Differentiation and ferritin synthesis in phvcomvces blakesleeanus; an ultrastructural and physiological study

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To my Son: Yassir

Differentiation and Ferritin Synthesis in *Phycomyces blakesleeanus*: An Ultrastructural and Physiological Study

by

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Declaration:-

The work described in this thesis has been carried out by the undersigned at the Department of Botany of Durham University and has not been previously submitted for any other diploma or degree.

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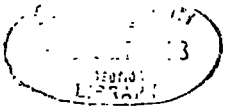
Contents

ABSTRACT

The first part of this investigation deals with the ultrastructural aspects of the dormant and germinating spores and the growing mycelia of the fungus Phycomyces blakesleeanus in submerged culture. Emphasis is focussed on the detection and behaviour of the protein ferritin in these stages particularly in the growing mycelia. The concomitant morphological and ultrastructural changes are described. Time course experiments on growing mycelia revealed that at certain stages of differentiation, detectable ferritin first disappeared in incipient germ hyphae. Its reappearance was found to depend upon the presence of added iron in the medium. A correlation between this reappearance and the availability of lipid droplets, in sections of mycelia, is described and discussed. Successive changes in the ultrastructure of mitochondria during growth and differentiation are also described.

In the second part of this study, the electron microscopic observations of ferritin behaviour are backed with tangible biochemical evidences by isolating the protein at different times and conditions from the growing mycelia. The effect of the presence of high iron in the medium and other factors on ferritin synthesis are described. A sixfold de novo increase in apoferritin synthesis is attained by iron



administration to growing mycelia. Factors affecting such a synthesis and radioactivity incorporation are described and discussed. By the use of polyacrylamide gel electrophoresis the subunit molecular weight of Phycomyces ferritin has been determined as 20,200 daltons.

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List of Abbreviations

The following abbreviations were used, where appropriate, to label all micrographs presented in this study:-

C	-	Cuticle
Ci	-	Cisternae
db	-	Dense Body
dbv	-	Dense Body-Containing Vesicle
edg	-	Electron Dense Granule
ER	-	Endoplasmic reticulum
F	-	Ferritin
fr	-	Food Reserve
G	-	Golgi cisternae
g	-	Glycogen
HW	-	Hyphal Wall
L	-	Lipid Droplet
Lo	-	Lomasome
M	-	Mitochondria
mg	-	Mitochondrial Granule
ms	-	Membranous System
mvb	-	Multivesicular Body
mw	-	Mitochondrial Whorl
N	-	Nucleus
Nu	-	Nucleolus
NW	-	New Wall
PM	-	Plasma Membrane

R	-	Ribosomes
SW	-	Spore Wall
V,v	-	Vesicles
VA	-	Vacuole

General Introduction

Phycomyces blakesleeanus, Burgeff belongs to the family Mucoraceae and to the order Mucorales and class Zygomycetes. It is thus one of the less-advanced (lower) fungi which used to be grouped together as *Phycomycetes*. Since its discovery in 1823 by Kunze, biologists have been attracted to *Phycomyces* for several reasons. The most persistent fascination has been its remarkable sensory responses mainly of its unique sporangiophore (Cerda-Olmedo, 1974). The sensory physiology of the sporangiophore has been extensively studied and reviewed (Bergman *et al.*, 1969). A general review and a review on the behavioural genetics of *Phycomyces* have been published (Cerda-Olmedo 1974, 1977). Recently, a comparative general study of *Mucor*, *Phycomyces* and other members of the Mucoraceae has been published (Ingold, 1978).

The occurrence of the iron protein: ferritin in *Phycomyces* adds a new dimension to the biological importance of this organism. Such an importance emerges from the fact that ferritin, as an iron storage protein, plays a vital role in animal physiology because of the iron it stores. Iron is known to be involved in storage and transport of oxygen, in electron transport, in the metabolism of nitrogen and hydrogen and in the reduction of DNA precursors (Neiland, 1972). In microorganism, beside ferritin, many iron-containing protein-

and enzymes of different functions have been reported (Neiland, 1974).

In the present study attempts were made to explore some aspects of this important protein in Phycomyces. During the process of germination and the subsequent differentiation, the behaviour of ferritin was observed by electron microscopy. This study was later extended to the isolation of this protein and investigation of its synthesis.

It is envisaged that the findings of this study would contribute to the knowledge on Phycomyces ferritin. In addition, a study on the role of iron in the metabolism of this fungus is greatly needed to unravelling the significance of the curious existence of such a specialized protein in such a saprophytic fungus.

