between the cell wall and plasmalemma
on the outside of the geotropic curvature

Fig. 32 'Lomasome-like' bodies between the
cell wall and plasmalemma on the inside of the
geotropic curvature

walls in those regions of the sporangiophore where
secondary wall material was being deposited (Fig. 33).
In these regions several extremely large plasmalemma
invaginations were observed (Fig. 34.) Moore and McAlear
suggested that these bodies might play some role

in the building of cell walls.

Discussion

Autoradiographic studies (Barton 1968) however
showed that in *Chara* species, which possessed
lomasomeelike bodies, when the cells are provided with
tritiated glucose it is the Golgi apparatus in which
the glucose appears before being extruded in the
Golgi vesicles and taking up its final position in

the cellulose fibres of the wall.

Despite Barton's findings and his suggestion
that lomasomes are probably of no significance in
wall synthesis, the presence of these lomasome-like
bodies in regions of active hyphal wall growth, their
apparent absence in non-growing regions and their
extensive development close to the outer wall of the
geotropic bend, where one would expect increased wall
synthesis, strongly suggests that they play some role
in wall synthesis of the sporangiophore of *Phycomyces*
blakesleeanus and that they play some part in bringing
about growth responses. Heath and Greenwood (1970) as
a result of their investigations on *Saprolegnia ferax*
and *Dictyuahus* also suggested that lomasomes and
plasmalemmasomes may not be involved in wall synthesis.
However the two organisms used by them were both
oomycetes which like *Chara* possess a substantial
proportion of cellulose in the cell wall. They may

Fig.33 Transverse section of a sporangiophore
centrifuged at 5 g for 5 min, 4 mm below the
sporangium. Note the development of secondary
wall material and the presence of 'lomasome'
like bodies.   X 7,180
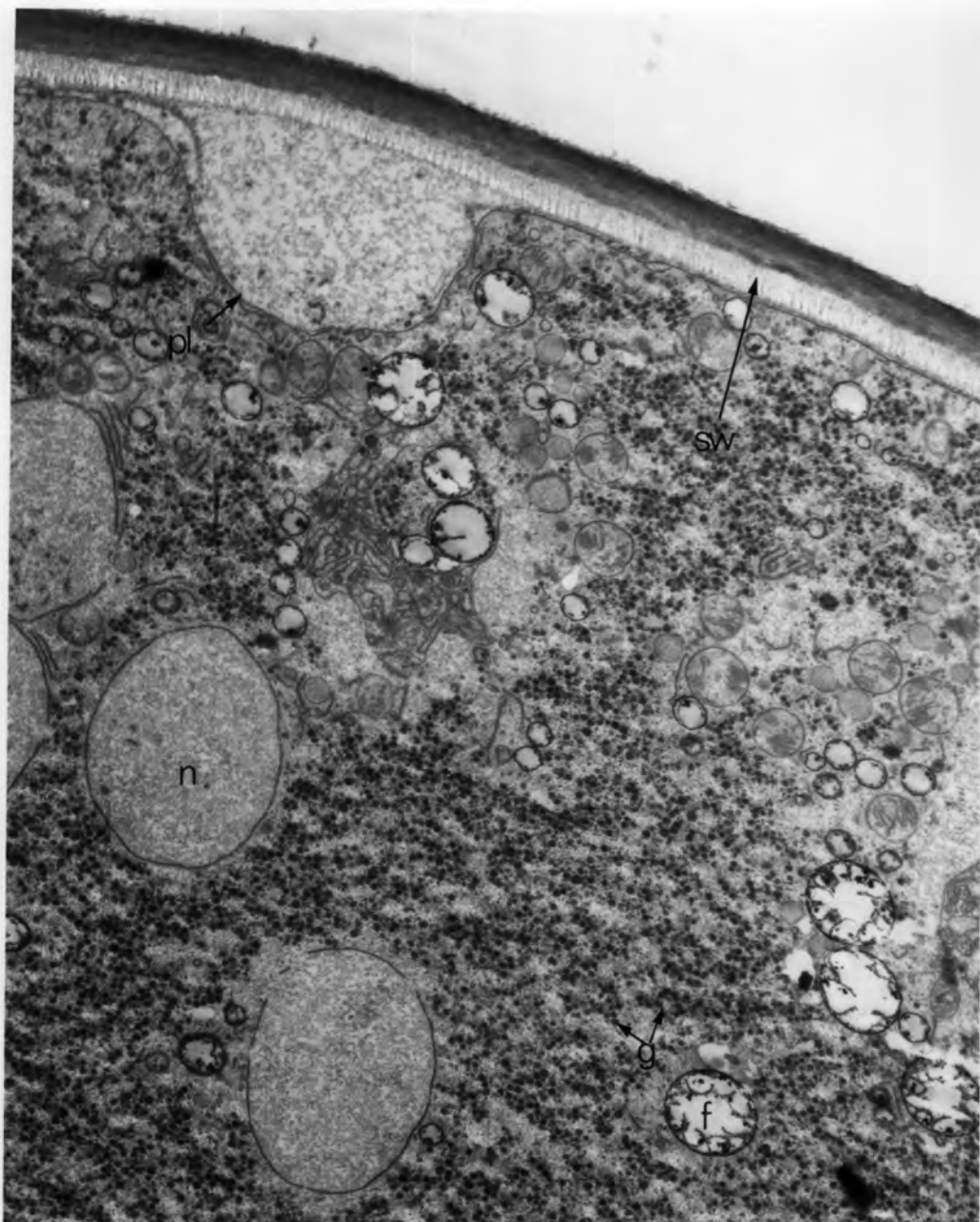
Fig. 34. Extensive invagination of pasmalemma in region of secondary wall development. Sporangiophore centrifuged at 5 g for 30 min. X 7,180

therefore possess a closer affinity to <u>Chara</u> in their mechanism of wall synthesis than they do to most other fungi which,like <u>Phycomyces</u>,have cell walls containing a substantial proportion of chitin.

The electron microscope evidence so far obtained for the mechanism of cell wall growth in <u>Phycomyces</u> <u>blakesleeanus</u> is somewhat confusing. The presence of both microfilaments passing from the cytoplasm into the cell wall and 'lomasome-like' bodies between the cell wall and plasmalemma suggest the involvement of a two phase system leading to the final deposition of wall material and the associated spiral growth of the sporangiophore. Future autoradiographic studies may help to verify the precise mechanism of wall synthesis in <u>Phycomyces</u> sporangiophores.

Section VII

Photoreception in Phycomyces

VIIa.Historical.

      The earliest investigations on the light growth responses of Phycomyces sporangiophores were made by Blaauw (1914) and later confirmed by Castle (1932), who observed among other things that there was a marked but transient increase in growth rate when sporangiophores were symmetrically illuminated from two or more sides. Blaauw (1918) developed a theory for the phototropic response of the Phycomyces sporangiophores. He interpreted the unequal growth that leads to a phototropic curvature as a result of an unequal light growth reaction in the proximal and distal sides of the unilaterally illuminated sporangiophore. He supposed that in the sporangiophores of Phycomyces there would be a tendency for light to focus on that side of the sporangiophore away from the source and this would consequently give a stronger positive light growth reaction on that side thus bringing about a positive phototropic curvature. Such a process would not increase the total amount of light received by the back wall but would only alter its spacial distribution. Castle (1933) assumed that the absorbing pigment was not located in or near the sporangiophore wall but was uniformly distributed throughout the protoplasm. He pointed out

that due to refraction, the average length of path
traversed by the light in the back half of the
sporangiophore is about 20% greater than that
traversed in the front half. Therefore the nett
absorption in the back half might well be in excess
of that in the front half of the sporangiophore
if the attenuation of intensity during traversal
is slight.

According to Banbury (1959) Buder argued that
Castle's assumption of a uniform absorption of light
throughout the cytoplasm was an unlikely one.
Allowing that the total flux of light reaching the
back wall of the sporangiophore will not be greater
than that striking the front wall, but in fact must
be somewhat smaller, Buder argued that due to lens
action photoreception would be concentrated in a
narrow zone in the back wall resulting in a narrow
concentrated growth reaction. This, Buder argued,
would give the growth response in the back wall a
mechanical advantage over the uniformly distributed
one in the front wall.

However Delbrück and Reichardt (1956) point
out that none of these theories account for the
fact that the phototropic response must be extremely
localised at the base of the growth zone, since
without such localisation the spiral growth so
characteristic of Phycomyces together with the tropic
response would cause the growth zone to bend into
a helix. Delbrück and Reichardt (1956) showed by
means of stimulating short sections of the growth
zone and measuring the growth response that there

was an independent response in each fraction of the growth zone. The full response of the totally stimulated growth zone was the sum total of the response of each component part of the zone. They were however unable to explain why the latent periods between the stimulation of the various zones and the responses are all alike if each stimulation produces its response at the base of the growth zone, although it is not impossible that stimulation leads to conduction of the stimulus to the base of the growth zone.

Banbury obtained results which supported the deduction of Delbrück and Reichardt that the tropic response is confined to the base of the growing zone. He obtained a phototropic curvature, in Stage I sporangiophores illuminated by a very narrow grazing pencil of light, which was in a plane almost perpendicular to the beam and at a distance of a millimetre or more behind the region of wall surface actually illuminated. There is thus a strong indication that there is a downward transmission of the stimulus although the experimental evidence is far from conclusive.

VIIb The Photoreceptor.

Numerous attempts have been made to identify the photoreceptor. Studies to find it have been based on the action spectra of the sporangiophores and, as a result of these, β-carotene, retinal attached to a protein, or a flavoprotein have been suggested as likely receptor pigments. These compounds

have light absorption spectra which closely resemble the action spectra of the phototropic curvature.

In vivo measurements through the growing zone of single sporangiophores show that β- carotene is the main absorbing pigment in the 400-500 nm region of the visible spectrum (Zankel, Burke & Delbrück 1967). Its absorption spectrum is very close to the action spectrum although displaced 5-10 nm to the red. There have, however, been several pigment mutant strains of <u>Phycomyces</u> discovered containing less than 1/1000th of the β - carotene possessed by the wild type which are still fully photosensitive (Meissmer and Delbrück 1968). These findings indicate that the bulk of the β - carotene is not the effective pigment, but do not necessarily mean that it plays no part in photoreception. The mutant type sporangiophores have been estimated to possess about 10^{10} molecules of β - carotene per sporangiophore while the requirements for effective tropic responses have been estimated at about 3×10^9 molecules of receptor pigment per growth zone (Bergman et al 1969). It is thus still possible that some specialised form of β - carotene may play some part in photoreception.

Retinal as a receptor pigment is most unlikely since wild type sporangiophores contain very small quantities, between 10^9 and 10^{10} molecules per sporangiophore which is about 1/10,000th that of the β-carotene content. Attempts to find retinal protein complexes which liberate retinal on exposure to light have proved negative and no retinal was found in the β - carotene

mutant car 5 which shows a phototropic response
(Bergman et al 1969).

Wolken (1969) obtained two distinctly different
absorption spectra for the growing zone 0.1 to 3 mm
below the base of the sporangium. One he found to be
more characteristic of a carotene whilst the other
was more characteristic of a flavin. When the absorption
spectra were combined they reproduced very closely the
action spectrum for the phototropic response. In the
albino car 10 (-) which is deficient in carotenes,
only the spectrum characteristic of 2 lumichrome or a
reduced flavine was found. In both the wild type
and albino car 10 (-) a c type cytochrome was isolated
which if coupled with a flavin could permit a photo
reversible oxidation - reduction system.

Wolken also observed birefringent crystals
aligned in the growing zone in which the photoreceptor
is believed to lie. Using microspectrophotometry he
was able to show that the absorption peaks of these
crystals were similar to those of riboflavin crystals
and to the absorption spectra of the sporangiophore.
These facts together with the isolation of a cytochrome
suggested to Wolken a possible scheme which would
provide a photo-reversible system such as from the
oxidised yellow riboflavin via cytochrome and light
to the reduced colourless form. He also suggests the
possible conversion of riboflavin in light and acid
pH to lumichrome. It therefore seemed to me well worth
while to attempt to determine the frequency of occurrence
and distribution of such crystals with the electron
microscope.

As a result of his work Wolken strongly suggests that β-carotene does not appear to be the primary photoreceptor molecule although it or one of the other carotenes present in the sporangiophore most likely functions as an accessory pigment molecule.

Heuckel (1927) claimed to have observed a greater density of protoplasm on the far side of unilaterally illuminated sporangiophores, than on the near side in Mucor mucedo and in Pilobolus crystallinus. Banbury (1952) was not able to detect any asymmetry in opacity in living sporangiophores of Mucor mucedo or Phycomyces blakesleeanus during or immediately after unilateral illumination.

However, it was thought that small aggregates of photo-absorbing material might become arranged in the cytoplasm in a distribution that was determined by the light flux and that a small concentration gradient might have important consequences in modifying the light absorption.

VIIc. Electron Microscope Investigation for a possible Photoreceptor

Method.

Cultures were grown in 2% malt agar in diffuse light at ambient room temperature until the sporangiophores were in Stage IV B of development, approximately 2 - 2.5 cm tall and growing vertically.

They were then transferred to the growth chambers in a dark room. In the growth chamber the sporangiophores were illuminated by one 6 volt 3 watt bulb on each of two opposite walls positioned so that the incident light

from each bulb made an angle of approximately 45°
with the growth zone. They were left for 30 mins to adapt
to the reduced light conditions. At the end of this
period one light was turned off so that the specimens
were now receiving unilateral illumination of half the
intensity of the original symmetrical bilateral
illumination. Within 30 mins the sporangiophores
had produced a positive phototropic curve that was
visible to the naked eye.

Preparation of Material for Electron Microscopy.

Once a visible curvature had been produced the
sporangiophores were immediately plucked from the
culture and prepared for electron microscopy in the
same way as were the sporangiophores subjected to
gravitational/centrifugal force.

The specimens were orientated in the chuck and
sections were cut between 1.5 and 2 mm below the
sporangium and stained as in the preparation of
material being examined for a primary gravity
receptor. (Section VI b iii)

VIId. Results of Examination of Material

Examination of the thin sections under the electron
microscope did not show any asymmetry of the cytoplasm
on the illuminated and non illuminated sides of the
sporangiophore and did not confirm Heuckel's view
(1927) that there was a difference in protoplasmic
density on the near and far sides of a sporangiophore
showing a phototropic curvature.

Estimates of the organelle distribution on the
far and near sides of the phototropic curvature gave

no evidence of any significant reorganisation of particular organelles. (Fig.35)

The only appreciable difference was that between the glycogen content of sporangiophores grown under normal daylight conditions and those under investigation. In the latter the glycogen content was significantly less than that of sporangiophores grown in normal daylight as judged by the electron density after staining.

VIIe.Discussion.

In consequence of these findings it seems most unlikely that photoreception has its origin in the organelle distribution of the growth zone. Despite Wolken's findings it also seems most improbable that the crystals, identified by means of their absorption spectra as being either riboflavin or a closely related compound, found within the sporangiophore are associated with photoreception. These crystals, which vary considerably in their size and form, appear to have no specific location within the sporangiophore. They were occasionally found within the growth zone, in large numbers in the older mature cytoplasm many millimetres behind the growth zone, and also in the large central vacuole. (Fig 36) The alignment of these crystals in the vacuole of the growth zone, as reported by Wolken (1969) was not commonly observed in my material. His findings may have been significant for his strains or may have been a matter of chance; they are not valid for my strain which gave a strong phototropic response.

Fig. 35. Transverse section of the growth zone of a
sporangiophore unilaterally illuminated for 30 mins.
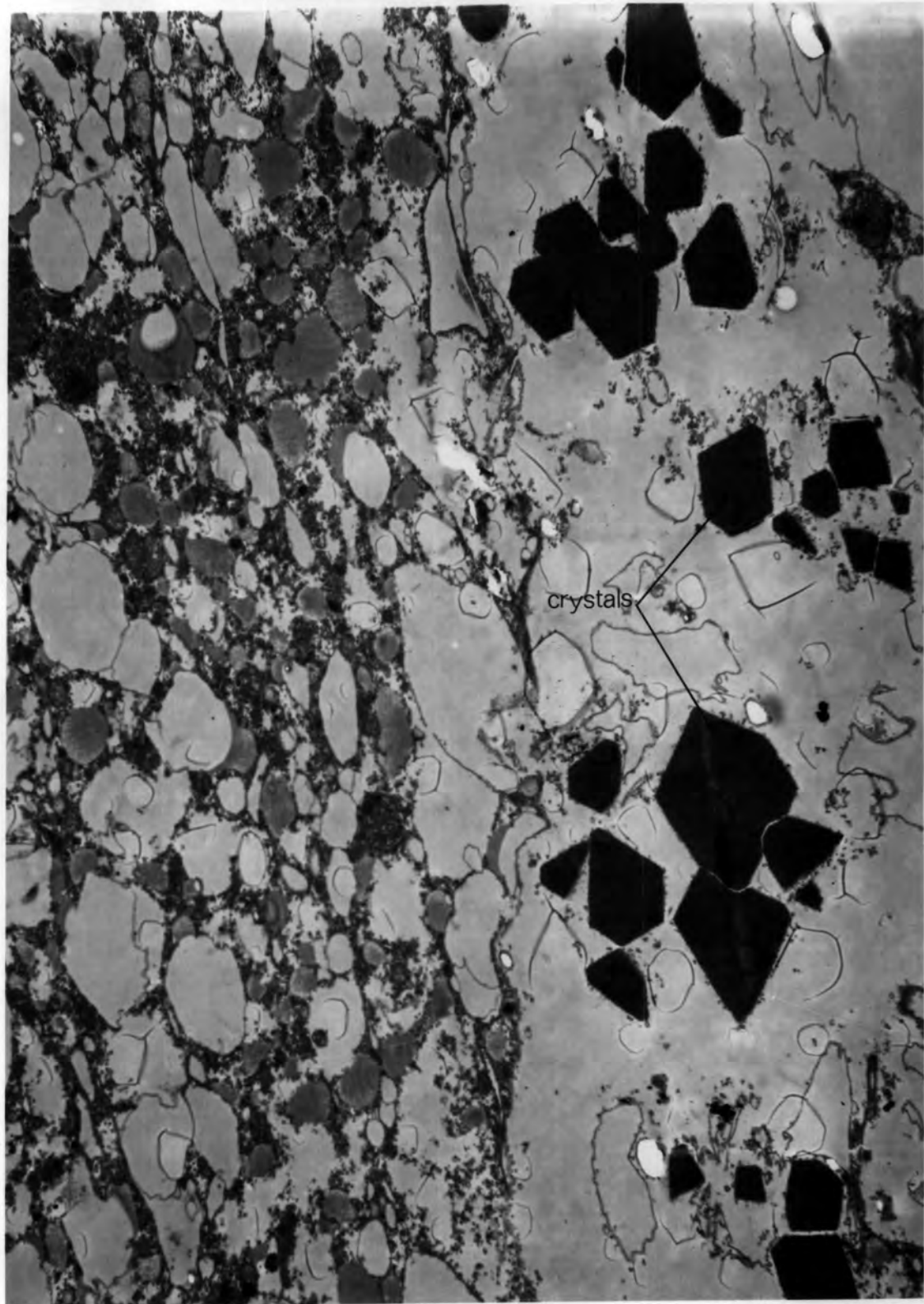Note the apparent absence of glycogen.   X 7,500

Fig.36. Crystals identified as riboflavin or closely related compound (Wolken 1969) in the vacuole of the mature region of a sporangiophore. Gluteraldehyde – Osmium fixation.   X 6,250

This random distribution, especially within the vacuole, makes them far from suitable as photoreceptors.

The results of these preliminary experiments do not suggest that the photoreceptor of _Phycomyces_ sporangiophores will be found in the macromolecular structure of the cytoplasm as revealed by electron microscopy.

Section VIII

Sporangium Development and Spore Germination
VIIIa. Review of Previous work on Sporangium Development

Columella formation

The earliest account that deals specifically with
the formation of spores in the Mucorineae is that of
Corda (1838)(as reported by Swingles 1903). He
investigated the development of the Sporangium of
Ascophora mucedo, now known as Rhizopus nigricans
and gave us the first description of the method of
columella and spore formation. He described the
columella as "at first being slightly arched in
the lower part of the sporangium and gradually pushing
upwards into the sporogenous tissue". The spores he
described as being formed in radiating rows from the
columella. Although this method of columella formation
was soon described as being "a matter of pure invention"
on the part of Corda (Fresenius 1850-63) it was generally
accepted and appeared frequently in the classical literature
until well beyond the turn of the century.

According to Swingles an accurate and detailed
description of the structure and development of a very large
number of the Mucorineae was later covered in a series of
papers by Van Tieghem (1873, 1875, 1876).

According to Schwarze, Brefeld (1872) studying Mucor
mucedo described the columella as not being something

that is primarily flat and then becoming
arched by increased pressure from below, but "has
from the first instance an arched form which may
subsequently and incidentally become modified".
Similar descriptions of columella formation were made
by several later workers (Leger 1896, Harper 1899,
Swingles 1903 and Schwarze 1923).

Spore formation.

The method of spore formation in sporangia was
studied by Pringsheim as early as 1859. It was however
Van Teighem (1873, 1875,1876),working on almost the
entire group of zygomycetes,who first indicated
that the spores may be formed by simultaneous division.
He believed that the method of spore formation was the
same in all genera producing a spherical sporangium.
He observed that the sporogenous protaplasm of
Sporadinia separated itself into two very different
substances. The spore-plasm which was always granular,
condensed into small polyhedral portions which rounded
off, secreted a cell wall and formed the spore, and
the interspore-plasm which was always homogeneus and
brilliant, and during spore formation dispersed itself
so that it occupied all the spaces between spores and
formed a layer between the outer spores and the
sporangial wall.