Chapter One: Electron Microscopy

Introduction

The gigantic, coenocytic sporangiophore of *Phycomyces blakesleeanus* has received considerable attention by electron microscopists who have studied its ultrastructure for different purposes (Roelofsen 1951; Peat & Banbury 1968; Thornton 1968a,b; Zalokar 1969; Tu *et al.*, 1971; Hankinson 1972; Ootaki & Wolken 1973; Tu & Malhotra 1975; Malhotra & Tu 1976; Tu & Malhotra 1976). Scanning electron microscopy has also been used to study the formation and development of *Phycomyces* zygospore (Tewari & Malhotra 1976; O'Donnell *et al.*, 1976, 1978).

In contrast to the several ultrastructural studies on the sporangiophore of *Phycomyces*, dormant and germinated spores and mycelia of this mucoraceous fungus were very briefly studied as a subsidiary part of a major investigation (Hankinson 1972; Tu & Malhotra, 1976; Grove, 1976).

Ultrastructure of germination of fungi has been extensively studied covering many of the principal genera of most classes of Eumycotina. These included:- *Blastocladiella emersonii* (Cantino *et al.*, 1963); *Phytophthora parasitica* (Hemmes *et al.*, 1971); *Mucor rouxii* (Bartnicki-Garcia 1968); *Aspergillus nidulans* (Border & Trinci, 1970); *Coprinus cinereus* (McLaughlin 1977) and *Trichoderma viride* (Rosen *et al.*, 1974) as representatives of Chytridiomycetes,

Oomycetes, Zygomycetes, Ascomycetes and Deuteromycetes respectively (more examples are listed in the appendix).

Fungal spores are diverse in form, function and origin. They can however be morphologically considered as single or many-celled structures ultimately cut off from the parent mycelium or thallus, in which cytoplasm, nuclei and other organelles are packaged (Cochrane 1974). These spores can be divided according to Gregory (1966) into two general groups:-

1. Xenospores are those which become detached and are dispersed from their place of origin and which usually germinate readily in suitable conditions.
2. Memnospores are those which remain attached to their place of origin, undergo dormancy and germinate only after the application of some specific stimulus or counter inhibitor.

Dormany can be defined according to Sussman and Douthit (1973) as: "any rest period or reversible interruption of the phenotypic development of an organism." There are two main types of dormancy:-

1. Constitutional dormancy: a condition wherein development is delayed due to an innate property of the dormant stage such as a barrier to the penetration of nutrients, a metabolic block or the production of a self inhibitor.

2. Exogenous dormancy: a condition wherein development is delayed because of unfavourable chemical or physical conditions of the environment.
Recent reviews on dormancyare those of Sussman (1969), Sussman & Douthit (1973) and Cochrane (1974).

The asexual sporangiospore of Phycomyces blakesleeanus is somewhat anomalous in its dormancy behaviour since many of the spores will germinate immediately in some nutritionally complex media without a dormant period (Cochrane 1974) while in a synthetic medium, it requires activation (Robbins et al., 1942; Bergman et al., 1969). On the basis of such a behaviour, sporangiospores of Phycomyces blakesleeanus would appear to behave either as xenospores or as constitutionally dormant spores depending on the nature of the medium.

Dormancy where applicable can be broken by an extensive range of treatments. The method commonly used for Phycomyces is heat shock (3 min at 50^{o}C), but activation may also be obtained by the use of many chemicals (Bergman et al., 1969). Dormant sporangiospores of Phycomyces whether dry or suspended in water can also be activated by an exposure to gamma rays (Van Assche et al., 1977). Activators and the mechanisms of activation in fungi were recently reviewed by Sussman (1976).

Trehalase is the enzyme responsible for the hydrolysis of the disaccharide reserve carbohydrate trehalose and the subsequent release of glucose from the latter. In Phycomyces blakesleeanus sporangiospores, trehalase exists in two different locations: inside the spore and in the periplasmic region. A more precise localization has not yet been determined (Van Assche et al., 1978). Upon activation of sporangiospores of Phycomyces, whether by heat shock (Van Assche et al 1972) or ammonium acetate (Delvaux 1973), trehalase activity is markedly increased. This however does not occur when gamma rays are used for breaking dormancy. This suggests that a rise in trehalase activity or glucose availability are not a prerequisite for breaking dormancy (Van Assche et al., 1977). Moreover, dormant spores of Phycomyces take up labelled glucose and metabolize it into various compounds (Van Leare & Carlier 1975). According to Rudolph & Furch (cited in Van Assche et al., 1972) increased glycolytic activity is more strictly required in activation of Phycomyces spores than is trehalose breakdown and the subsequent glucose availability.

Once an effective activation is applied, a programmed series of developmental changes is initiated at the biochemical, morphological and ultrastructural levels. Heat activation of Phycomyces sporangiospores affects markedly the metabolism of

the dormant spores as indicated by the increase in the respiratory rate and an output of CO_2, acetaldehyde, pyruvic acid and ethanol during the activation process. Immediately after activation the spores show a rapid glycolysis which leads to accumulation of pyruvic acid and its subsequent release in the medium (Rudolph et al., 1966). Soon after activation the amount of cytochromes gradually increases up to 8 hours (Keyhani et al., 1972). RNA and protein synthesis increase immediately after activation but DNA synthesis begins only when the germ tube is already formed (Van Assche & Carlier 1973). Changes in the free nucleotide pattern also occur during Phycomyces germination (Furch 1974). Nucleic acids and protein synthesis during fungal spore germination were recently reviewed (Bramble et al., 1978).

Lipids were also studied in the sporangiospores of Phycomyces and found to account for about 3% of the dry weight of the spore with triglycerides being the major constituents. Heat activation causes a striking 300 fold increase in the level of glycerol one hour after such activation (Furch et al., 1976).

Acetate activation of Phycomyces sporangiospore is accompanied by a transient rise in trehalase activity followed by an accumulation of glucose in the surrounding medium.

At the same time pyruvate, acetalydehyde, ethanol and lactate can be detected in the culture medium. During acetate treatment most of the CO_2 produced is supplied by the turnover of endogenous material (Delvaux 1973).

The cell wall of Phycomyces blakesleeanus undergoes major changes during differentiation and morphogenesis. This can be demonstrated by the carbohydrate composition of the cell walls from sporangiospores, mycelium and sporangiophores. The major change is in the aminosugar content which dramatically increased from 10% in spores to 45% in mycelia and 90% in sporangiophores. This increase is due to the formation of two enzymes involved in the synthesis of glucosamine-6-phosphate and N-acetyl-glucosamine-6-phosphate (chitin precursor) during early germination. These enzymes can be inhibited by both 5-fluorouracil and cycloheximide which suggests that they are synthesized on mRNA formed during germination (Van Laere et al., 1976, 1977)

Tu & Malhotra (1977) studied the effect of exogenous cyclic adenosine monophosphate (cAMP) on germinating spores and mycelia of Phycomyces blakesleeanus. They observed that the time required for germ tube emergence was reduced and the diameter of the mycelium increased. They also observed that the cell wall of the mycelium exhibited some 5-fold thickening which led them to suggest that cAMP enhanced chitin

synthesis. Jan (1974) studied chitin synthetase in Phycomyces and suggested that the enzyme is bound to the plasma membrane of hyphae. He also found that both Mg^{2+} and N-acetyl-D-glucosamine stimulate the enzyme activity, but the presence of the antibiotic polyoxin D (0.1mM) inhibits this activity so that germinating spores develop into protoplast-like structures. Recently the properties of this enzyme were further studied by Van Laere & Carlier (1978) who reported its absence in dormant spores and that it is synthesized de novo in germinating spores from 4 hours of germination onwards.

The presence of adenyl cyclase in Phycomyces was investigated histochemically in situ in dormant and germinated spores and in the growing zone of the sporangiophore (Tu & Malhotra 1973). This enzyme was found to be in association with the plasma membrane within the mitochondrial membranes and the nuclear membranes of all stages studied. They suggested that the enzyme might play a role in the initiation of spore germination and that it might be stimulated by heat shock. Recently, Furch & Gooday (1978) studied sporopollenin, an oxygenated polymer of carotenoids known for its chemical resistance. It was found associated with the zygospore wall of a carotene-containing member of the mucorales (Gooday et al., 1973). Furch & Gooday (1978) reported it to be present in the sporangiospores, sporangiophores and zygospores of Phycomyces

blakesleeanus in varied amounts: 0.6%, 3.6% and 1.4% respectively. Its absence in mycelia and its association with wall of spores and sporangiophores of Phycomyces imply a protective role for it.

In this part of the present investigation, the electron microscope was used as a useful tool in detecting the presence of the iron storage protein ferritin during cellular differentiation and morphogenesis of the germinating spores and the growing mycelia of Phycomyces blakesleeanus. Because of its electron opacity and its specific binding to lipid droplets, ferritin is thus comparatively conspicuous in electron micrographs. The concomitant morphological and ultrastructural changes during differentiation are described.

Materials and Methods

Phycomyces blakesleeanus Burgeff, wild type, minus strain 1555 NRRL (Northern Regional Research Laboratory) was kindly provided by Mr. G.H. Banbury and was used in the early stages of this work.* It had been grown on a slope of 2% malt agar.

1. Cultures and Media

1.1 Cultures and inoculum

Stock cultures were obtained by inoculating Petri dishes containing 2% autoclaved malt agar with small blocks of original stock bearing mycelia. Plates were incubated at room temperature (c 22°C) in a cupboard receiving a low level of diffused light. New stocks were maintained by routine sub-culturing onto either malt agar or solid minimal medium every four weeks. After 2 days of incubation, mycelia covered the surface of the agar and this was followed by the initiation of sporangiophores which elongated to a potential height (3-10 cm) and gave rise to mature black sporangia containing vegetative spores.