Strasburger (1880) studying zoospore formation in
Saprolegnia described spore formation as being
simultaneous. "In den Zoosporangien der Saprolegnien
wird, wie aus zahlreichen angaben bekannt, eine grosse
Angahe Schwarmsporen simultan aus dem gesammten
Protoplasmatischen Inhalte des Sporangium gebildet"
Similar accounts of simultaneous spore formation were given

by several other authors including Dangeard (1890)
working with <u>Synchytrium taraxaci</u>, Cornu (1872) working
with <u>Olpidiopsis</u> & Thaxter (1897) working with
<u>Syncephalastrum.</u> (as reported by Schwarze 1923)

Schwarze (1923) however, claims, after studying
cleavage in the sporangia of a number of fungi, that
the evidence for simultaneous spore formation was
far from adequate, but rather that there was an
accumulation of evidence in support of the view that
the process was a progressive one resulting from
furrowing either from the periphery of the sporangia
or from vacuoles within the spore-plasm.

Swingle (1903) made the first detailed study of
spore formation in <u>Phycomyces</u> species, an account of
which is given in a paper in which he compares the processes
occurring in <u>Phycomyces nitens</u> with that occurring in
<u>Rhizopus nigrᶦᶜans</u>. In this paper he gives a lucid account
of the development of the cleavage vesicles, the zonation of
the sporangial contents in the early stages of
development, and the differentiation of the columella and
spores, as seen in material prepared by using the
mixtures of Flemming, Herman and Merkel as fixing fluids
together with Flemming's triple stain. Swingle, however,
claimed that the cleavage vesicles were present within
the cytoplasm at the onset of sporangium development.
He also claimed to have observed two layers in the
sporangium wall from a comparatively early stage in
development but only one layer in the wall of the
sporangiophore. Another feature which Swingles describes
in his paper, is the contraction of the spore-plasm during
cleavage, a process he believes to be responsible for
the enlargement of the cleavage vesicles and hence
cleavage.

Numerous electron microscopical studies have to
date been made on fungal material. Some of these
have been concerned with spore germination. (Hawker and
Abbott (1963) and Hawker and Hendy (1963), but only one
detailed electron microscopical study of sporangium
development has been made (Bracker 1966).

VIIIb. Sporangium Development in Phycomyces.

Two to three days after inoculation of a 2% malt
agar plate with mycelium the culture is usually seen to
possess sporangia in all stages of development. The
developmental sequence can for convenience sake be
divided into five stages (Fig.37)(Castle 1942 based
on Errera).

Stages of Development.

Stage 1. The Sporangiophore grows up from the mycelium
as a simple tube tapering rather sharply at its apex,
where it grows at a rate of 1-2 mm./hour. Unlike later
stages the apex shows little, if any, rotation around the
vertical axis.

Stage II. The onset of Stage II is influenced by a number
of environmental factors which include the chemical
composition of the medium and illumination. It represents
a change in the morphology of the sporangiophore. The tip
of the sporangiophore swells and a bright yellow sporangium
is formed. During the enlargement of the sporangia,
the sporangiophore does not lengthen or rotate.

Stage III. This is a period lasting for several hours
during which there is neither sporangium enlargement
nor sporangiophore growth.

Stage IVa. The sporangiophore resumes elongation and
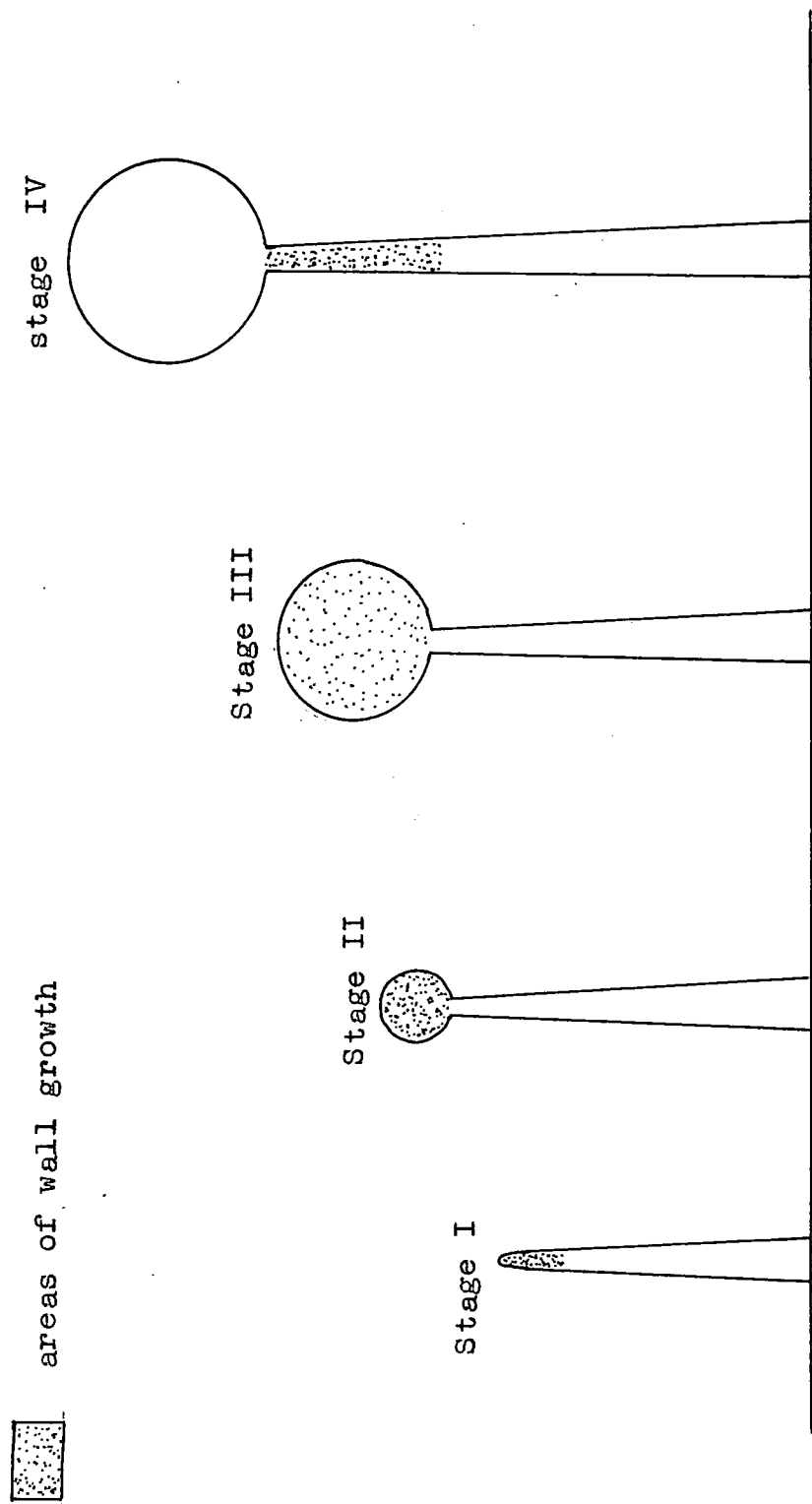twists in an anticlockwise direction viewed from above.

Fig. 37. Stages in development of the sporangium of Phycomyces (after Castle 1942)

The sporangium turns brown.

Stage IVb. About 90 minutes after the resumption of growth anticlockwise rotation slows down and is gradually replaced by rotation in a clockwise direction soon reaching a rate of one complete rotation in 30 minutes. The sporangiophore continues growth for many hours maintaining a steady rate, once attained, of about 3 mm per hour.

The apical zones of all stages of development were examined and during investigation very many sporangia were sectioned in several places.

VIII6. Preparation of Phycomyces Sporangia for Electron Microscope Investigation.

Sporangiophores bearing sporangia at Stages 1-4 of development (Castle 1942) were plucked from the mycelium at the point of junction of the sporangiophores and mycelium. These were put in separate vials containing 2% $KMnO_4$ and one part per thousand of detergent. The material was left in the fixative for varying periods of time.

The fixation times were as follows:-

Stage	I	1 hour
Stage	II	2-4 hours
Stage	III	5-6 hours
Stage	IV	12 hours

These fixation periods were chosen after several trial attempts. Fixation using gluteraldehyde followed by post fixation with osmic acid was attempted but it gave extremely poor results.

After dehydration in a graded series of alcohols the Stage I sporangiophores and the sporangia were embedded in T.A.A,B. embedding resin.

Section of the order of 60nm in thickness were cut. After uranyl acetate and Reynold's lead citrate

staining these were examined and photographed on an
A.E.I EM6B electron microscope.

VIIId.   Results.

(i) The Stage I apex.

The apical zone of the Stage I sporangiophore has
been examined by Peat and Banbury (1967) and Thornton
(1968). My findings were in general similar to theirs.
At the growing tip is an area about 20 μm in length
which is devoid of nuclei and possesses very few
mitochondria, the latter only beginning to make their
appearance at approximately 9.2 μm behind the apex.
I was not able to confirm the findings of Peat and
Banbury that these mitochondria differed markedly in the
form of their cristae from those in other regions of the
hyphae. The plasmalemma in this apical region is
extremely irregular often showing extensive and much
folded ingrowths passing deep into the cytoplasm.
The cytoplasm within the first 9 μm contains numerous
small pieces of membrane forming a variety of configurations
within the ground plasm. The wall of the hyphal apex
measured approximately 1.1 μm in thickness and
was surrounded by an electron dense cuticle (Fig. 38).

This apical zone is followed by a region about
100 μm long which contains numerous nuclei which are
so closely packed together as to be almost touching each
other. These nuclei are often jointed together by extensions
of the nuclear membrane, and many of them appear to be in
a state of division (Fig. 38a) Scattered mitochondria
are found interspersed between the nuclei together with
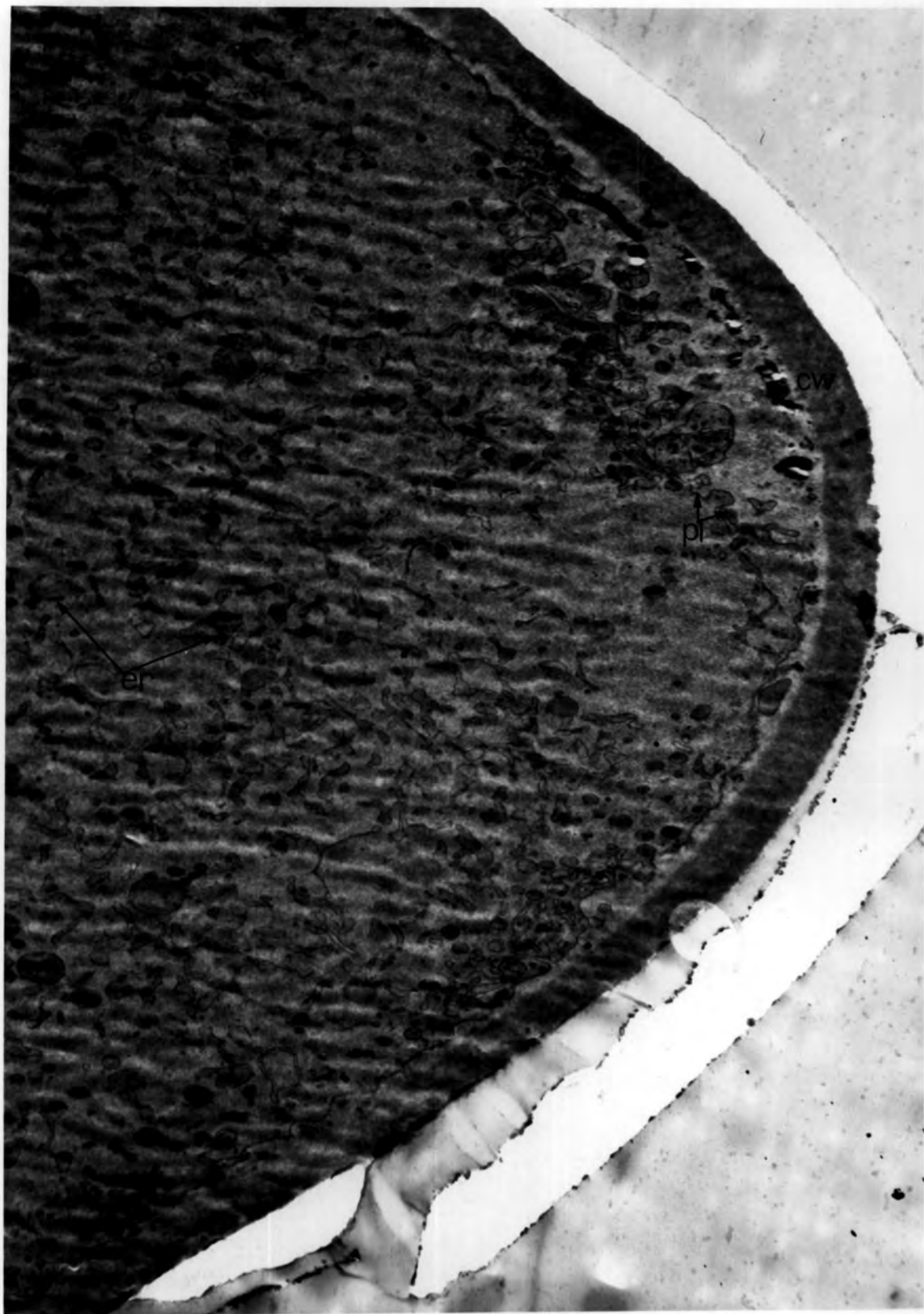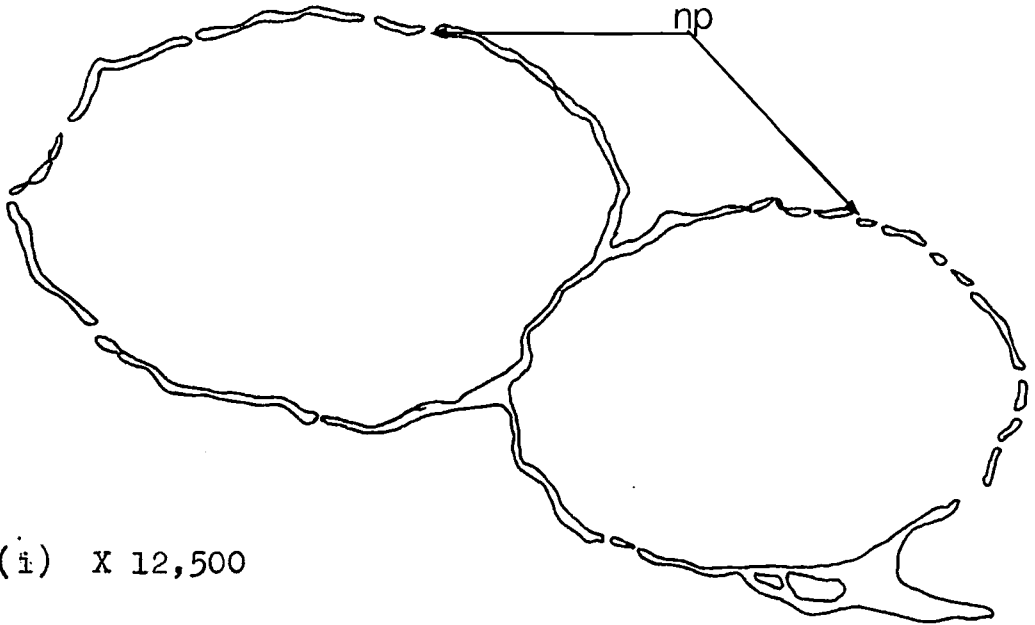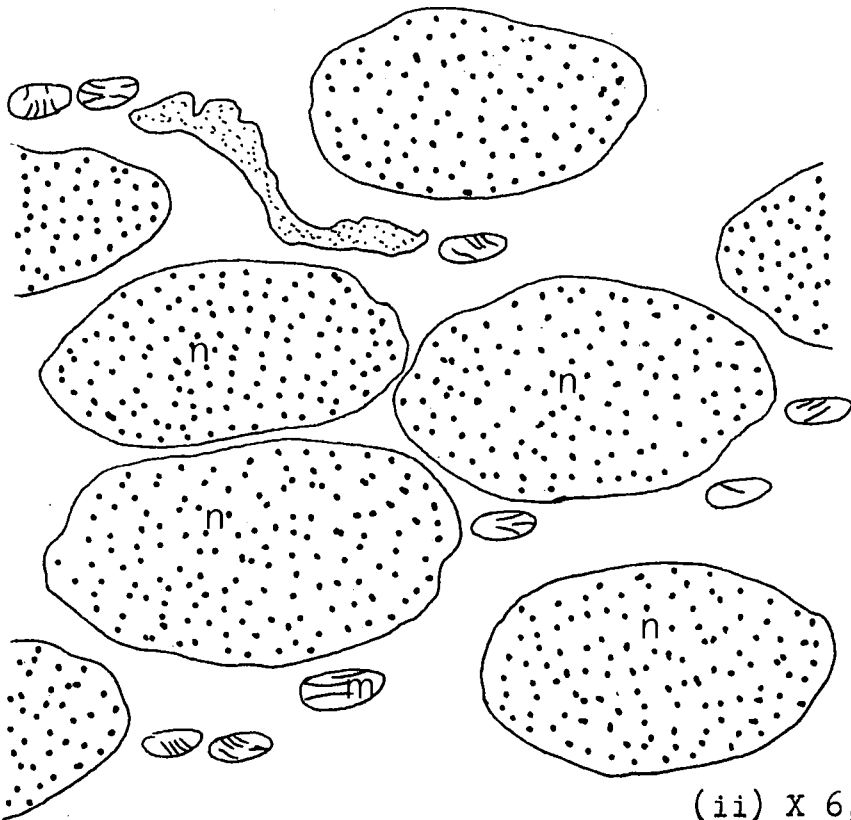membrane bound "pockets" of granular cytoplasm.

Fig. 38. Longitudinal section of a Stage I sporangiophore.
tip. Note the extensive invagination of the plasmalemma
and development of endoplasmic reticulum.   X 87,250

(i) X 12,500

(ii) X 6,250

Drawings from electron micrographs to show,
Fig. 38a (i) two nuclei with dividing membrane and
(ii) close aggregation of nuclei, between 20 μm
and 100 μm behind the apex of a Stage I
sporangiophore

The cytoplasm surrounding this central core of nuclei and lying between it and the cell wall is almost devoid of nuclei, but contains numerous mitochondria, fat droplets and a concentration of glycogen granules. The plasmalemma in this region still possesses numerous invaginations surrounding vesicles between it and the cell wall.

About 100 μm below the sporangiophore apex large vesicles, similar to those appearing during the cleavages of the Stage III sporangium and described later, are present. Further down the sporangiophore these enlarge and eventually coalesce to form the large central vacuole characteristic of the older parts of the sporangiophores.

Thornton (1968) attributes the formation of the central vacuole to the development of autophagic vesicles which he suggests originate by extension and pinching off of parts of the nuclear membranes. In support Thornton published micrographs showing vesicles containing mitochondria and other cell constituents in various stages of breakdown. I have not been able to obtain any corresponding micrographs with the exception of some of materials showing a considerable distortion of organelles indicating poor fixation. In all these instances the fixative was gluteraldehyde followed by osmium tetroxide , the fixative used by Thornton.

(ii) The Stage II apex.

Stage II begins with the enlargement of the apical zone. This continues until the terminal swelling is about three times the maximum diameter of the

sporangiophore and a bright yellow sporangium is formed. During this stage there is neither any elongation nor twisting of the sporangiophore.

The initial sporangium is bounded by a minutely sculptured sporangial wall which measures between 0.9 μm and 1.8 μm in thickness; it is thus very similar in dimension to that of the Stage I sporangiophore apex, a feature which suggests the intersusception of additional wall material between the existing wall constituents during the period of apical swelling. The sculpturing of the sporangium wall is a new feature not found in either the Stage I or later stages of sporangium development. There was no evidence with the techniques employed for the presence in this wall, or that of the Stage I sporangiophore, of a well defined microfibrillar structure such as is recognizable in the wall in other parts of the sporangiophore (Fig.39). This does not however exclude the possibility of fibrillar material being present. The electron dense cuticle is still present.