For inoculating liquid or solidified minimal media, a spore suspension was used. The inoculum was prepared by flooding a culture in a petri dish with sterile distilled water or minimal medium with agitation to release spores either from intact sporangia or broken ones. The spore suspension was collected in a sterile flask and was subjected to heat shock

* See page 17A

All the subsequent studies were made on Phycomyces blakesleeanus from the central mould culture collection at Baarn, since this strain grew more vigourously.

in a water bath at 50°C for 5-10 minutes with occasional stirring. The suspension was cooled down to room temperature before use for inoculation. Routinely 3-4 plates (2-4 week old cultures) were used for each inoculum, this gave between 2.4-4 x 10^7 spores/ml. Liquid cultures were inoculated with one millilitre of spore suspension per 100 ml medium.

1.2 Media

At the beginning of the work malt agar was the sole medium used for maintaining stocks of Phycomyces by growing it in either petri dishes or 250 ml flasks having approximately 20 or 50 mls of 2% autoclaved malt agar respectively. This medium was replaced by a defined artifical medium which was found to secure maximum growth (Ødegard 1952). It was also found to be poorly buffered in the pH range 5-3. This minimal medium (MM) was made as follows:-

D-Glucose	30 g
L-Asparagine	3.5 g
KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g
$CaCl_2.2H_2O$	80 mg
$FeCl_2.6H_2O$	0.32 mg
$ZnSO_4.7H_2O$	0.44 mg
Thiamine	50 µg
Distilled water	one litre

Ca^{2+}, Fe^{3+}, Zn^{2+} and thiamine were prepared individually and kept in a refrigerator as stock solutions. Appropriate volumes of each were used to bring them to the required final strength.

Modifications to this medium were undertaken only with respect to the concentrations of iron and zinc and for the following purposes:-

1. In some experiments growth of mycelia had to be either partially or completely in the absence of added iron. Iron as an impurity in distilled water was measured by atomic absorption and found to be 0.02µg/l. Details will be shown later in descriptions of experiments.
2. The concentration of iron recommended for this minimal medium was not employed because much higher concentrations (50 fold) were needed to show obvious effect on the role of iron as an inducer of ferritin synthesis.
3. Apart from some few specific experiments on the effect of high level of zinc in the medium either in the presence or absence of iron, the recommended concentration (0.44 mg/l) was used.

Throughout this investigation, mycelia of Phycomyces were grown in Pyrex conical flasks of different capacities as follows:-

150	ml	flask	contained	25	ml	MM
250	"	"	"	50	"	"
1000	"	"	"	200	"	"
2000	"	"	"	500	"	"

The smaller flasks were used for studying germination for electron microscopy while the larger ones were used in order to obtain substantial yield of mycelia for ferritin isolation. All flasks were plugged with cotton wool and autoclaved for 15 min at 1 Kg/Cm^3. When inoculated small flasks (150-250 ml) were put in a specially designed orbital shaker running at about 60 rev./min while the bigger ones (1L and 2L) were put in another orbital shaker, running at approximately 150 rev./min., in a separate room. Thermostatic control was not in operation with these shakers.

2% aqueous malt extract was also used for a comparative study of germination and was dealt with as above except that it had to be filtered through Whatman No. 1 filter paper before autoclaving in order to remove insoluble material which could interfere with subsequent sampling for electron microscopy.

2. Sampling

2.1 Dormant spores

A suspension of dormant spores was centrifuged at low speed (500xg) using MSE clinical centrifuge and conical test tubes. The supernatant was discarded and fixative was added

directly to the pellet and the tube was covered with Parafilm. Before dehydration, the pellet was suspended in 1% molten agar (45-50°C). After setting, small blocks (not more than 3mm) of agar were subjected to dehydration, resin infiltration and embedding.

2.2 Germinating Spores

Two techniques were employed here:-

1. Germinating spores were collected at various stages of germination by filtration through Millipore filter discs (pore size 0.22-0.45μm) and briefly washed with distilled water. The resulting layer was gently scraped with a tooth pick to make a compact, concentrated sample which was covered with a drop of 1% molten agar. After setting, the agar was cut into small pieces which were dipped in a vial containing fixative and processed as before. Sampling by this rather harsh technique was later abandoned because of unusual features observed under the electron microscope which indicated signs of mechanical injury and post mortem effects similar to injury effects caused by chilling of germinated spores of Rhizopus stolonifer (Buckley et al., 1968).

2. Another technique was a modification of that employed by Bracker (1971). Germinating spores in liquid medium were collected by filtration through Millipore filter discs and the latter were then transferred to a vial containing cold fixative. Removal of fixative and washings was achieved

by careful pipetting or low speed centrifugation. Finally before dehydration the pellet was suspended in 1% molten aqueous agar and cut into small pieces which were subjected to dehydration, resin infiltration and embedding.

2.3 Mycelia

These were filtered off using either Whatman No. 1 filter paper or for older mycelia, Miracloth. A weft (not more than 4mm in diameter) was taken with a tooth pick and was immersed in a vial containing cold fixative. No agar coating was needed for processing of this particular material.

3. Fixation

The following fixatives were used:-

1. 2.5% (v/v) glutaraldehyde in 0.1M sodium phosphate buffer pH 7
2. 4% (v/v) glutaraldehyde in 0.1M sodium phosphate buffer pH 7
3. 1.5% (v/v) formaldehyde + 2.5% (v/v) glutaraldehyde in 0.1M sodium phosphate buffer pH 7
4. 1% (w/v) Osmium tetroxide in distilled water
5. 2% (w/v) filtered potassium permanganate in distilled water

Samples (dormant or germinated spores or mycelia) were fixed in cold aldehyde fixative [(1) (2) or (3) above] for 2-4 hours at +4^{o}C followed by 2-4 washings with cold buffer. They were stored at 4^{o}C for subsequent simultaneous osmication with cold aqueous osmic acid for 2 hours followed by brief washing

with cold distilled water prior to subsequent dehydration.

Permanganate fixation was achieved at room temperature. Vials were kept in a light-tight black box for one hour followed by removal of fixative and several washings with distilled water prior to subsequent dehydration.

In two experiments where formaldehyde-glutaraldehyde was the fixative, following osmication and subsequent washing the samples were stained with 0.5% aqueous uranyl acetate overnight prior to dehydration according to a procedure recommended by Hess (1966). In a third experiment using the same fixative this latter step was omitted because such treatment led to over-staining of sections intended to reveal the familiar ferritin arrays and rendered them inconspicuous.

4. Dehydration

Agar blocks of fixed dormant or germinating spores were dehydrated through 2 changes of a graded series of ethanol as follows:-

50, 75, 95 and 100% 15 min each

Fixed mycelia were dehydrated in a quicker way to minimize loss of lipids using the following series of ethanol:-

50, 75 and 95% (one change), 10 min each

100% (two changes), 5 min each

5. Resin infiltration and embedding

A stock of all components of Spurr's (1969) resin (modification B) was chosen in preference to the standard medium because it gave reasonably hard castings. The medium was made up by weighing and thoroughly mixing the following components:-

10 g	ERL-4206,	Vinyl Cyclohexene dioxide (epoxy resin)
4 g	DER 736,	Diglycidyl ether of polypropylene glycol (flexibilizer)
26 g	NSA ,	Nonenyl succinic anhydride (hardner)
0.4 g	S 1 ,	Dimethylaminoethanol (accelerator)

5.1 Resin infiltration into dormant and early germinating spores:-

After many trials, including the procedure devised by Spurr (1969), the following procedure was found satisfactory:-

20% resin (with accelerator) in dry ethyl alcohol (v/v) for 1-2 hours

40% resin (with accelerator) in dry ethyl alcohol (v/v) for overnight

60% resin (with accelerator) in dry ethyl alcohol (v/v) for overnight (cap on)

60% resin (with accelerator) in dry ethyl alcohol (v/v) for one day (cap off)

100% resin (with accelerator) in dry ethyl alcohol (v/v) for one day or longer

Samples were finally embedded in complete resin in various forms of containers (e.g. caps of vials, or of milk bottles). Castings were polymerised in an oven at 70°C for 8-24 hours after which they were allowed to cool down before trimming and sectioning.

5.2 Resin infiltration into mycelia.

This was achieved by adopting Spurr's procedure but using defined concentrations as follows:-

50% resin in 100% ethanol for 30 min
75% " in 100% " " 30 min then cap off for overnight or longer
100% resin in 100% ethanol for one day or longer

Casting and polymerisation were as previously described

6. Sectioning and Staining

Sections of various thicknesses (between 500-900 Å) were cut on an LKB ultratome using glass knives. They were picked up on Formavar-coated grids. Sections were stained by floating grids on a drop of a saturated aqueous solution of uranyl acetate for 15-30 min at room temperature followed by washing with a jet of distilled water, blotting and then further staining on a drop of lead citrate (Reynolds 1963) for 10-15 min followed by washing as before.

7. Electron Microscopic Examination

An AEI EM 6B electron microscope operating at various voltages was used. A sample of fixed, unsectioned dormant spores was collected by filtration, (Millipore filter disc), dried, coated with gold-pallidium grains (c 300 Å thick layer) and viewed under a scanning electron microscope (Cambridge S600 SEM) operating at 7.5 KV.

8. Photography

Plates (Ilford and Kodak) were printed on appropriate grades of Veribrom (Kodak).