The plasmalemma situated just within the wall showed numerous invaginations containing vesicles varying in size and number. These invaginations were similar to those described earlier and reported by several authors (Weisenach and Kessel 1965, Marchant, Peat and Banbury 1967, Caloqne, Fielding and Byrde 1969 and Chet, Henis and Kisley 1969) as occurring in regions of wall synthesis in several fungi, and I see no reason to suppose anything other than their involvement in the maintenance of wall thickness during sporangial enlargement.
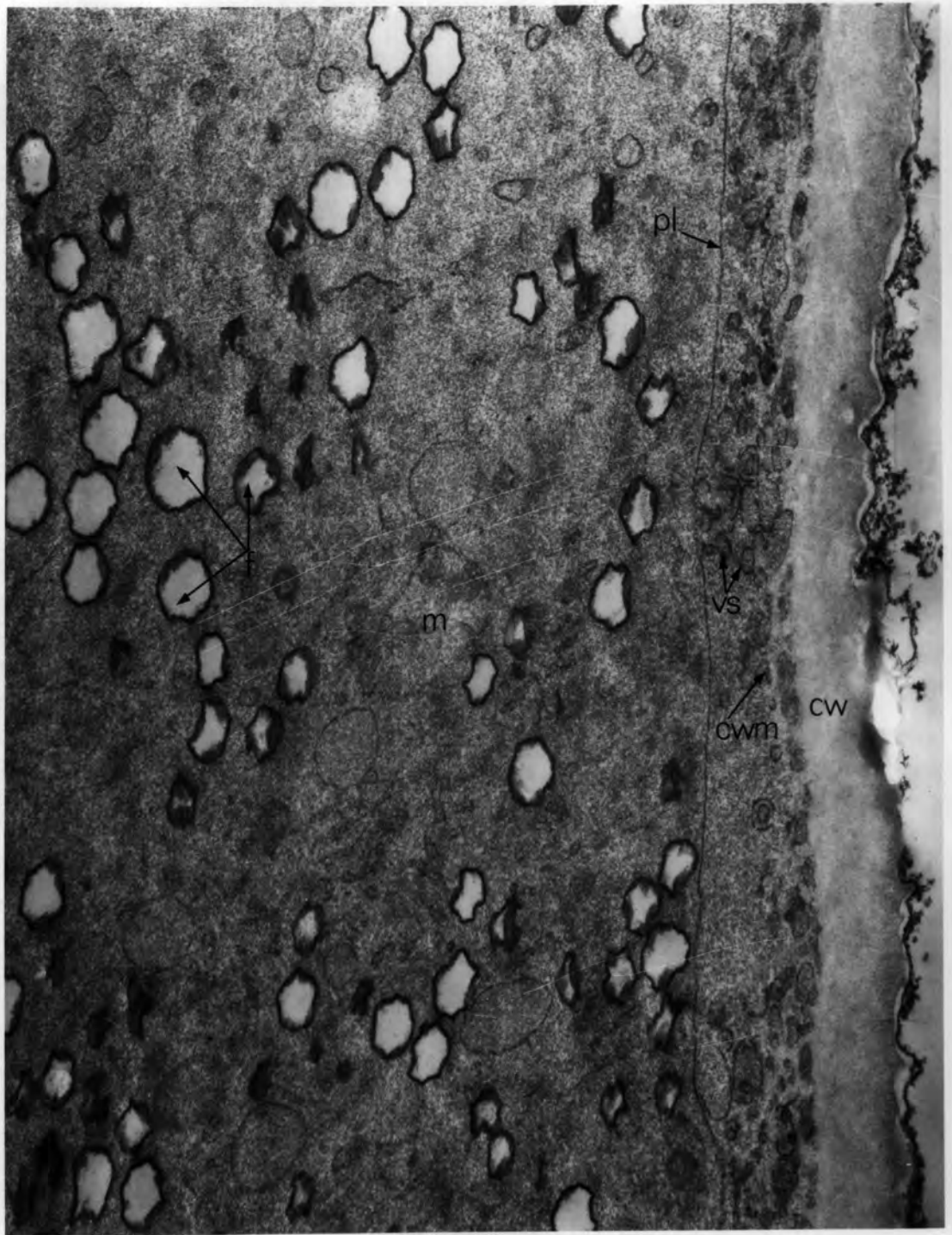
Fig. 39. Part of the wall of a Stage II sporangium showing development of new wall material and the vesicles apparently associated with its deposition.

X 24,600

The cytoplasmic organelles of this initial
sporangium when examined in longitudinal section
exhibited a marked zonation forming three distinct
areas (Fig.39a).

Outer Zone.

Immediately within the spherical sporangial wall
is a zone about 15 μm deep. The cytoplasm of this area
is completely lacking nuclei but contains all the other
organelles found within the Stage I sporangiophore.
This zone itself was stratified into three layers.
The outer and inner regions (A_1 and A_3 Fig. 40), of
this zone each contained a well developed membrane system,
numerous vesicles and ovoid bodies of low electron density
internally but with an electron dense bounding layer.
On the basis of their fixation these latter bodies
are probably lipid globules consisting of a central
core of saturated fats surrounded by unsaturated fats
and phospholipids or alternatively the two forms of fat
uniformly mixed with a reaction occurring at the surface.

The membranes forming the endoplasmic reticulum
of the region appear to bud off vesicles at their
extremities and in some instances the sections
revealed chains of small vesicles comparable in length
to the membranes themselves. In the A_3 region similar
membranes were often grouped together to form structures
showing some resemblance to the Golgi bodies of higher
plants and the dictysomes of Pythium debaryanum (Hawker
and Abbott (1963).

The vesicles.

In all the specimens examined the vesicles
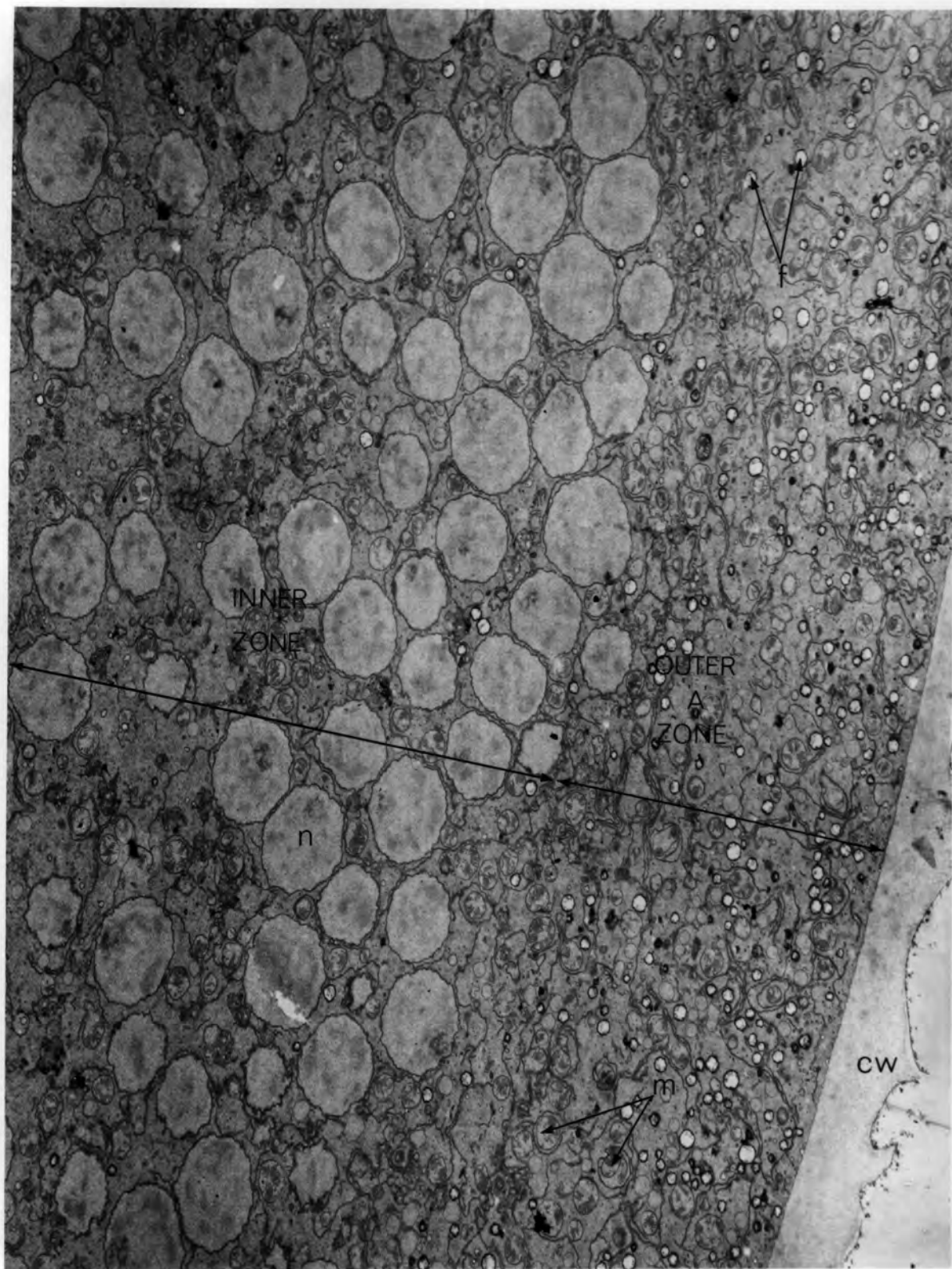associated with the endoplasmic reticulum appear to be

Fig. 39a. Section through the Stage II sporangium to
show the organized distribution of organelles and
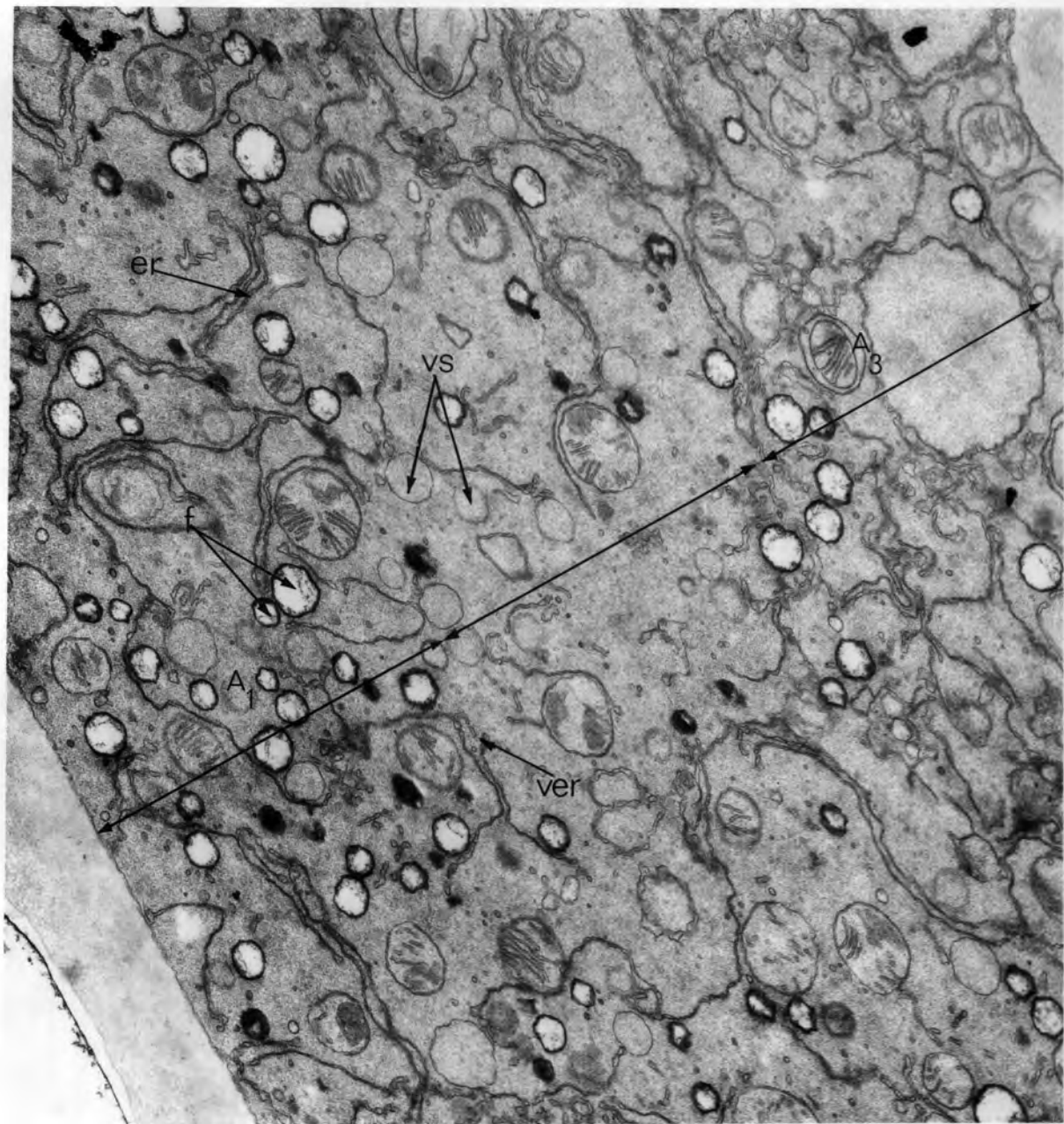the sculptured sporangium wall   X 6400

Fig. 40. The outer zone of the Stage II sporangium
X 15,900

of four types: microvesicles about 4-8 nm in diameter, spherical vesicles,pleomorphic vesicles and multivesicular bodies.

The micro vesicles although common throughout this outer zone were particularly abundant just within the plasmalemma particularly in the older Stage II sporangia (Fig.41.) In dimensions they were close to those vesicles found within the lomasome like bodies in various parts of the sporangiophores and developing sporangia. It is not improbable that these vesicles could be derived from the vesicular membranes, although one cannot totally exclude the possibility that they may represent either transverse or oblique sections of minute tubules, even though no clear evidence has been found for the existence of tubules of the appropriate dimensions within the developing sporangium. The spherical vesicles were the most conspicuous. They ranged in size from about 30 nm to 90 nm in diameter, the largest being about half the size of the largest mitochondria. These vesicles were bounded by a simple membrane and contained fine granular material very similar to the ground plasm.

The pleomorphic vesicles and multivesicular bodies were rarely found in the outer A_1 region but were numerous in the inner A_3 region. The pleomorphic vesicles appeared to be formed from swellings in isolated strands of the endoplasmic reticulum. They contained a small number of electron dense granules about 13 nm in diameter which sometimes appeared clumped together. The multivesicular bodies were less frequent than any of the other three kinds of vesicles. In form and dimension they were usually similar to the mitochondria.
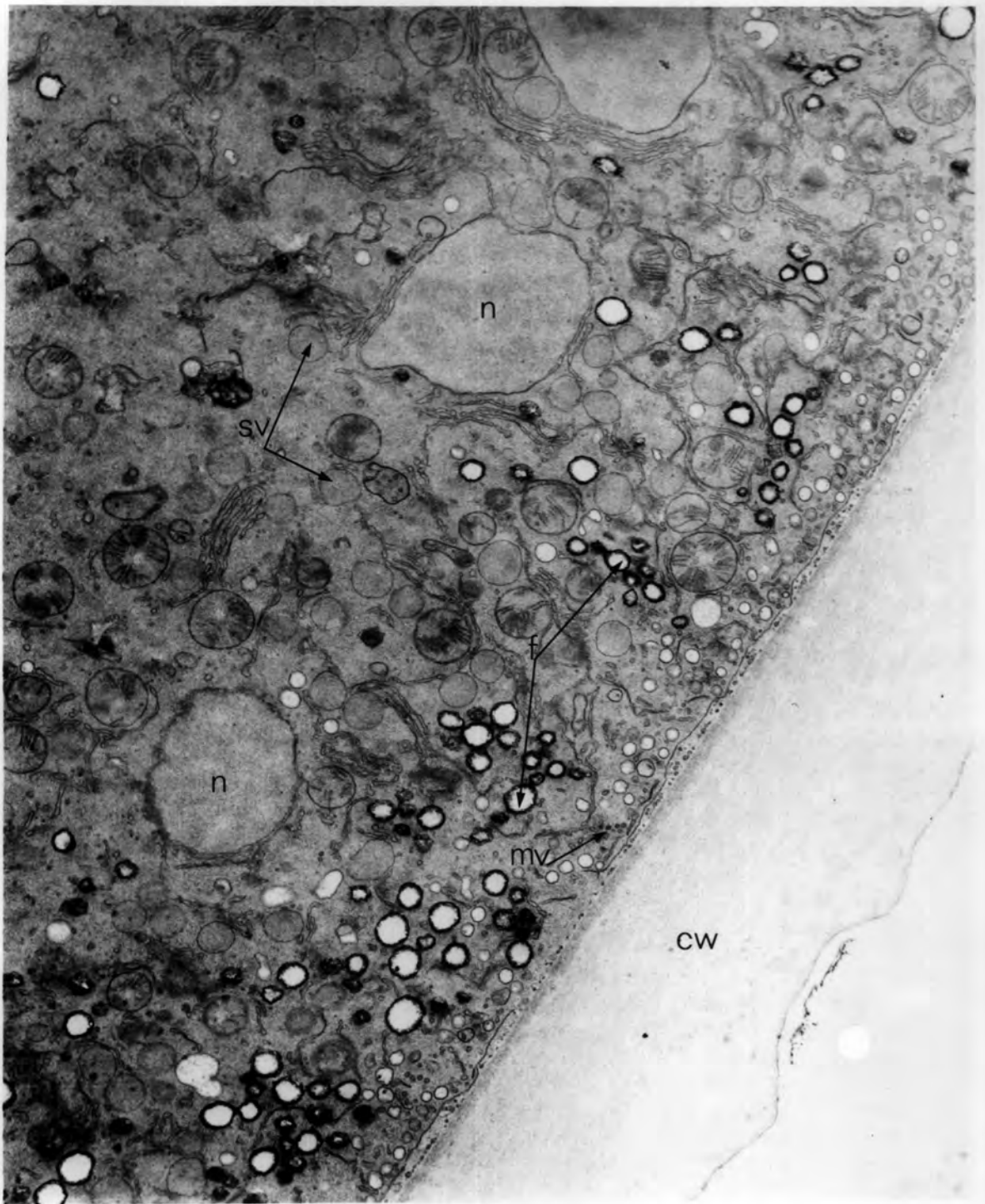
Fig. 41. Late Stage II sporangium showing numerous microvesicles just within the plasmalemma X 18,300

Each contained many small hollow vesicles. Some of them appeared, like the pleomorphic vesicles, to be formed by the distension of the isolated strands of endoplasmic reticulum. Cisternae (Fig.42.)(Bracker 1967) of varying configurations and size were also found in this zone although they were not of frequent occurrence.

Inner Zone.

Situated within this outer zone is a crescent shaped zone approximately 8 μm-11 μm thick. It was formed by a close aggregation of nuclei (Fig.42.). The nuclei were about 2-7 μm in length and most possessed an electron dense body, lying asymmetrically, which was probably the nucleolus. Other irregular dense areas probably indicated the position of chromocentres. Each nucleus was bounded by a well defined nuclear envelope with many simple nuclear pores. Almost every nucleus was surrounded by at least one incomplete sheet of endoplasmic reticulum which often had interconnections with the nuclear envelopes. Often several nuclei were connected by means of these sheets of endoplasmic reticulum. The small areas remaining between the nuclei were occupied by either mitochondria or spherical vesicles or more frequently by a pleomorphic vesicle and occasionally a multivesicular body or cisterna (Fig.43.)

Central Zone.

The characteristic feature of the central zone was the membrane system. For the most part this appeared in the sections as stacks of membranes with vesicular swellings at their edges, and adjacent, presumably budded off, free vesicles. These stacks were not unlike
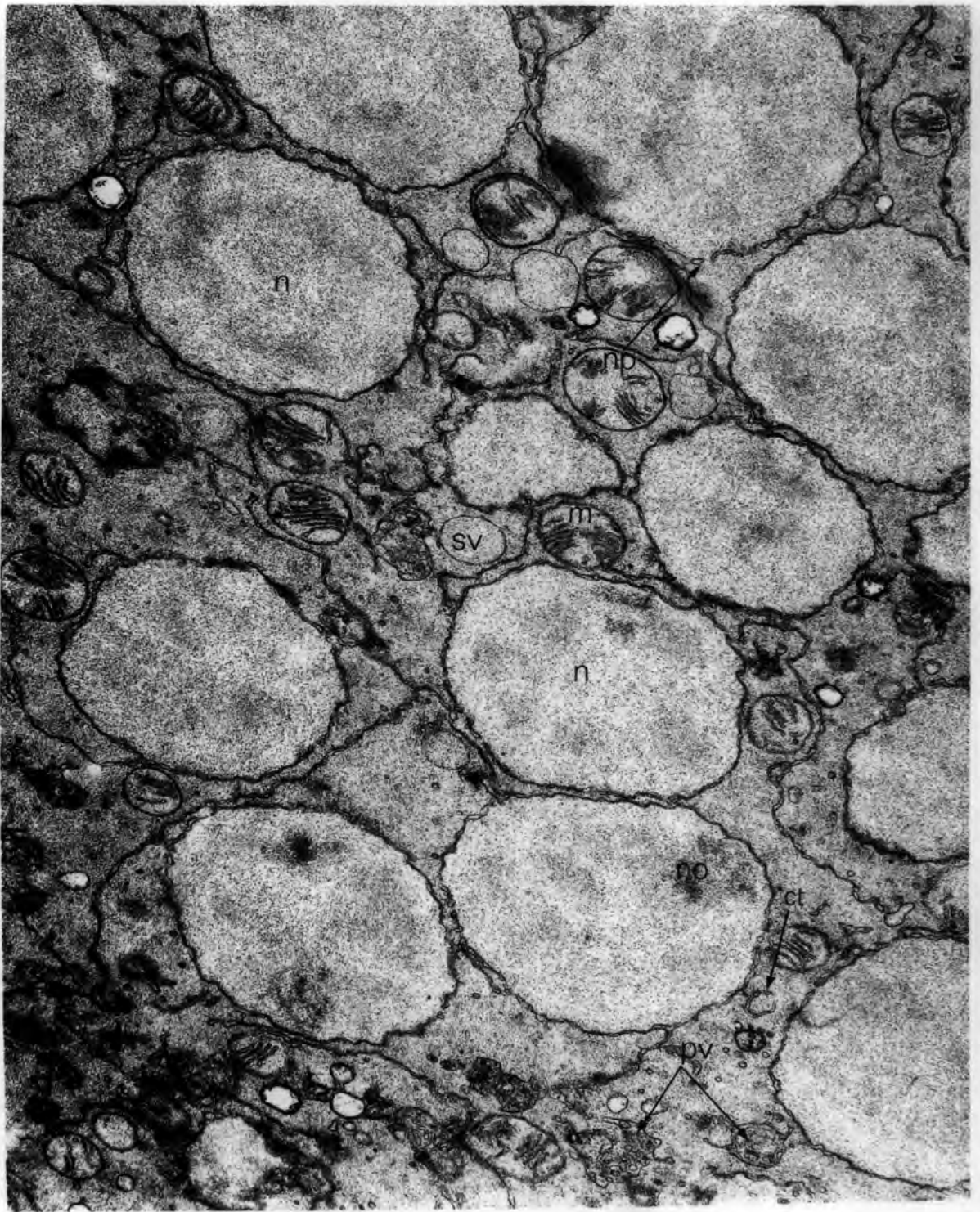
Fig. 42. Part of inner zone of the Stage II sporangium
formed of closely aggregated nuclei.   X 18,300
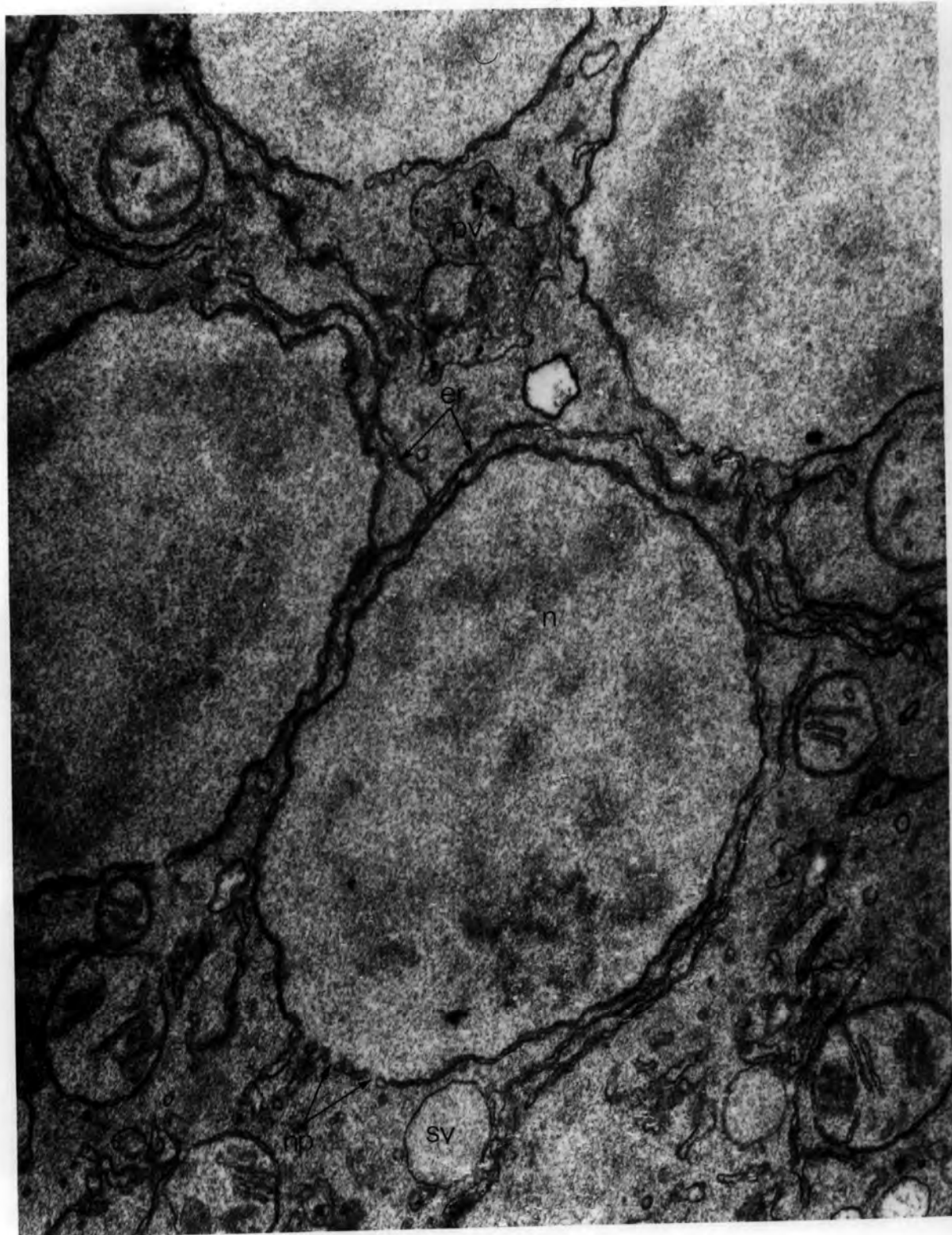
Fig. 43. Nuclei and associated endoplasmic reticulum
of the Stage II sporangium.   X 36,600

the membrane stacks of the outer zone, although each
group did contain many more membranes. Between the
stacks lay isolated strands of endoplasmic reticulum,
mitochondria and electron dense granules which were
about 8 nm in diameter. Numerous large pleomorphic
vesicles and microvesicles also occurred in this
region. (Fig.44).

Situated at the boundary of the nuclear and
central zones and laying about 3-5 μm apart were several
groups of closely packed irregular shaped vesicles with
thick electron dense bounding membranes. I have never
found similar aggregations of vesicles anywhere else
in Phycomyces. There was little evidence of glycogen
granules in any of the three zones. Indeed, I have found
little evidence of the presence of glycogen in the Stage I
apex, although its presence has been reported by others
(Thornton 1968); glycogen is however present in the
older parts of the Stage I sporangiophore between the
nucleated region and the vacuole.

VIIId.(iii) Stage III sporangium.

This is the period during sporangium development
when there is neither an increase in volume of the
sporangium nor any elongation of the sporangiophore.
It is a period of spore development within the fully
extended sporangium, its termination is indicated by
resumed activity in the growth zone of the sporangiophore
and its subsequent elongation. During this phase the
sporangium contents undergo division to produce the spore
initials. At the completion of this phase, the
sporangium contains numerous multinucleate spores.
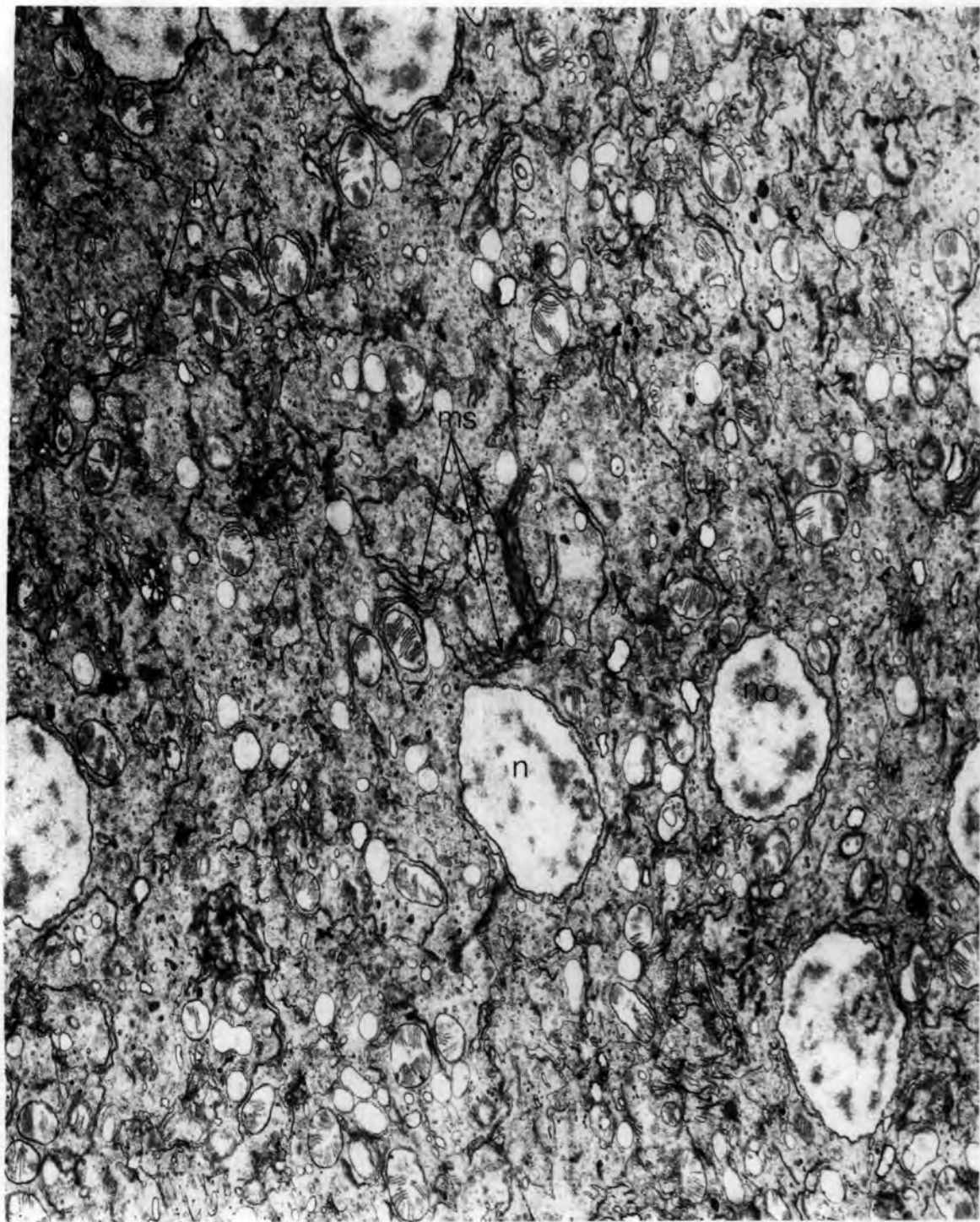
Fig. 44. Central zone of the Stage II sporangium.
Note the extensive development of the membrane
system.   X 12,200

At the onset of cleavage, numerous small vesicles
containing course granules become apparent (Fig.45);
the origin of these vesicles is uncertain but since the
pleomorphic vesicles and multivesicular bodies
disappear at about this time, it seems not unlikely
that the granular vesicles originate from one or other,
or both, of these organelles.

On the micrographs closely associated with these
cleavage vesicles an extensive development of folded
endoplasmic reticulum can be seen. From this, straight
membranes extend out into the surrounding ground plasm
and sometimes serve to join together two or more cleavage
vesicles. Some of the vesicles are, at the same time,
joined to one or more nuclei by membranes which appear
as extensive evaginations of the outer nuclear
membrane (Fig.46).

By fusion of these initial cleavage vesicles a
conspicuous system of large irregular shaped cleavage
vesicles is produced. As they develop these enlarged
vesicles continue to possess a coarse granular
matrix containing many rod like bodies. They do however
eventually lose their irregular form and the associated
endoplasmic reticulum disappears, presumably by its
incorporation into the bounding membranes of the
vesicles.

During the phase of cleavage vesicle development,
when the latter are extremely irregular in form, there
appears to be present within the cytoplasm a second type
of vesicle. These often appear to have a quite smooth
bounding membrane, to be more or less eliptical in
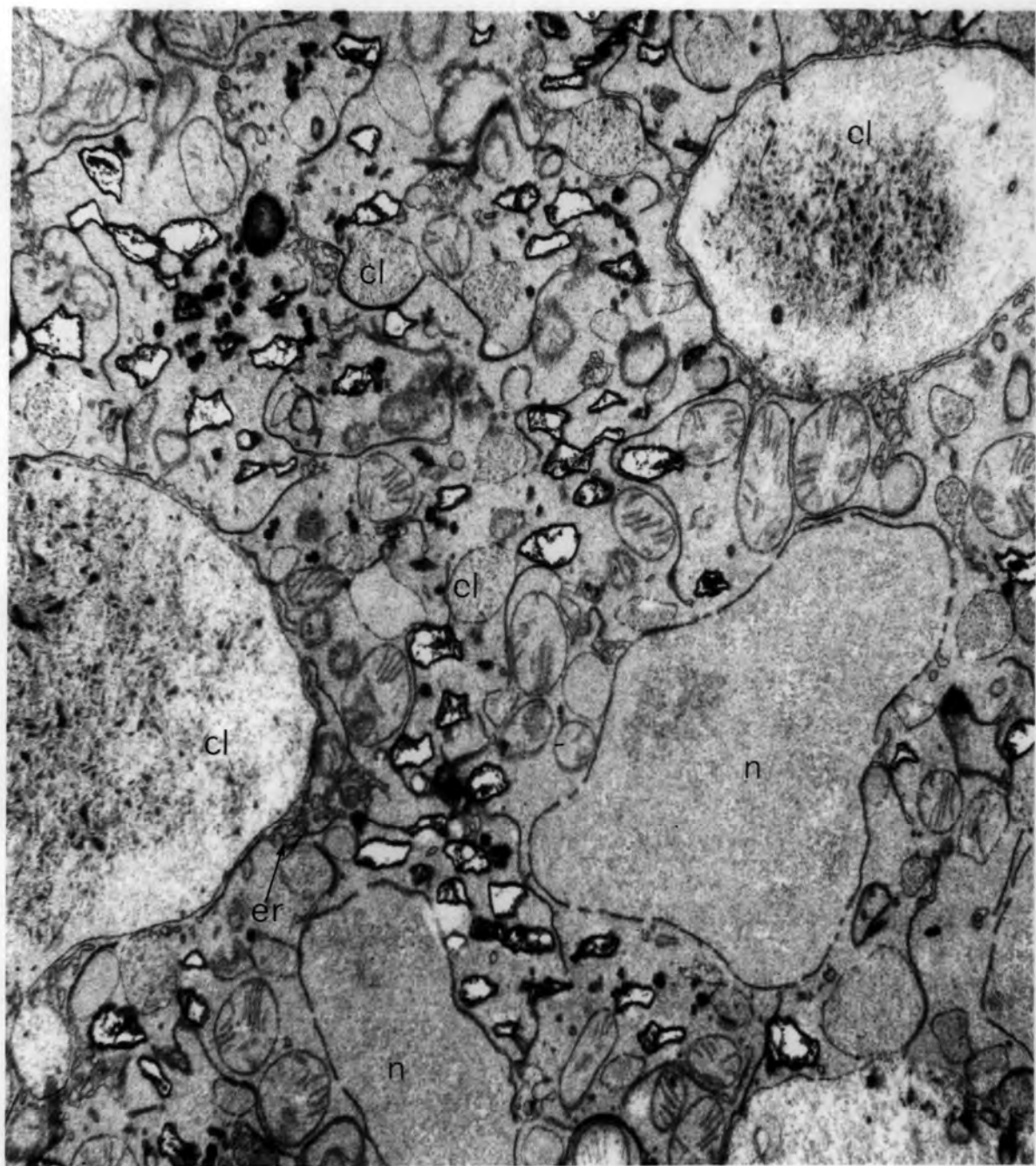section, and to contain eliptical bodies enclosing

Fig. 45. The Stage III sporangium showing many
small cleavage vesicles and two large cleavage
vesicles. Note the presence of folded endoplasmic
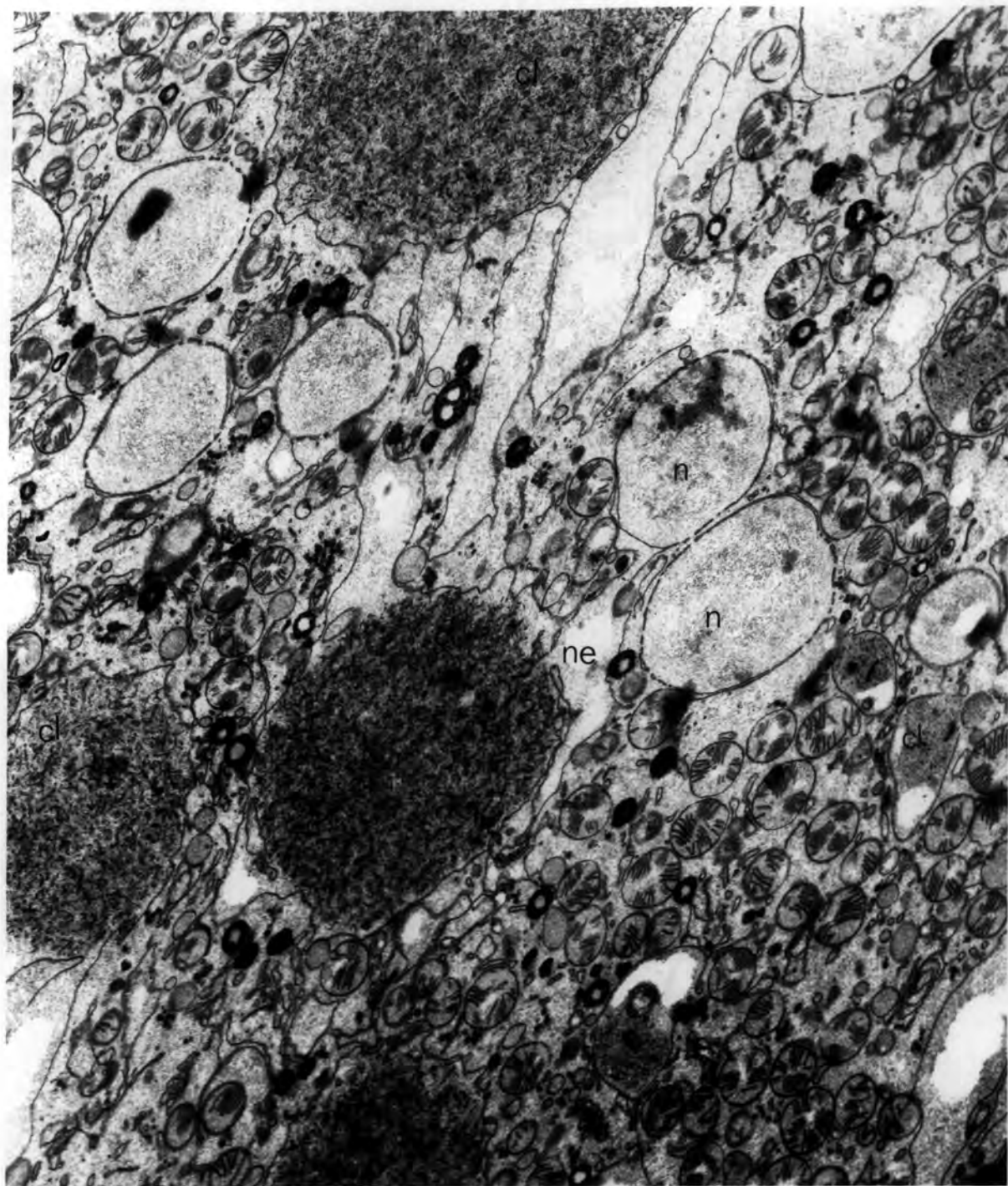reticulum round the cleavage vesicles. X 16,900

Fig. 46. Part of a Stage III sporangium showing the developing cleavage vesicles. The membrane of one vesicle is continuous with a nuclear membrane. X 11,875

various membrane configurations (Fig.47). These bodies were at first difficult to interpret, but examination of several sporangia in the initial stage and later stages of cleavage revealed no organelle from which they could have been derived, nor to which they could give rise. Re-examination of the micrographs containing both the irregular cleavage vesicles and the eliptical bodies leadsme to suggest that the latter are not discrete organelles of the developing sporangial mass, but sections across the cleavage vesicles incorporating areas of groundpham almost completely surrounded by two or more of the elongated finger like extensions of the cleavage vesicles (Fig.48).

During the development of the cleavage vesicles, fat droplets are the most conspicuous storage material, with small isolated areas of glycogen granules.

The interassociation of the cleavage vesicles, endoplasmic reticulum and nuclei is in some ways similar to the situation that exists in Gilbertella persicaria (Bracker 1966) and, as in the latter fungus, probably determines the directional growth of the cleavage vesicles and hence the boundaries of the initial spores. This predetermination of the cleavage planes will ensure that the resulting spore initials will each contain at least one nucleus.