9. Chemicals

All material used for growth medium were of Analar grade. Glutaraldehyde, paraformaldehyde and osmium tetroxide were of EM grade. Potassium permanganate, buffer material and ethanol were of analytical grade.

Results

1. General Observations

Obtaining nearly synchronous germination, in culture, was a major aim to be achieved. Age of spores proved to be an important factor, and spores 1-2 months old were found to show poor synchrony. Variability in germination was much reduced by using only spores between 1-2 weeks old. It was also found that prolonging heat activation, over 30 min at 50°C, delayed germ tube emergence and caused a reduction in synchrony, so the shock was confined to 5 min at 50°C.

After activation, the microscopic appearance of the spores began to change. About one hour after incubation swelling could be observed and this continued with a concomitant transformation from the ellipsoid to the globose shape between 3-8 hours. During this phase (spherical growth), several minute vacuoles appeared, at between 2-4 hours, but 2 hours later they coalsced to form a single large vacuole. By 8 hours most of the spores developed 1-2 short germ tube which later elongated to form the hyphae. These hyphae later branched and by 24 hours a mycelium was established.

2. Electron Microscopy

2.1 The Dormant Spore

The dormant sporangiospores of Phycomyces blakealeeanus are single, non motile and generally ellipsoid. However,

their size is variable: 8-13 by 5-7.5μm (Bergman et al 1969). Plate 1 shows to a certain extent this variation. It also reveals the relatively smooth surface feature and the lack of any ornamentation or a spore pore.

Many trials aimed at adequately fixing and sectioning unhydrated dormant spores were not successful although resin infiltration seemed not to be the problem. Sections of spores were relatively intact with the content present but the protoplast was too dense to reveal any detail in the structure present. Only slightly hydrated spores (1-2 hours) showed some detail, and none better than those in Plate 2 a,b could be obtained. A complete image by the double fixation procedure (using glutaraldehyde followed by osmic acid), was not obtained, since membranes appeared negatively stained. This appearance suggested that osmic acid penetration had been impaired, although lipid droplets seemed to be well preserved (Plate 2b). This ambiguous situation was also observed by Grove (1976). Plate 2b show the structure of the spore wall to consist of 3 layers: an outer electron dense layer, a fibrillar or mosaic layer and an inner electron-transparent layer. In most sections examined there were one or two nuclei per section. Those in Plate 2b appear lobed and surrounded by a nuclear envelope, in Plate 2a some of their contents (chromatin) was densely stained. Endoplasmic

reticulum (ER) is scattered in the cytoplasm and occurs as small strands close to the cell wall (Plate 2b). Mitochondria are fairly large, lobed with inconspicuous cristae and tend to occur near the periphery (Plate 2a, 2b) but in another section (Plate 19, Fig. A), at higher magnification, the lobed configuration is maintained with well developed cristae. Lipid droplets are scattered in the cytoplasm and show a negatively stained membrane-like profile surrounding them (Plate 2b). Ferritin was bound to some lipid droplets though inconspicuous, but sometimes it was found to bind to residual body structures (black arrow in Plate 2a). Some membrane-bound vesicles with contents of different electron opacity are scattered in the cytoplasm near the periphery (white arrow in Plate 2a). All these features suggests a stage of inactive cell typical of a dormant spore.

2.2 The Germinating Spore:-

The sequence of germination was studied by sampling at approximately one hour, 4, 6, and 8 hours after heat activation. The resolution of cytoplasmic detail was much enhanced after activation whether samples were fixed with glutaraldehyde or permanganate. The first striking feature which can be observed in glutaraldehyde fixed material is the deposition and build up of a new wall layer (NW) between the innermost layer of the spore wall and the plasma membrane

in a uniform way all around the protoplast. This new wall has an electron density characteristic and distinguishable from other wall layers (Plate 3).

Another noticeable change that occurred after activation was the appearance of electron dense convoluted membranous structures resembling myelin figures. These disappeared as germination proceeded (Plates 3 and 5). Plate 3 shows also some electron dense aggregates designated as food reserves because of their resemblance to glycogen aggregates seen in permanganate fixed material (Plate 13). Perhaps, because of a partial hydrolysis, these appeared later on as patches of electron-translucent material (Plates 4 and 6).

As germination proceeds there is a near doubling in the number of nuclei per section suggesting that nuclear division occurred (Plates 4, 14 and 15). The centre of a germinating spore is occupied by a large vacuole with membranous inclusions implying a lytic role (Plates 4, 6 and 14). Ribosomes are very conspicuous and abundant in samples fixed with glutaraldehyde (Plate 5). The endoplasmic reticulum is clearly shown in samples fixed with permanganate; it is more abundant near the periphery and in close proximity to the nuclei (Plates 14 and 15). Numerous vesicles of various sizes and shapes are scattered in the cytoplasm throughout the spherical growth phase. In particular some

large, single membrane-bound vesicles filled with contents of medium opacity are found in the cytoplasm of spores fixed both with glutaraldehyde or permanganate (triangles in Plate 5, white arrows in Plates 14, 15 and 33). Their presence near the periphery (before germ tube emergence) and their resemblance to peroxisomes suggests that they might be involved in some processes taking place at the wall. Multivesicular bodies are also present before and during germ tube emergence (Plates 4, 6 and 33) and occasionally lomasome-like structures occur (Plate 33). The number of lipid droplets is greatly decreased as compared with ungerminated spores and where found some ferritin arrays seem to be associated (Plates 4 and 6). Mitochondria exhibit profound changes throughout the germination sequence and they will be described under separate title.