As the cleavage vesicles enlarge (Fig.49) they eventually coalesce with each other and reach the plasma - membranes of the sporangium, with which the vesicle membrane coalesces (Fig.50), thus forming a membrane - bound network throughout the sporangium. The innermost cytoplasm of the sporangium during cleavage is isolated from the outer spore-forming cytoplasm by a crescent shaped series of vesicles (Fig. 51).
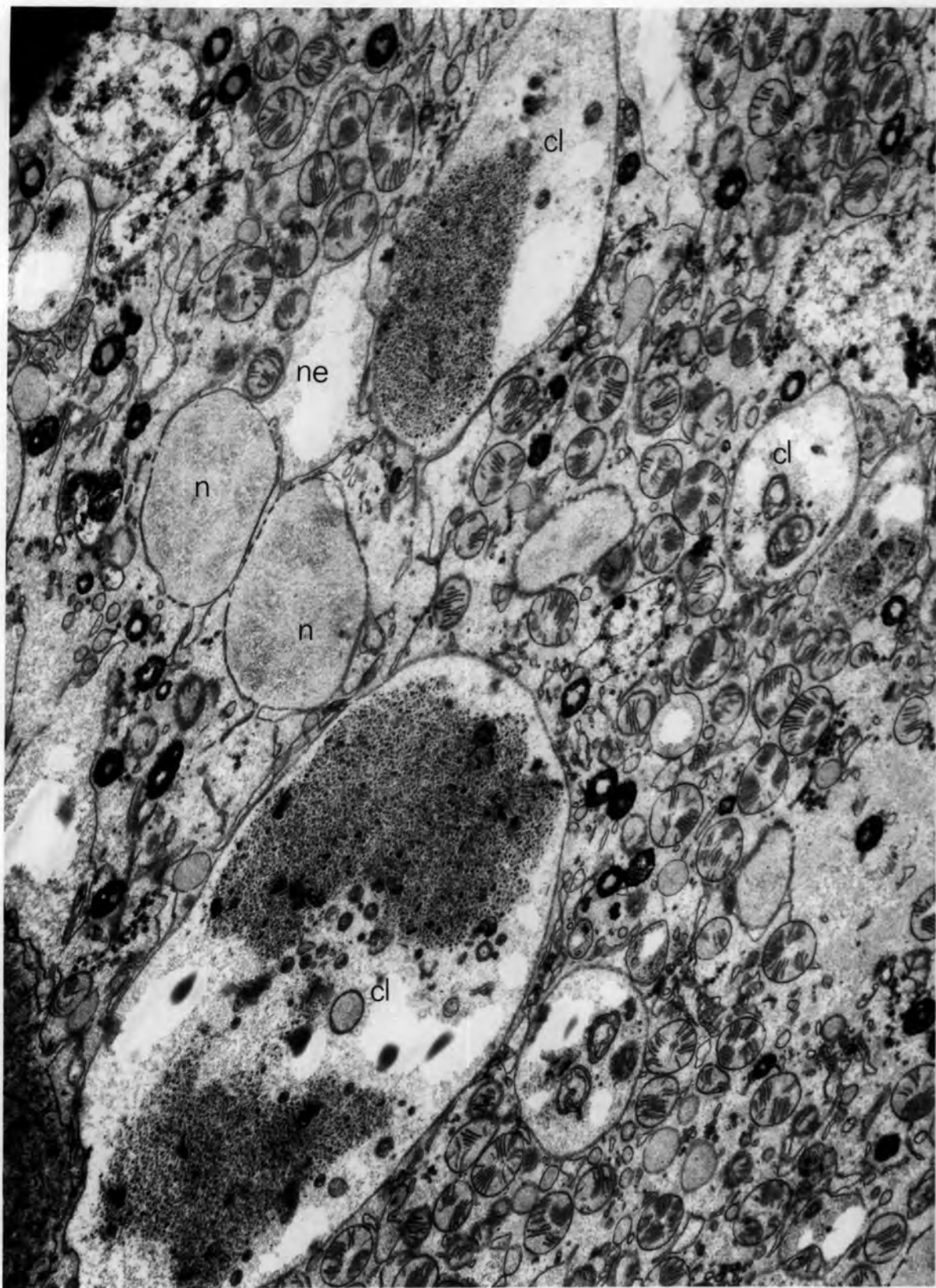
Fig. 47. Part of Stage III sporangium showing what appeared to be a second type of cleavage vesicles. X 12,800
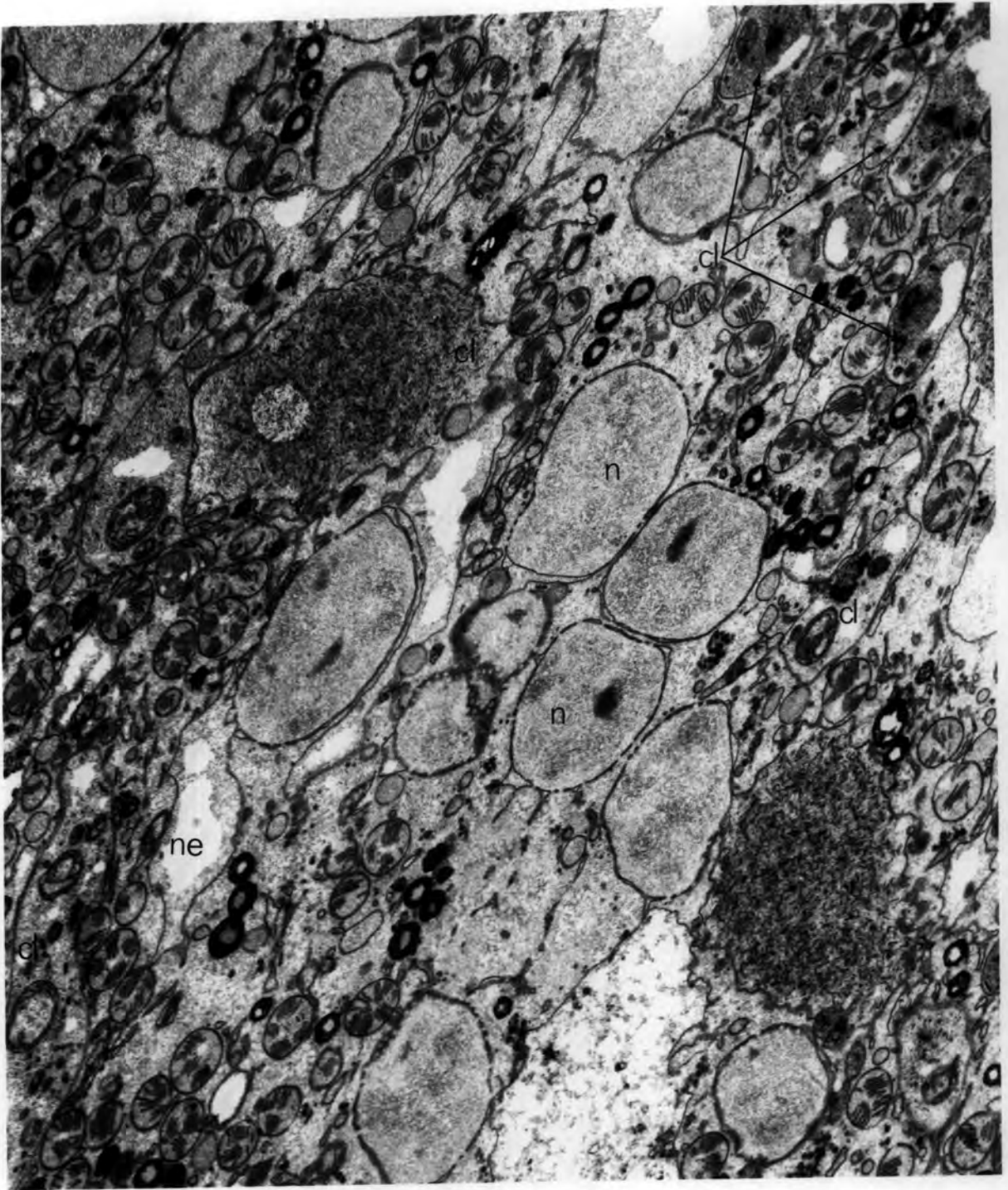
Fig.48. Part of Stage III sporangium showing two types of
vesicles.   X 11,875
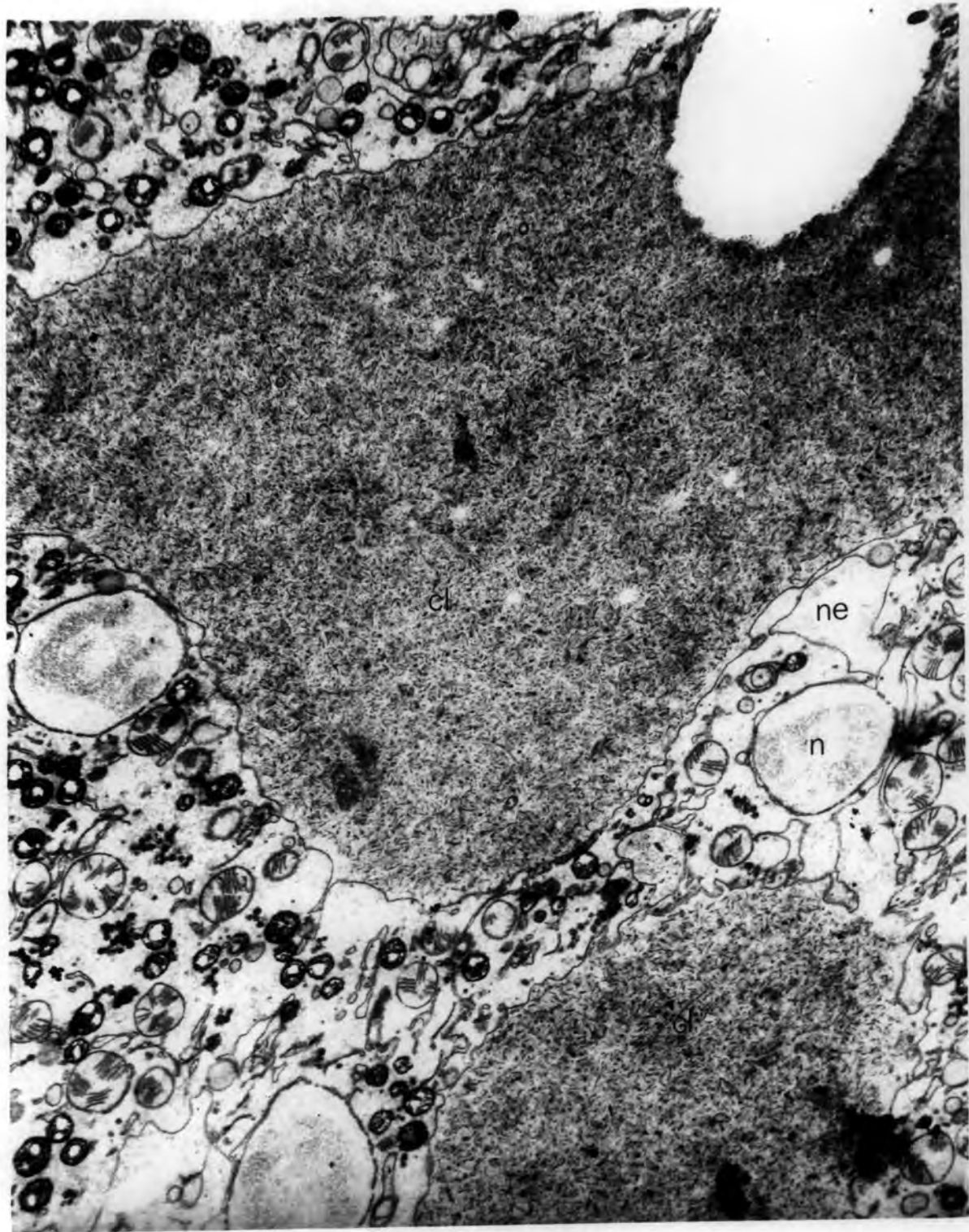
Fig.49. Two large cleavage vesicles. Note the evagination from the nucleus lying between the vesicles. X.11,875

Fig. 50. Drawing from an electron micrograph showing
the fusion of two cleavage vesicles with the plasma-
membrane of the sporangium.   X 12,500

Fig.51. Drawing from an electron micrograph showing the
fusion of the cleavage vesicles which isolate the columella
from the sporangium contents. Note the accumulation of
Glycogen in the columella.   X 12,500

These on coalescence form a dome shaped septum which fuses with the sporangium plasma membrane at the base of the sporangial swelling. The columella is thus formed.

VIIId. (iv) Possible Mechanism of Cleavage Vesicle Enlargement.

The evidence from the electron micrographs would support Swingle's view that the cleavage vesicles are present at the onset of sporangium development, His view that the contraction of the sporeplasm is responsible for the enlargement of the cleavage vesicles must of necessity be at least partially correct since cleavage, brought about by the enlargement of the vesicles, is not accompanied by an increase in the total volume of the sporangium.

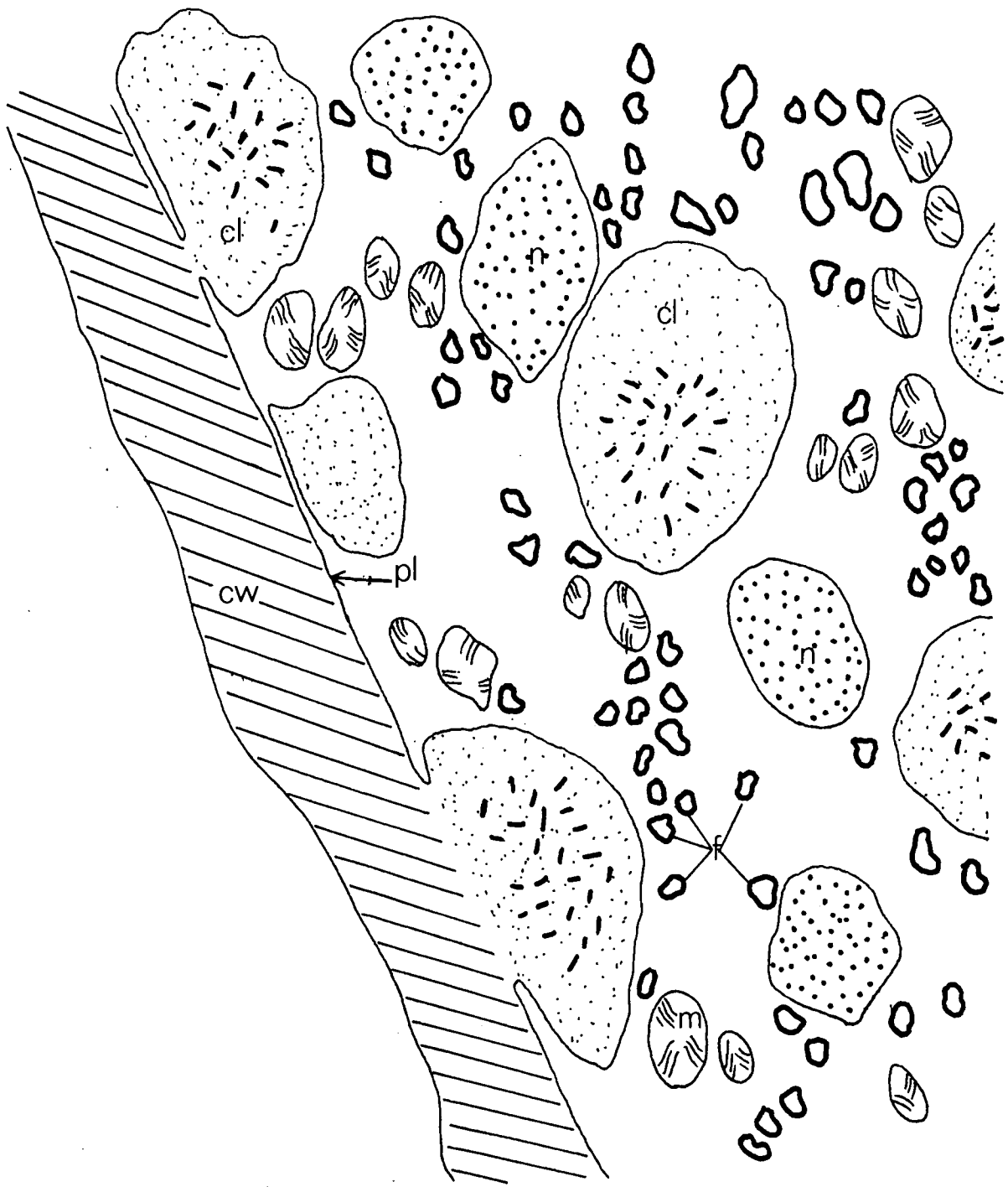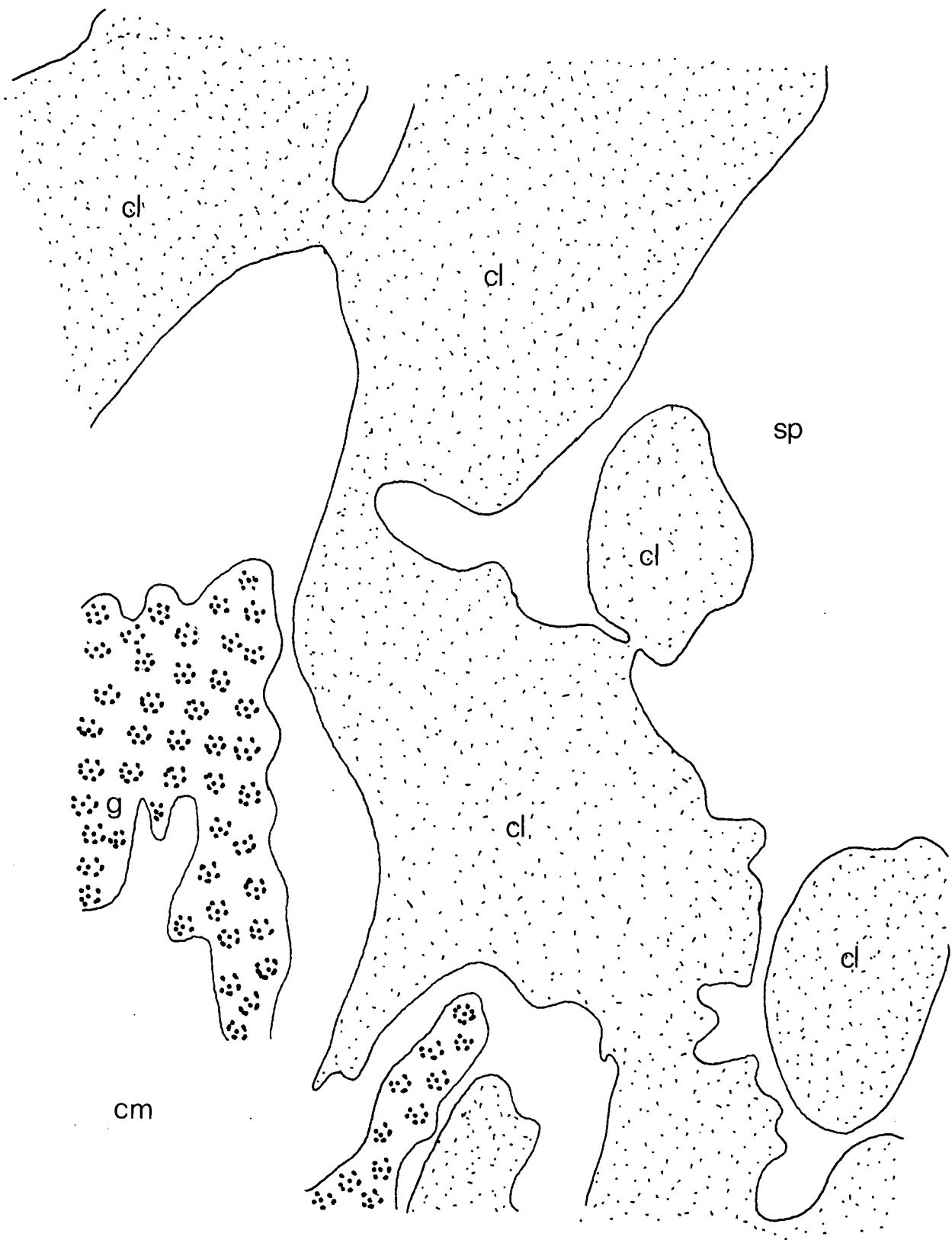Examination of the micrographs of the sporangium during cleavage shows clearly a pronounced compacting of such organelles as nuclei and mitochondria (Fig.52) in the areas destined to be spores as the cleavage vesicles develop, There is not however any marked increase in the electron density of the ground plasm which might perhaps be expected if the sporeplasm was contracting.

The precise mechanism by which the sporeplasm contracts and the cleavage vesicles enlarge is difficult to envisage. The one which most readily presents itself is the osmotic withdrawal of water from the sporeplasm into the cleavage vesicle. For this to take place however, there must develop at the onset of cleavage a high osmotic potential within the cleavage vesicle with respect to the ground plasm. The osmotic difference between the cleavage vesicle content and the ground plasm must then be maintained until at least the time of completion of the process.
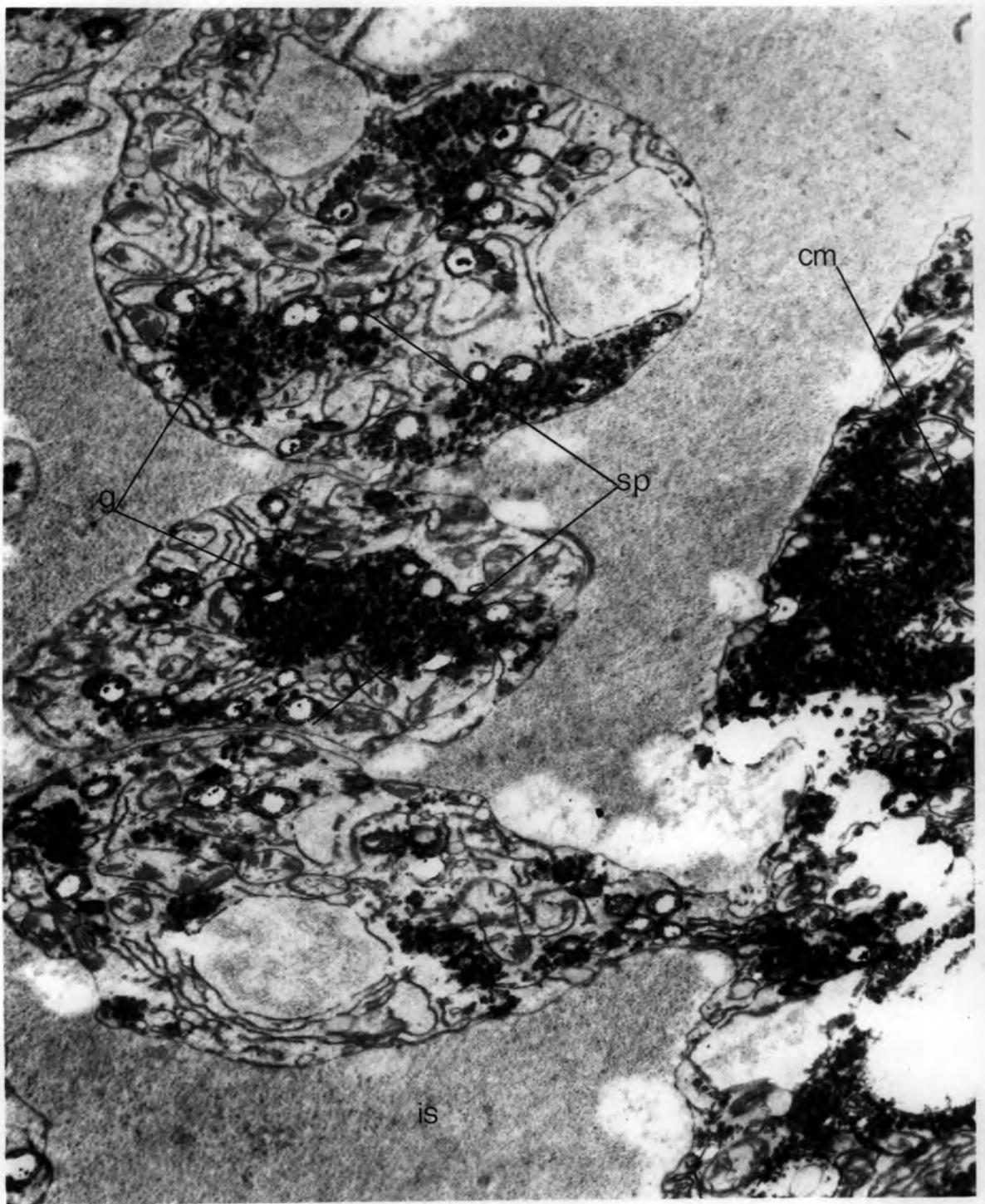
Fig. 52. Part of columella and three newly formed spores from a late Stage III sporangium. Note the close aggregation of organelles and the appearance of glycogen.   X 11,250

The development of such an osmotic difference between
the two would imply a rather specialized activity on
the part of the cleavage vesicle membrane.