Germ tube emergence is preceded by some changes, mainly in the pattern of the new wall which tends to become thicker in one or two locations than the rest of the wall, thus giving rise to a more polarized deposition of wall material (Plate 15). This is followed by the protrusion of the initial germ tube through the ruptured spore wall (Plate 6). Associated with this emergence is the presence of different vesicles in close proximity. One vesicle, having electron-dense content analogous to the new wall, appears to be fusing with the plasma membrane

(Plates 6 and 22). Later on, about 8 hours after heat activation, the germ tube has elongated further with its new wall which appears to differ from the spore wall (Plate 16). Plate 6 shows the plasma membrane as if it were pulled away from the cell wall, thus showing membrane fragments and fibrous material. Similar features are shown in Plate 32, where vesicles, having fibrillar contents, fuse with the plasma membrane (Fig. A and B are sections of one block, Fig. C section of independent preparation). It is likely however that these distortions are signs of stress or effects of a 'plasmolytic' phenomenon.

2.3 The Growing Mycelia

The exploration of ferritin presence or absence was extended further to include the growing hyphae until the establishment of mycelia and their subsequent growth. Samples of 12, 18, 24, 36 and 48 hour-old mycelia were used for this purpose and a brief description of the ultrastructural organisation at these stages will be given.

There is an overall increase in cellular organelles shortly after germ tube emergence (Plates 7 and 17), noticeably in the stainability of mitochondria (Plate 8). Despite numerous attempts to obtain a longitudinal section through a growing hyphal tip, this was not achieved. Nevertheless, vesicles similar in appearance to those

observed in the hyphal tips of many fungi (Grove, 1978) are found scattered in the cytoplasm of mycelia at different developmental stages, particularly near the periphery (Plates 9 a,b and 24 Fig. A). Vesicles of various sizes and shapes were found throughout the sections and can be grouped in two main types:-

a) membrane bound vesicles with stainable contents (Plates 9, 11, 24 Fig. A. and 34).

b) membrane bound vesicles with non or slightly stainable contents (Plates 9, 10, 11 and 24 Fig. A).

A third type was occasionally seen. This possessed a double membrane with membranous inclusion resembling the autophagic vesicles found in the sporangiophore of this fungus (Thornton, 1968a) (Fig. A Plate 35). Cisternae and cisternal rings (Plates 10, 18, 34 and 35A) are believed to be the functional equivalent of the Golgi apparatus (Bracker, 1967) and they were found to be sparse in the cytoplasm. These cisternae and smooth endoplasmic reticulum were particularly abundant prior to the appearance of lipid droplets (Plates 10 and 24 Fig. A). This might imply their involvement in lipid synthesis in a manner resembling plant and animal cells (Novikoff & Holtzman, 1976), Lomasome-like structures were easily discernible particularly in late development (Plate 11) but sometimes similar structures were noticed in young mycelia

(arrows in Plate 18). Multivesicular structures were rarely found, between the hyphal wall and the plasma membrane (Plate 35 Fig. B).

The most striking feature closely related to the present investigation which was observed during mycelial development was in the behaviour of lipid droplets. These almost disappeared in 24 hour-old cultures but reappeared one day later (Plate 11): this was accompanied by a gradual change in the colour of mycelia from pale yellow at 24h to very bright and distinct yellow in 48 hour-old mycelia. This observation was of particular importance since the presence of ferritin was only detected in the mycelial cultures up to 18 hour-old (Plate 8) and could not be seen in older mycelia (24 hour-old) apparently due to the absence of lipid droplets. Most lipid droplets such as those seen in Plate 11 would normally be partially covered with ferritin arrays like those shown in Plate 31 if the mycelia were grown in a high iron supplemented medium. This would support the specificity of binding and elucidates the inter-relationship between the existence of lipids and the recognisable presence of ferritin in EM preparations.