VIIId.   (v) Sporangium Membranes.

The fusion between the sporangium plasma membrane
and the cleavage membranes suggest that the two may
possess a structural similarity which differs from the
membrane structure of other organelles within the
sporangium.   Nevertheless, as suggested by Bracker (1967),
this structural similarity cannot be the only factor
governing the fusion of the membranes; if it were so,
then one might expect to observe frequent and gross
fusion between the organelles.   It may be significant
that fusion between the plasma membrane and the cleavage
membranes has only been observed at late cleavage, suggesting
the possible role of a time factor in triggering off
a transient change in the membranes concerned, resulting
in a temporary period of compatibility between them.

The whole sequence of events during cleavage strongly
suggests the existence of not one critical stage as
postulated by Bracker (1967) but a series of such
stages resulting in several transient changes which
culminate in cleavage.

VIIId.   (vi) The Newly Formed Spore.

When first formed the cytoplasm immediately within
the columella membrane and the spores lying nearest to
the columella are rich in glycogen.   This appears as
large dense aggregations of granules about 0.12 μm in
diameter (Fig.52)

Each multi-nucleate spore contains several fat
droplets, mitochondria and fragments of endoplasmic
reticulum, and is bounded by an apparently incomplete
membrane.

The interspore material remains coursely granular,
most of the granular material appearing as electron
dense rod like bodies about 0.08 μm in length (Fig.53)

VIId.   (vii) Spore Maturation.

At the onset of maturation, the spore. membrane
loses its irregular form and become much smoother so that
the spores appear spherical or eliptical in section.
This change in shape could be attributed to an increase
in turgor as the metabolically active spores absorb
moisture from the interspore material, or to the
interaction of an increased turgor and the laying down
of an electron dense wall layer about 25 nm thick. In
spores that are still irregular in form, pockets of
small vesicles each about 45 nm in diameter have been observed
in what might be considered as invaginations in the spore
membrane (Fig.54). When first recognisable these vesicles
appeared to arise in the interspore material that was
originally the contents of the cleavage vesicles. I should
like to suggest that these vesicles play some part
in the deposition of the primary wall material which
develops around the spores and is about 25 nm thick.

Spore Lomasomes.

Lomasome-like bodies were commonly observed in
spores that had developed the primary wall layer (Fig.55)
They appeared as groups of small vesicles lying between
the invaginated membrane and the primary wall layer.
Many spores contained up to seven or eight such bodies
in one section. These lomasome-like bodies when seen
in cross sections were either dome shaped (about 200 nm
from apex to base x 480 nm along the base) or tubular
(about 240 nm from apex to base x 140 nm along the base)
in appearance. Similar bodies were rarely found in older
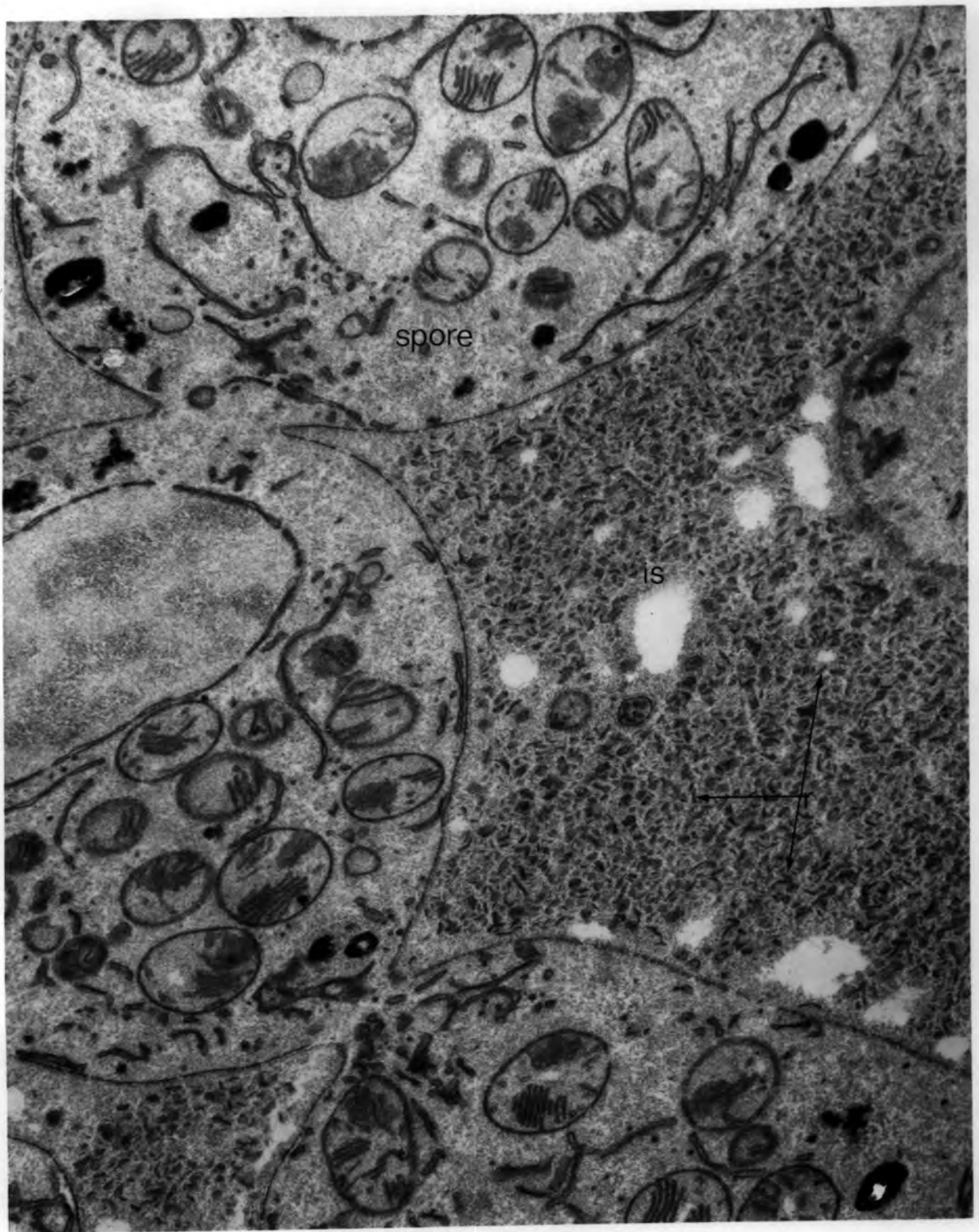
Fig. 53. Parts of four newly formed spores surrounded by
the interspore plasm, originally the contents of the cleavage
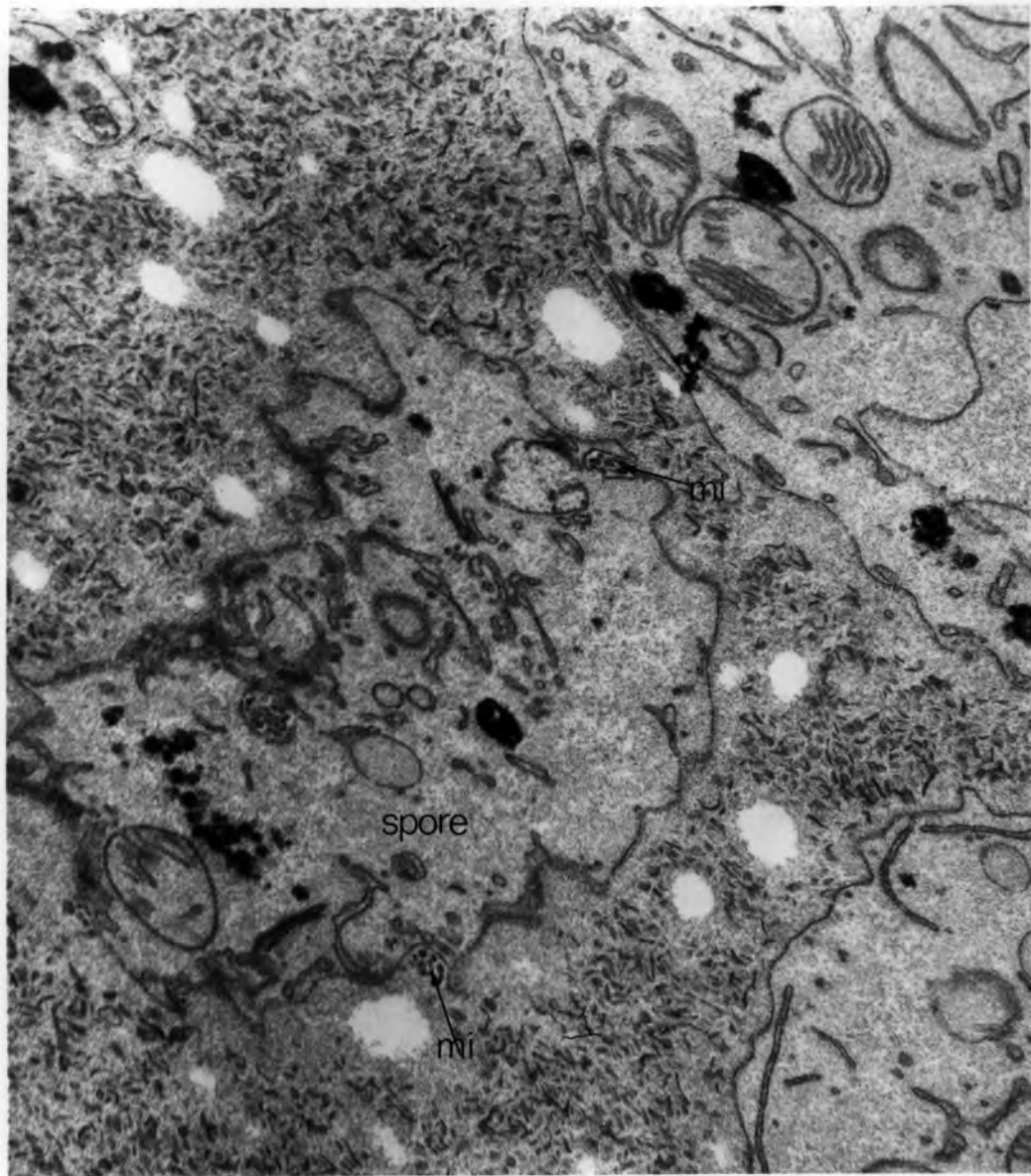vesicles, containing numerous rod shaped bodies.

X 18,750

Fig. 54. Newly formed spores showing invaginations of the spore membrane to form pockets containing vesicles. X 28,750
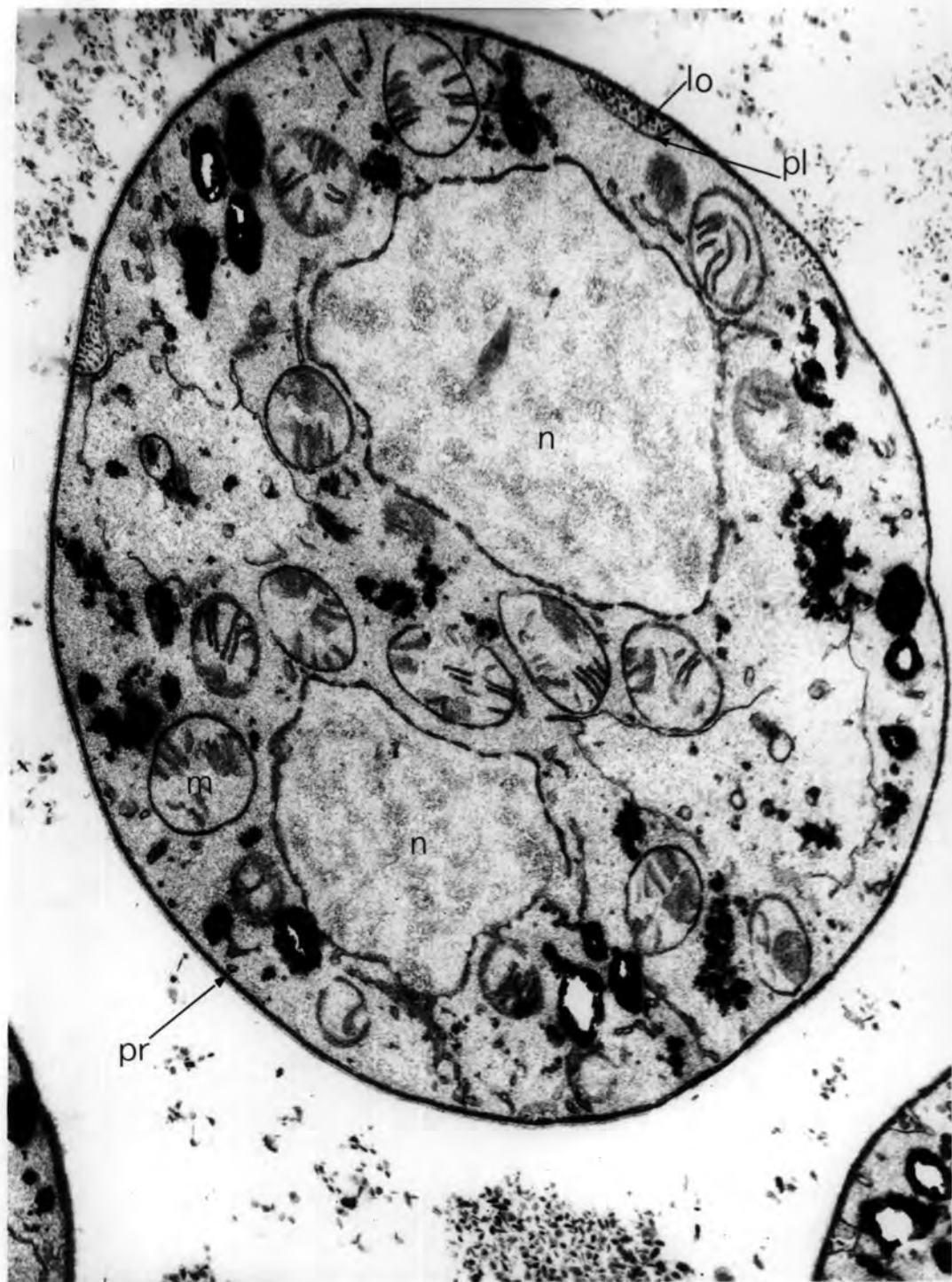
Fig. 55. Spore with electron dense primary wall layer
and lomasome-like body lying between the plasma-
membrane and the wall layer.   X 17,800

spores which had started to lay down secondary wall
material.

Reticulate tubular wall systems.

As the spores continued their maturation, the
lomasome-like bodies disappeared. Their place was then
taken by a series of wall tubules of various configurations
(Fig.56). Most appeared as extensive ingrowths of the
plasmalemma, forming reticulate systems of various forms
and dimensions. Some were globose while others were
flattened with their long axis extending parallel to the
spore wall. Several similar membrane-bound tubules,
some resembling the multivesicular bodies reported by
Marchant et al (1967), were found free within the cytoplasm
of the spores. From examination of serial sections,
it was found that these bodies were actually part of the
extensive reticulate wall systems and were in fact linked
at some point to the plasmalemma. These latter systems
were more numerous in spores which had produced
secondary walls, or at least contained some secondary wall
material.

Storage Material.

The spore initials are generally rich in glycogen
as are the spores at the primary wall stage of development.
With the appearance of the reticulate tubular wall
systems and the development of secondary wall material,
the glycogen store within the spore diminishes and often
disappears altogether. At the same time, large irregular-
shaped vacuoles appear within the cytoplasm (Fig.56).
In many instances the tubular systems are associated with
those vacuoles at one or more points.

The large fat droplets are the only storage materials
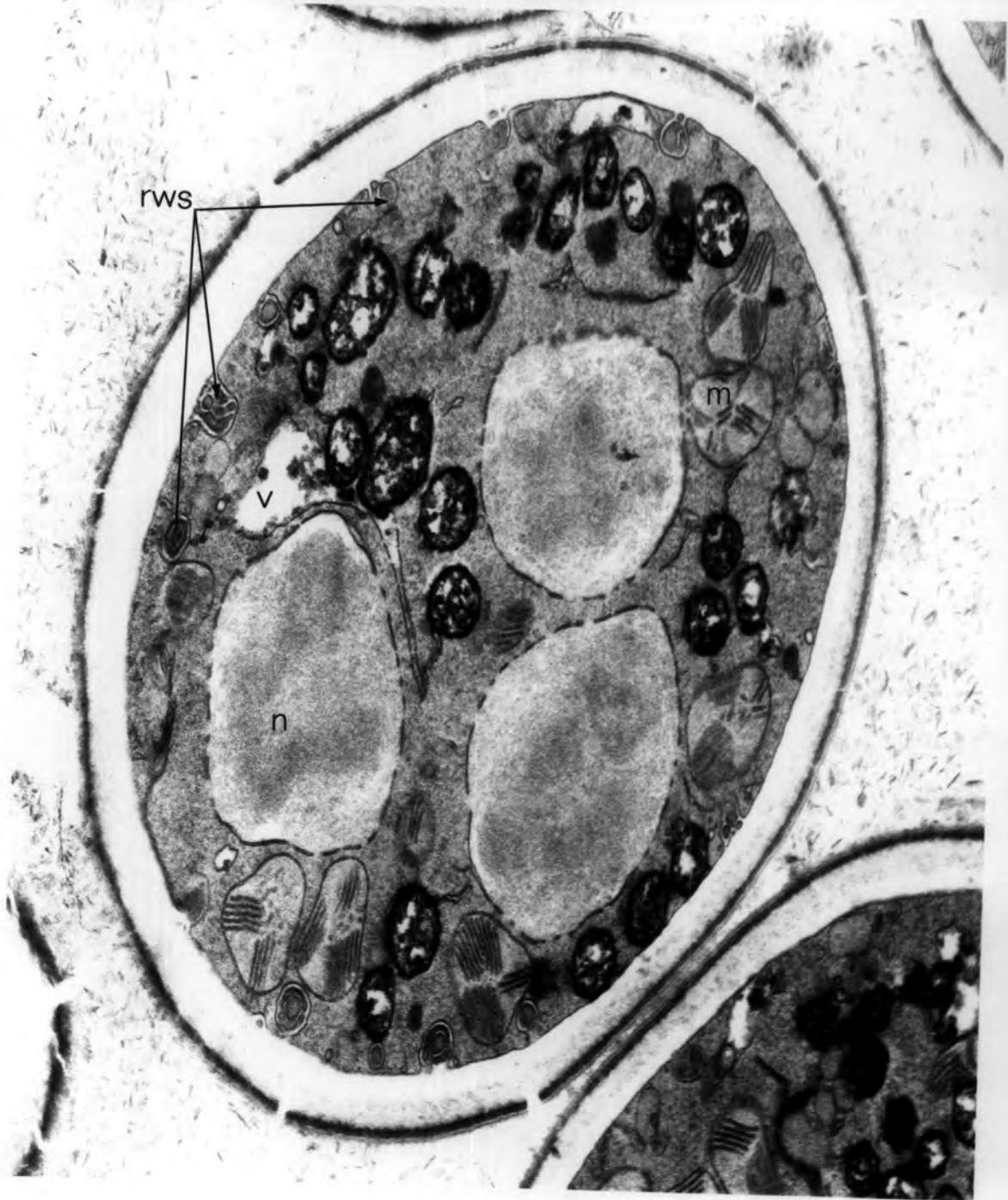evident within the spores when they are fully matured.

Fig. 56. A spore at the late stage of maturation showing the reticulate wall systems. Note the absence of glycogen and the presence of a large irregular shaped vacuole.

X 23,120

The reticulate wall systems are similar to the bodies reported by Chet et al (1969) in hyphae of Sclerotium rolfsii and considered by them to be the vehicle by which wall material is elaborated. The disappearance of glycogen and some fat at the same time as the appearance of these systems and the initial development of the secondary wall layer suggests a relationship between the three phenomena. The relationship might well be the utilization of glycogen and some fat in the synthesis of the material of the secondary wall. The reticulate wall systems might represent a pumping or transport mechanism by which materials are transferred from the storage regions to the regions of wall synthesis. Such a system would of necessity depend upon the presence of a suitable enzyme systems. On the other hand, it might conceivably be that these systems are the actual sites of enzyme synthesis of wall components to which material is transferred by some other mechanism.

Marchant et al (1967) postulate the formation of primary and secondary wall layers in fungal hyphae by two distinct mechanisms. It may be that the involvement of two structurally distinguishable systems in the synthesis of a two layered wall is a characteristic feature of fungal wall synthesis.

The Mature Spore.

When fully mature the spores possess a two layered wall. The outer wall which was the first to be deposited is extremely electron dense and about 25 nm ∴ thick. The inner wall is not so electron dense, and is slightly thicker than the outer wall being about 14 μm thick. Each mature spore contains several nuclei, numerous mitochondria, fragments of endoplasmic reticulum, usually some fat droplets and one or more vacuoles. In all

the mature spores examined the contents did not fully

occupy the total volume within the spore wall, but
this can probably be attributed to a degree of shrinkage
during the preparation of the material.

VIIId   (viii) Sporangium Development.

Within the developing sporangium, cleavage of
the sporangium contents always appeared to be total
so that the spores were formed simultaneously.
Nevertheless it was frequently observed that the inner
spores, those closest to the columella appeared to
mature far earlier than the outer spores, the former
frequently having developed the secondary wall whilst
the latter still only possessed the primary wall.(Fig.57).

The Columella.

Simultaneous with spore wall development, wall
material is deposited on the outside of the columella
membrane thus forming a rigid barrier between the
columella and the sporangium cavity. On completion the
columella wall extends for some distance, about 0.05 mm
down the sporangiophore wall, gradually decreasing in
thickness until it finally coalesces with the sporangiophore
wall.

On completion of development the area of the
columella membrane is extensively increased by the
development of irregular ingrowths of wall material which
frequently branch and anastomose to form a complex
network of wall material. The plasma membrane closely
follows the contours of the wall material and cytoplasm
is frequently found occupying the spaces between the
framework. (Fig. 58).

Ingrowths of wall material have been reported by
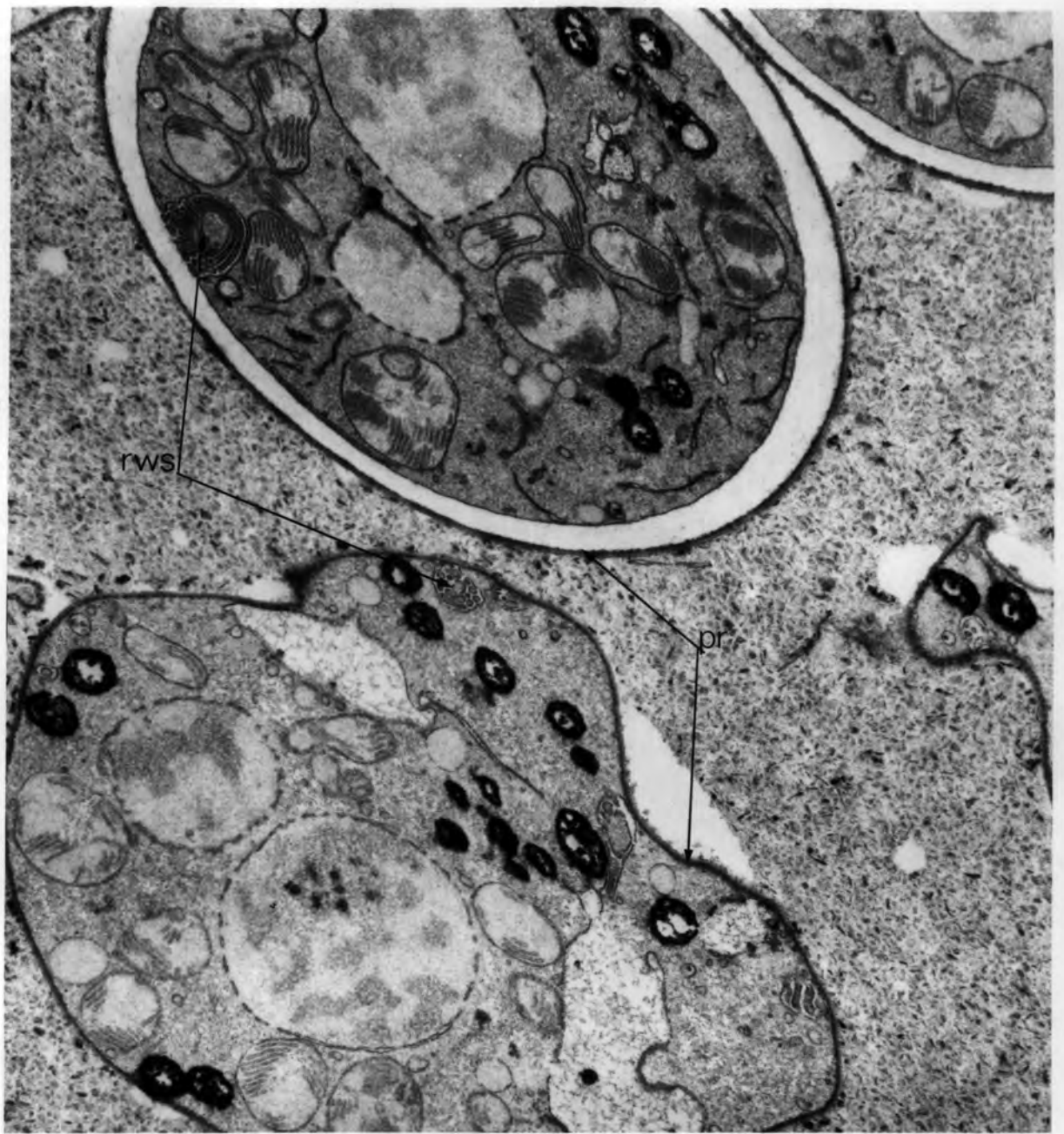numerous authors as occurring in a wide variety of plant

Fig. 57. Two spores at different stages of maturation.
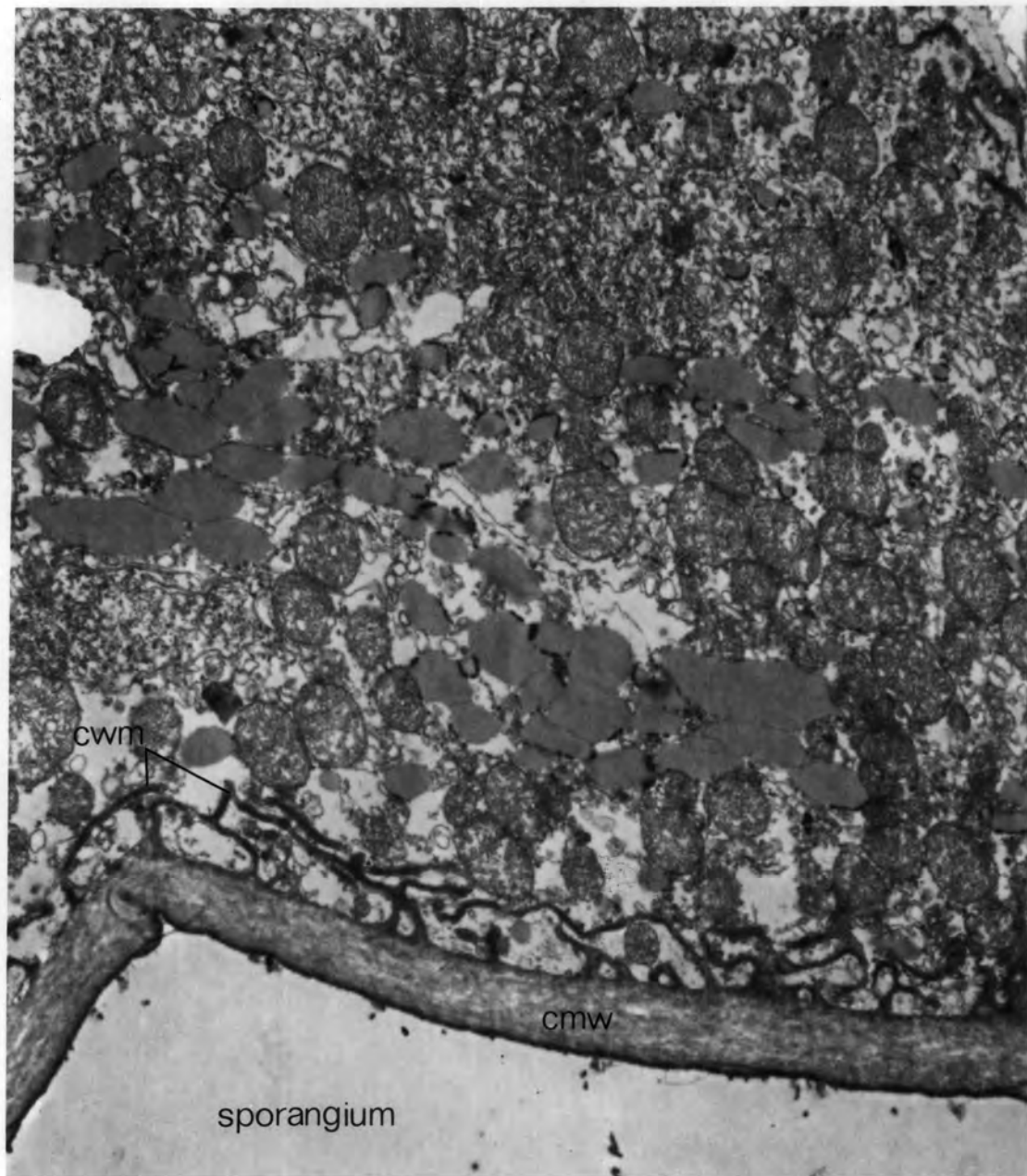Both contain at least one reticulate wall system. X 18,570

Fig. 58. Part of columella from within a Stage IV
sporangium. There is an extensive development of wall
material on the sporangiophore side of the columella
septum.   X 14,770

material including organs of several members of the
Pteridophyta and Bryophyta and a few lichens. These
findings have been reviewed by Gunning and Pate (1969)
but in their paper entitled "Transfer Cells", they
make no reference to the occurrence of such structures
in fungi.

Transfer Cells (Gunning and Pate 1969) which are
characterized by extensive wall ingrowths, are apparently
restricted to areas where adverse surface area/volume
relationships exist between adjacent cells forming part
of a transport pathway, or to areas where the transported
solutes are accompanied by a minimal flow of solvent.
If the columella zone of the sporangiophore of Phycomyces
is comparable in any way to transfer cells, then one has
to postulate the transport of solutes across the
columella membrane. Since these wall ingrowths only
develop at a very late stage in sporangium development
that is, when the spores are more or less fully developed,
it is difficult to envisage any reason for the mass
movement of solutes from the columella into the sporangium,
since at this stage it is most unlikely that they will
be incorporated into the sporeplasm. However, it is
generally accepted that spore discharge in many fungi
is dependent on either atmospheric or substrate water,
and that osmotic forces are involved. This most likely,
also applies to Phycomyces even though the final
rupturing of the wall only results when contact is made.
The development of the essential increase in turgor
can only result from the build up of highly osmotic materials
in the sporangium relative to the columella. It may
therefore be that this extensive plasmalemma develops as

a means by which solutes can, by active membrane transport, be rapidly moved from the columella into the sporangial mass, where they will serve to maintain a concentration gradient leading to the build up of a turgor pressure sufficient to facilitate the eventual rupture of the sporangium wall.

In immature asci and in the immature active layer of Sphaerobolous spp. (Cochrane 1958) glycogen is abudant. Its disappearance at maturity has led to the suggestion that the uptake of water is mediated by enzymatic conversion of the osmotically inactive polysaccharides to osmotically active sugars, and that such a mechanism might be extended to cover all discharge mechanisms which depend on increasing turgor of cells.

Glycogen an abudant reserve in immature sporangia of Phycomyces as in asci and Sphaerobolus Spp. disappears at maturity. There is however, adequate evidence from the electron micrographs to support the view that the disappearance of glycogen from the spores of Phycomyces is the result of its involvement either directly or indirectly in the synthesis of spore wall material, and does not merely imply hydrolysis to produce a high concentration of osmotically active sugar that would facilitate the uptake of water.

In only one respect is this hypothesis at variance with the findings of Gunning and Pate on transfer cells in multicellular organisms. Transfer cells are restricted to situations where the flow of solvent is minimal to the flow of solutes. In Phycomyces I suggest that the activities of the large membrane area might, in fact, be responsible for initiating and maintaining a flow of solvent sufficient to bring about sporangium

dehiscence. Despite this fundamental difference I see no reason at this stage to discard this possible function of columella wall ingrowths.

It remains however, somewhat of a conjecture until such times as it is possible to investigate experimentally both the quantitative and qualitative solute content of the mature sporangium over a period beginning with the appearance of the columella wall ingrowths and ending just prior to sporangium dehiscence.

Another possible reason for the increased area of the columella plasmalemma might be the transport back into the sporangiophore of solutes remaining in the interspore material after the completion of spore formation. Such a mechanism would serve to prevent excessive loss of valuable nutrient materials which might otherwise occur during sporangium

dehiscence. Although one cannot disregard the possibility of solute transport in this direction, it would seem highly improbable. As a result of cleavage the entire content of the sporangium becomes either incorporated within the spore or the columella. The remaining interspore material is the residue occurring within the cleavage vesicles themselves and it seems most improbable that these would possess valuable nutrients in quantities of economic importance to the mycelium.

Control of Wall Development.

The development of the wall ingrowths like that of the columella wall is in all events influenced by the unique morphological and physiological state that exists within the sporangium at the

termination of cleavage. Cross walls are not
of common occurrence within the mycelium of <u>Phycomyces</u>
and with a few exceptions are found only in this
unique situation. It seems therefore not unreasonable
to suppose that the stimulus initiating such an
unusual event as the formation of the columella wall,
might well also be responsible for the initiation of
the development of the wall ingrowth.

It has been stated that in certain species e.g.<u>Pisum</u>,
<u>Impatiens</u>, <u>Sedum</u> and <u>Ligularia</u> the frequency and intensity
of wall ingrowths in transfer cells is influenced by the
functions of the cell and its neighbours, and in others e.g.
<u>Vicia faba</u> that their production is often synchronized with
the onset of intensive transport (Gunning and Pate 1969)
This being so, the development of ingrowths of
the columella wall of <u>Phycomyces</u> might perhaps be
regarded as another example of a modification in the
evolutionary development of the wall membrane apparatus
capable of facilitating a rapid short distance transport
of materials to assist in the completion of maturation and
dehiscence of the sporangium. There is however no data
on which to base a theory of functional influence on the
development of columella wall ingrowths. Nevertheless their
presence might well have some significance.

<u>VIIIe.Germination of the Spores.</u>

The vegetative spores of <u>Phycomyces,</u> because of their
low metabolic rate, may remain dormant for long periods
of time when placed in an environment unsuitable for
growth. Air dried spores placed in loosely stoppered
tubes in a refrigerator have been kept viable for as long
as four years (Bergman et al 1969) and lyophilized spores have
been known to remain viable for as long as twenty three years

(Raper and Alexander 1945, Ellis and Robertson 1968 as
reported by Bergman et al 1969).

Under suitable conditions the spores will germinate,
although very few dormant spores will germinate immediately
when provided with the materials required for growth. The
most commonly used method of activating spores is heat shock.

VIIIe (i) Preparation of material for germination.

Spore suspensions were collected, incubated and
germinated in the manner described previously in Section
III.d. Eight to nine day old cultures of Phycomyces were
usually used as the source of the spores and the spores
usually produced from one to three germ tubes in about
5 hours after incubation.

Germination.

About one hour after incubation the spores begin to
swell. The swelling was most pronounced at the ends of the
long axis of the spore so that the latter gradually assumed
a dumbell shape, but as the swelling continued the spores
become more globose. About 4-5 hours after incubation
the spores were about twice their dimensions at the
dormant state

4.1 μ x 7 μm—dormant spore
8.2 μ x 11 μm at the onset of germ tube emergence

As the swelling developed usually two vacuoles became
apparent. These enlarged and coalesced at about the same
time that the germ tube emerged. At this stage a single
large vacuole occupied almost the whole of the spore volume.

VIIIe (ii) Electron Microscope Investigations.

Samples of spores were examined under the electron micros-
cope at:-

a) the end of 30 minutes after the initial incubation period
b) the end of 2 hours after the initial incubation period
c) on the emergence of the germ tube

Each sample was prepared for examination under the
electron microscope in the manner previously described
in Section III e.

Results.

The spores examined 30 mins after incubation were
in ultrastructural detail similar to the dormant spores.
At 2 hours after incubation, well formed vacuoles were apparent,
the storage fat droplets had often completely disappeared,
the cytoplasm was extremely dense and there was often a
pronounced development of strands of endoplasmic reticulum.
Just prior to the emergence of the germ tube the ultrastructural
changes that had occurred within the swollen spore were
for the most part changes in wall structure. The thin
electron dense outer wall layer was still very much
in evidence, but the inner wall layer appeared now in several
places as a much more loosely bound structure than it did
in the dormant spore; in some areas it had disappeared
completely. Surrounding the cytoplasm a third layer of wall
material, showing a fibrous nature in several places, was
developing (Fig.59). At one or more points the plasma
membrane was thrown into a series of irregular
invaginations into the cytoplasm. In these areas the space
thus formed between the plasma membrane and the third wall
layer was occupied by a number of irregular shaped vesicles
of varying sizes (Fig.60). At a later stage in the
development the original spore walls completely disappeared
from these areas, which were obviously destined to be the growing
points of the germ tubes and subsequent first hyphae
(Fig.61). The cytoplasm immediately behind the growing
point was rich in mitochondria,which were often very much
enlarged and elongated, small vesicles and pieces of endoplasmic
reticulum, all features indicating an area of intense
metabolic activity.

As a result of germination the spores of _Phycomyces,_

Fig.
59.

Fig. 60.

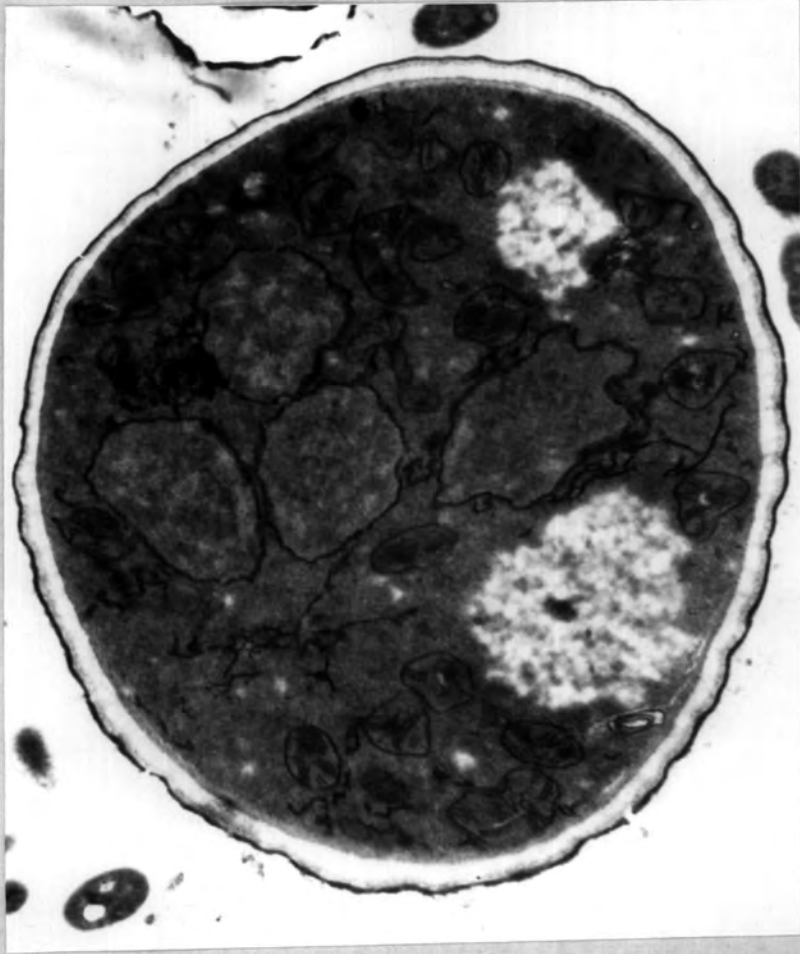Fig.59. Spore 2 hours after incubation in potato
extract at 20°C a third wall layer is just visible
within the original spore wall.   X 15,000
Fig.60. The emergence of the germ tube - the
original spore wall has disappeared. Note the
numerous, large mitochondria near the growing
lip.   X 15,000
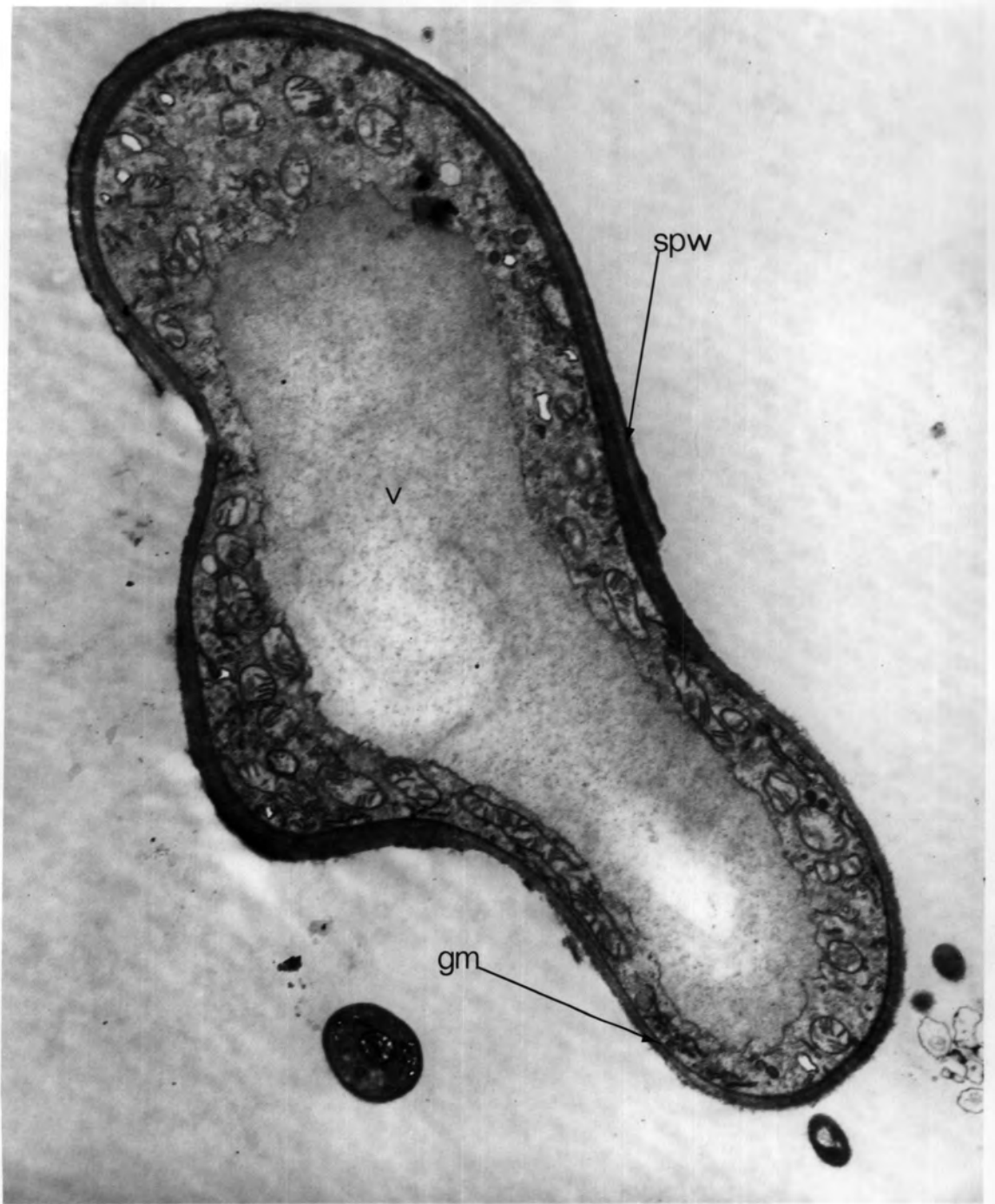
Fig. 61. Germinating spore five hours after incubation.   X 11,560

like those of many germinating fungal spores, show a
general increase in cell organelles. There is a rapid
build up of endoplasmic reticulum and a marked increase
in mitochondrial population. It could not however be claimed
that the increase in mitochondrial population in the
germinating spores is accompanied, as in the case of some
spores, (Luck, 1963) by a reduction in mitochondrial size,
since some of the largest mitochondria ever observed by me
in Phycomyces occurred in the germinating spores. If the
increase in mitochondrial population is obtained by the
division of the existing mitochondria, this division must
be accompanied by a rapid process of organelle enlargement.
Since the development of the third wall layer within the
spore, and the subsequent germ tube wall, is accompanied
by the disappearance of the fat globules it would seem
reasonable to suggest that the latter is in some way
involved in the production of wall material. The chitin
wall of Phycomyces has been shown to contain up to 25% of
waxy or fatty compounds readily extracted by carbon
tetrachloride (Kreger, 1954) so it may thus be that at least
part of the fatty material within the cytoplasm of the
spore is used strictly as a constituent of the wall material
during wall synthesis.

Attempts were made to germinate spores in a medium
containing tritiated glucose in the hope that it might be
possible to learn something of the mechanism by which wall
material is synthesised in Phycomyces spores. Unfortunately
the spores constantly showed a very low uptake of the
radioactive material so that these attempts proved
unsuccessful, perhaps to some extent because the spores
always contained wall constituents or their precursors in
adequate supply in the storage materials. It is more likely,

since spores will not germinate unless a nutrient medium
is supplied, that glucose is not normally taken up from
the growth medium although it may be required for germination.
The results of Ekundayo and Carlile (1964) and Ekundayo (1966)
indicate that in Rhizopus glucose could be metabolised on
the surface of the organism to bring about permeability
changes necessary for cell extension. The uptake of water
by the spores of Rhizopus arrhizus required the presence
of glucose. The rate of water uptake per unit area of spore
surface remained unchanged, as the volume of spore increased.
These facts suggest that the process was an active one
and not osmotic.

The glucose might be used to generate $NADH_2$ which
could reduce the disulphide linkages in the protein component
of the wall to sulphydryl groups, and so increase the
plasticity of the wall.

The exact chemical composition of the wall of
Phycomyces is still unknown (Bergman et al 1969); nevertheless
the chitin fibrils, which are known to make up about 25%
of the wall material, consist of polymers of 1-4 glucoside
linkages, like cellulose; the glucose residues being
N-acetylglucosamine instead of glucose. Work on the
synthesis of chitin in certain crustacea has shown that
it is glucosamine which is readily incorporated into the
chitin fibrils.

Nevertheless even if the appropriate amino acids
and nitrogen bases required for the synthesis of wall
materials were not present in the growth medium provided
it is difficult to explain the apparent absence of glucose
uptake by the spores.

The results obtained suggest that the germinating
spores and isolated hyphae possess sufficient reserves of

glucose or materials from which glucose can be derived
to make them independent of the external medium.

Vllle (lii) The Germ Tubes.

The young germ tubes (Fig.62) contain dense cytoplasm,
a relatively high proportion of endoplasmic reticulum, and
numerous mitochondria; also in transverse section several
small electron dense bodies were apparent. The cytoplasm
was bounded by a single wall layer about 13 nm thick.
There was no evidence of this wall material ever having
been surrounded by the cuticle commonly found in
Phycomyces sporangiophores.
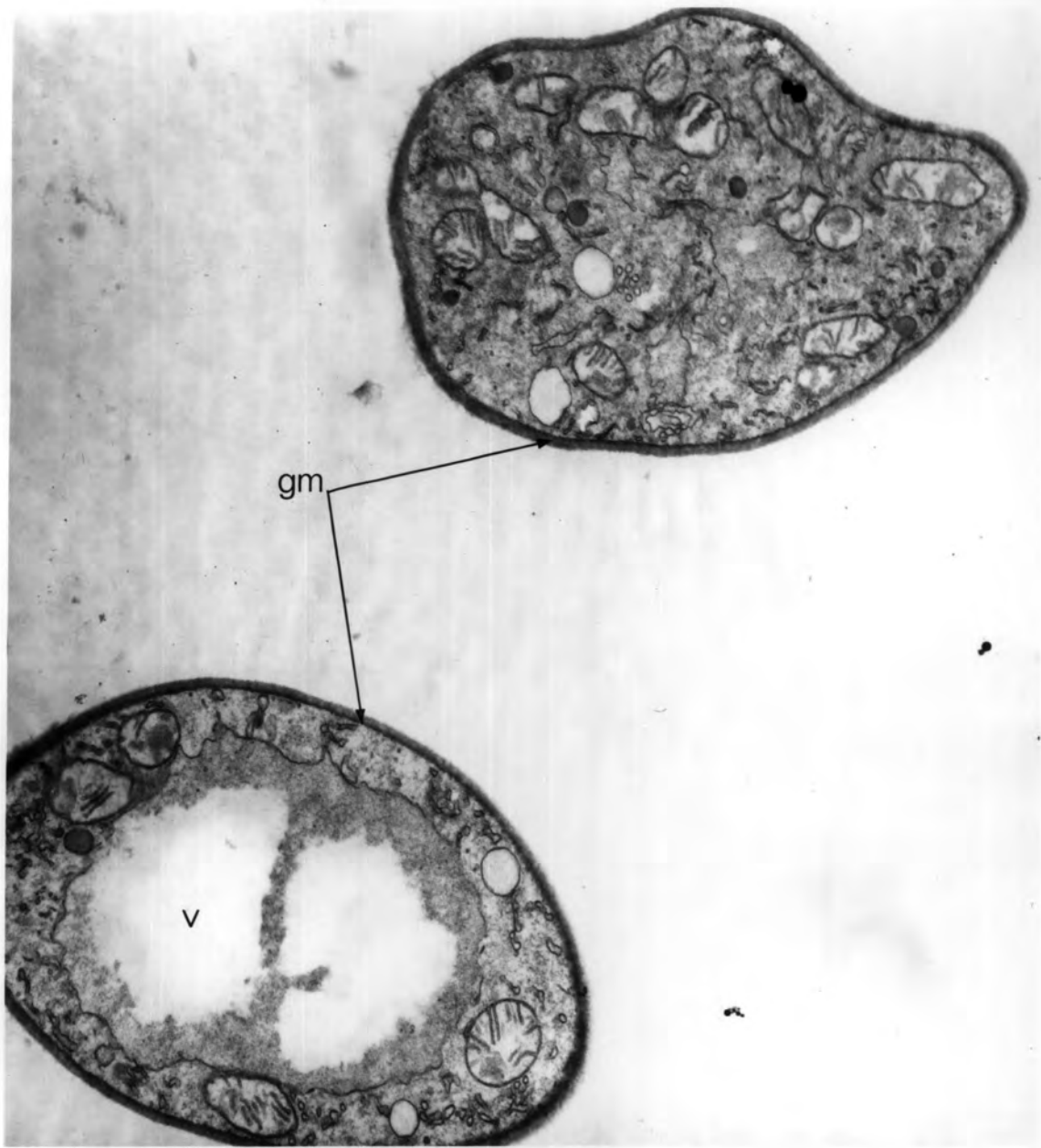
Fig. 62. Transverse sections of a very young and older germ tube. There is no indication of the presence of a cuticle.   X 15,460

Abbreviations.

cl – cleavage vesicle; cm – columella; ct – cisterna;
cmw- columella wall; cvs – central vacuole of
sporangiophore; cw – cell wall;   cwm– cell wall material

er – endoplasmic reticulum;

f – fat droplet;

g – glycogen; gm – germ tube wall;

i – interspore material;

lo– lomasome like body;

m – mitochondrion; mf – microfilament; mi– membrane
invagination; mv – microvesicle; ms – membrane stack;

n – nucleus; ne– nuclear evagination; no – nucleolus;
np – nuclear pore;

pl– plasmalemma; pr – primary wall layer; pv–pleomorphic
vesicle

r – rod like body; rws – reticulate wall system;

sp – spore mass; spw – spore wall; sw – secondary wall
layer; sv – spherical vesicle;

v – vacuole; ver – vesicle of endoplasmic reticulum;
vs – vesicle;

BIBLIOGRAPHY.

AUDUS, L.J., The Mechanism of the perception of gravity,
by plants. Sympos. Soc. Experiment.
Biol 16, 197-226   1962

BANBURY, G.H., Physiological Studies in the Mucorales.
Journal of Experimental Botany. 3, 77-94   1952

BANBURY, G.H., Phototropism of lower plants. Encyclopedia
of Plant Physiology. Springer-Verlag. Berlin xvii/i
530-578   1959

BARTON, R., Autoradiographic Studies on wall formation
in Chara. Planta (Berl.) 82, 302-306 1968

BENJAMIN, C.R., & HESSELTINE, C.W., Studies on the
genus Phycomyces. Mycologia 51, 751-771   1959

BERGMAN, K., et al., Phycomyces. Bacteriological Reviews
33. 1., 99-157 1969

BLAAUW, A.H., Licht und wachstum I.Zeitschrift Botan
6, 641-703 1914

BLAAUW, A.H., Licht und wachstum III.(Die Erklärung
des Phototropismus) Mededeelingen Landbouwhoogeschool
Wageningen 15, 89-204 1918

BLAKESLEE, A.F., Sexual reproduction in the Mucorineae Am.
Acad. Arts. Sci. 40 205-319   1904

BRACKER, C.E., Ultrastructural aspects of sporangiospore
formation in Gilbertella persicaria. In "The Fungus
spore." Proc. Sym. Colston Res. Soc., 18th Bristol,
England. (Madelin, N.F., Ed. Butterworths, London)
39-60 1966

BRACKER, C.E., Ultrastructure of Fungi.Ann. Rev. Phytopathol
55, 343-367   1967

BUCKLEY, P.M., SJAHOLM, V.E., and SOMMER, N.F., Electron
Microscopy of Botrytis cinerea conidia. Journal
of Bacteriology 91, 2037-2044 1966

BURGEFF, H., Uber arten und artkreuzung in der gattung
Phycomyces. Kunze. Flora 118-119, 40-46 1925

BURGEFF, H., Pflanzliche avitaminose und ihre behebung
durch vitaminzufuhr. Ber. Deut. Botan. Ges. 52
384-390 1934

CALOGNE, F.D., FEILDING, A.H., & BXRDE, R.J.W., Multivesi-
cular bodies in Sclerotinia functigena and their
possible relation to extracellular enzyme secretion.
Journal of General Microbiology. 55, 177-184 1969

CASTLE, E.S., Dark adaptation and the dark growth response
of Phycomyces. Journal of General Physiology 16,
75-88 1932

CASTLE, E.S., The refractive indices of whole cells.
The physical basis of the positive phototropism of
Phycomyces. Journal of General Physiology 17,
41-62 1933

CASTLE, E.S., Spiral growth and the reversal of spiraling
in Phycomyces and their bearing on primary wall
structure. American Jonrnal of Botany 29, 664-672
1942

CASTLE, E.S., The topography of tip growth in a plant cell.
Journal of General Physiology 41, 913-926 1958

CASTLE, E.S., Growth distribution in the light growth
response of Phycomyces. Journal of General Physiology
42, 697-702 1959

CHET, I., HENIS, Y., & KISLEV, N., Ultrastnucture of
sclerotia and hyphae of Sclerotium rolfsii (Sacc).
Journal of General Microbiology 57, 143-147 1969

CRAWLEY, J.C.W. , Fine structure and cytoplasmic streaming
in Physarum polycephalum. Journal of the Royal
Microscopical Society 85, pt III, 313-322 1966

COCHRANE, V.W., The physiology of fungi. John Wiley and
Sons, N.Y. 1958

DAVIS, L.E., Intramitochondrial crystals in _Hydra_. Journal
    of UltrastructuralResearch <u>21</u>, 125-133   1967

DELBRÜCK, M., & REICHARDT, W., Systems analysis for the
  light growth reaction of _Phycomyces_. In "Cellular mechanisms
    in differentiation and growth". Ed. Runich, D.,
    Princeton University Press 3-44   1956

DENNISON, D.S., Tropic responses of _Phycomyces_
    sporangiophores to gravitational and centrifugal
    stimuli. Journal of General Physiology. <u>45</u>, 23-38 1961

EKUNDAYO, J.A., & CARLILE, M.J., The germination of
    sporangiospore of _Rhizopus arrhizus_; Spore swelling
    and germ tube emergence. Journal of General
    Microbiology <u>35</u>, 261-269   1964

EKUNDAYO, J.A., Further studies on germination of
    sporangiospores of _Rhizopus arrhizus._ Journal of
    General Microbiology <u>42</u>, 283-291   1966

FRANKE, W.W., KRIENS, S., & BROWN M.R., Jr.
    Simultaneous gluteraldehyde-osmium tetroxide fixation
    with postosmication. Histochemie. <u>19</u>, 162-164   1969

GLAUERT, A.M., & GLAUERT, R.H., Araldite as an embedding
    medium for electron microscopy. Journal of Biophysics,
    Biochemistry and Cytology 4, 115   1958

GUNNING, B.E.S., and PATE, J.S., "Transfer Cells" Plant
    cells with wall ingrowths specialized in relation to
    short distance transport of solutes - their occurrence,
    structure and development. Protoplasma <u>68</u>, 107-133 1969

HARPER, R.A., Cell division in sporangia and asci. Annals of
    Botany <u>13</u>, 467-525   1899

HAWKER, L.E., Fine structure of fungi as revealed by
    electron microscopy. Biological Reviews <u>40</u>, 52-92   1965

HAWKER, L.E., and ABBOTT,P.McV., An electron microscope
    study of maturation and germination of sporangiospores
    of two species of _Rhizopus._ Journal of General
    Microbiology <u>32</u>, 295-298   1963

HAWKER, L.E. and HENDY, R.J., An electron microscope study
    of germination of conidia of _Botrytis cinerea_. Journal
    of General Microbiology <u>62</u>, 129-137   1963

HAWKER, L.E., and ABBOTT, P.McV., Fine structure of the
    young vegetative hyphae of _Pythium debaryanum_. Journal
    of General Microbiology <u>31</u>, 491-494   1963

HEATH, I.B., and GREENWOOD, A.D., Structure and Formation of lomasomes. Journal of General Microbiology 62, 129-137 1970

HEUCKEL, A., Le phototropisme des Mucorini. Bull. Inst. Rech. biol. St. Biol. Univ. Perm. 5, 307-310 1927

ISAAC, P.K., Cytoplamic streaming in filamentous fungi. Canadian Journal of Botany 42, 787-792 1964

JAHN, T.L., and BOVEE. E.C., Protoplasmic movements within cells. Physiological Reviews 49, 793-862 1969

JONES, R.C., The feulgen reaction as a cytological technique with Allomyces arbuscula. Mycologia 34, 109-112 1947

KAMIYA, N., Protoplasmic streaming. Protoplasmatologia. viii/3/a Springer-Verlag Wein. 1959

KANE, R.E., The mitotic apparatus - Fine structure of the isolated unit. Journal of Cell Biology 15, 279-287 1962

KORSON, R., A differential stain for nucleic acid. Stain Technology 26, 265-270 1951

KAVANAU, J.L., Protoplasmic streaming as a process of jet propulsion. Developmental Biology 7, 22-37 1963

KAY, D., (edt) Techniques in electron microscopy. Blackwell Scientific Publications Ltd., Oxford 65-67 1961

KREGER, D.R., Observations on cell wall of yeasts and some other fungi by X-ray diffraction and solubility tests. Biochem. Biophys. Acta 13, 1-9 1954

LEDBETTER, M.C., and PORTER, K.R., A 'microtubule' in plant cell fine structure. Journal of Cell Biology 19, 239-250 1963

LUCK, D.J.L., Formation of mitochondria in Neurospora crassa quantitative radioautographic study. Journal of Cell Biology 16, 483-499 1963

LUFT, J.H., Permanganate - a new fixative for electron microscopy. Journal of Biophysics, Biochemistry and Cytology 2, 799-801 1957

MARCHANT, R., PEAT, A., and BANBURY, G.H., The ultrstructural basis of hyphal growth. New Phytologist 66, 623-629 1967

MEISSNER, G., and DELBRÜCK, M., Carotenes and retinal
in _Phycomyces_ mutants. Plant Physiology 43, 1279-1283
1968

MERCER and BIRBECK. Electron Microscopy. Blackwell Scientific
Publications. 1961

MOHR, W.P., and COCKING, E.C., A method of preparing highly
vacuolated, senescent or damaged plant tissue for
ultrastructural study. Journal of Ultrastructural
Research 21, 171-181 1968

MOORE, R.T., and McALEAR, J.H., Fine structure of mycota.
V Lomasomes-previously uncharacterized hyphal
structures. Mycologia 53, 194-200 1961

NAGAI, R., and REBHUN, L.I., Cytoplasmic microfilaments
in streaming _Nitella_ cells. Journal of Ultrastructural
Research 14, 571-589 1966

OORT, A.J.P., and ROELOFSEN, P.A., Spiralwachstum wandbau und
plasmaströmung bei _Phycomyces_. Koninkl. Ned. Akad.
Wetenschap. Proc. 35, 898-908 1932

ORTEGA, J.K.E., and GAMOW, R.I., _Phycomyces_: habituation of
the light growth response. Science 168, 1374-1375 1970

PEAT, A., and BANBURY, G.H., Ultrastructure, protoplasmic
streaming, growth and tropisms of _Phycomyces_ sporangio-
phores. New Phytologist 66, 475-484 1967

PEAT, A., and BANBURY, G.H., Occurrence of ferritin-like
particles in a fungus. Planta 79, 268-270 1968

PEAT, A., and WHITTON, B. A., Environmental effects on the
structure of the blue-green alga _Chlorogloea fritschii_
Archive für Mickrobiologie 57, 155-180 1967

POP, L.J.J., Protoplasmic streaming in relation to spiral
growth in _Phycomyces_. Proceeds of the Med. Akad. Wet.
41 ii, 661-672 1938

ROELOFSEN, P.A., Cell wall structure in the growth zone of
Phycomyces sporangiophores. II Double refraction and
electron microscopy. Biochem. Biophys. Acta. 6,
357-373 1951

ROELOFSEN, P.A., The plant cell wall. Encyclopedia of plant
anatomy III (4) 298-305 Berlin 1959

ROTH, L.E., Primitive motile systems in cell biology
        p.527 Eds. Allan, R.D., and Kamiya, N., Academic
        Press New York, 1964

SABNIS, D.D., and JACOBS, W.P., Cytoplasmic streaming
        and microtubules in the coenocytic marine alga
        Caulerpa prolifera. Journal of Cell Science 2,
        465-472 1967

SCHOPFER, W.H., Versuch über die Wirkung von reinen
        kristallisierten vitamin B auf Phycomyces Ber. Deut.
        Botan. Ges. 52, 308-312   1934

SCHWAB, D.W., JANNEY, A.H., and SCOLA, J., Preservation
        of fine structure in yeast by fixation in dimethyl
        sulfoxide - acrolein - gluteraldehyde solution.
        Stain technology. 45; 4 143-147 1970

SCHWARZE, C.A., The method of cleavage in sporangia of
        certain fungi. Mycologia 14, 143-172   1922

SITTE, P., Einfaches verfahren zur stufenlosen gewebe-
        entwässerung für die electronenmikroskopische
        präparation. Die Naturwissenschaften 17, 402-403
        1962

STRASBURGER, E., Zellbildung und Zelltheilung 3rd ed. 1880

SWINGLE, D.B., Formation of the spores in the sporangia
        of Rhizopus nigricans and of Phycomyces nitens. U.S.
        Department of Agriculture Bulletin 37, 5-40 1903

THORNTON, R.M., The fine structure of Phycomyces. I
        Autophagic vesicles. Journal of Ultrastructural
        Research 21, 269-280 1968

THORNTON, R.M., The fine structure of Phycomyces. II
        Organization of the stage I sporangiophore apex.
        Protoplasma 66, 269-285 1968

VILLIERS, T.A., Intranuclear crystals in plant embryo
        cells. Planta (Berl) 78, 11-16   1968

WILSENACH, R., and KESSEL, M., The role of lomasomes in wall
        formation in Penicillium vermiculatum. Journal of
        General Microbiology. 40, 401-404 1965

WOHLFARTH-BOTTERMANN, K.E., Differentiation of the ground
       cytoplasm and their significance for the generation
       of motive force of amoeboid movement. In "Primitive
       motile systems in Cell Biology." Eds Allen, R.D.,
       and Kamiya, N., Academic Press New York. 79   1964

WOLKEN, J.J., Microspectrophotometry and the photoreceptor
       of Phycomyces I.   Journal of Cell Biology 43, 353-360 1969

ZANKEL, K.L., BURKE, P.V., and DELBRUCK, M., Absorption
       and screening in Phycomyces. Journal of General
       Physiology. 50,1893- 1906 1967

A C K N O W L E D G M E N T

I wish to thank Mr. G. H. Banbury for his help and
encouragement throughout this work.

I would also like to thank Mr. A. Cobb and
Mr. R. Swinhoe for their assistance with the
photography.