2.4 The Abundance of Ferritin in Germinating Spores

David (1974) found by using labelled iron that ferritin was selectively incorporated in the sporangiospores of

Phycomyces and that ferritin iron was later released to a soluble pool during germination. This release was found to occur quicker in iron-poor spores than in iron-rich ones. This led David (1974) to conclude that ferritin-iron release was controlled by the cytoplasm of the germinating spores. It was found interesting to investigate ferritin behaviour in those spores. For this purpose iron-rich (ferritin-rich) and iron-poor (ferritin-poor) spores were obtained from cultures respectively grown on an iron supplemented medium (15 µg/ml) and one without iron supplementation. In addition spores obtained from malt extract medium, having an undetermined level of iron, were also included in this comparative study. Each of the first two types was studied in the presence of iron supplemented minimal medium and MM when iron was present only as impurity in the medium. The third type was studied in malt extract natural medium. The behaviour of ferritin in each type was studied during germination and the subsequent developmental changes up to 48 hours from heat activation.

When ferritin rich spores were germinating on high iron medium, ferritin was greatly abundant around and on lipid droplets. Some peculiar strands of ferritin were also found free in the cytoplasm (Plate 30 Fig. A). These strands were not detectable later during germination (before germ

tube emergence) and most the remaining ferritin was lipid-bound. The presence of ferritin in this pattern of germination was very frequent and lasted longer than the others (Plate 31). This ferritin was hardly detectable by 24 hour but it reappeared one day later when lipid droplets were again plentiful. A similar pattern was obtained when ferritin-rich spores were allowed to germinate on MM without iron supplement except that ferritin did not reappear abundantly at 48 hours. In ferritin-poor germinating spores in which ferritin was not very abundant it was always seen bound to lipids (Plate 30 Fig. B) whether the spores were germinating in the presence or absence of iron. In both cases it was not detectable at 24h, and it reappeared at 48h only in mycelia growing in high iron containing medium. Spores obtained from malt extract culture followed a similar pattern when germinated in natural medium but ferritin behaviour resembled that of the ferritin-rich spores (Plate 26A). Finally it was frequently observed that lipid droplets occurred partially or fully within the central vacuole as if they had been engulfed together with their bound ferritin (Plates 25, 26B).

2.5 Mitochondrial Changes

The most profound and striking feature observed during germination sequence and throughout the subsequent stages of development and differentiation was in the successive changes

in mitochondrial profiles. The consistency of these changes in the many repeated experiments has led to the belief that they might reflect respiratory states of the fungus.

In dormant spores, despite the difficulty of fixation, mitochondria appeared lobed, fairly large and contained seemingly parallel cristae (Plate 19 Fig. A). Shortly after dormant spores were heat shocked, mitochondrial profiles were of a smoother contour with organised cristae. Occasionally a whorl or dense inclusions were seen in the electron-lucent matrix (Plate 19 Fig. B). As the spherical growth proceeded, mitochondria exhibited some swelling but the general outline was maintained (Plate 20). In aldehyde fixed material, some mitochondria showed an organisation of variable electron density in their matrices (arrow in Fig. A Plate 20), this was shown in permanganate fixed material as hyaline rings (Plate 21).

At a stage prior to germ tube initiation, two types of mitochondria were commonly observed in the same section, both with aldehyde fixation (Plate 5) and permanganate (Plate 21). The main difference between them was in the density of their matrices. At the onset of germ tube formation, mitochondria were uniform in their profiles. The main character of this stage was in the uniform density of matrices of large mitochondria with smooth outlines (Plate 22). After the

protrusion of the germ tube and its subsequent outgrowth, it was noticeable that the mitochondrial population had increased. Mitochondria at this stage exhibited the typical features observed in mitochondria of growing hyphae (Grove 1978) in being longer than before, with dense matrices and organised cristae.

As hyphae differentiated further into mycelia, this was accompanied by a dramatic change in the mitochondrial population. This was manifested first in the presence of two types of mitochondria which were distinguished by their stainability and the organisation of their cristae (Plates 8 and 23 Fig. A). The climax of these mitochondrial changes occurred about 24 hours after heat activation, when nearly all the mitochondria were swollen with patchy matrices and distorted cristae (Plates 9a,b and 18). An extreme case is shown in Plate 23 Fig. B in which osmiophilic material is deposited in the intermembrane space. However, this stage was very transient because by 30 hour mitochondria appeared very abundant with characteristics resembling the ones seen in incipient hyphae in their tendency to elongation and their electron-dense matrices. At this stage some mitochondria had some variation electron-dense granules in their matrices which showed/in their electron opacity, suggesting the presence of an osmiophilic and amorphous material (Plate 24). A coincident observation at this stage was the abrupt increase in vesicles, not seen

before, which were characterized by their stainable debris-like inclusions. This deposit tended to occur around the periphery of the inner side of the vesicle delimiting membrane (Plates 10 and 24A). This last feature of mitochondria prevailed during subsequent stages of development and was seen in mycelia up to 4 days old. These mitochondrial changes were also studied in germinating spores and growing mycelia in a different nutritional medium and found to follow closely similar patterns (Plates 25-29).

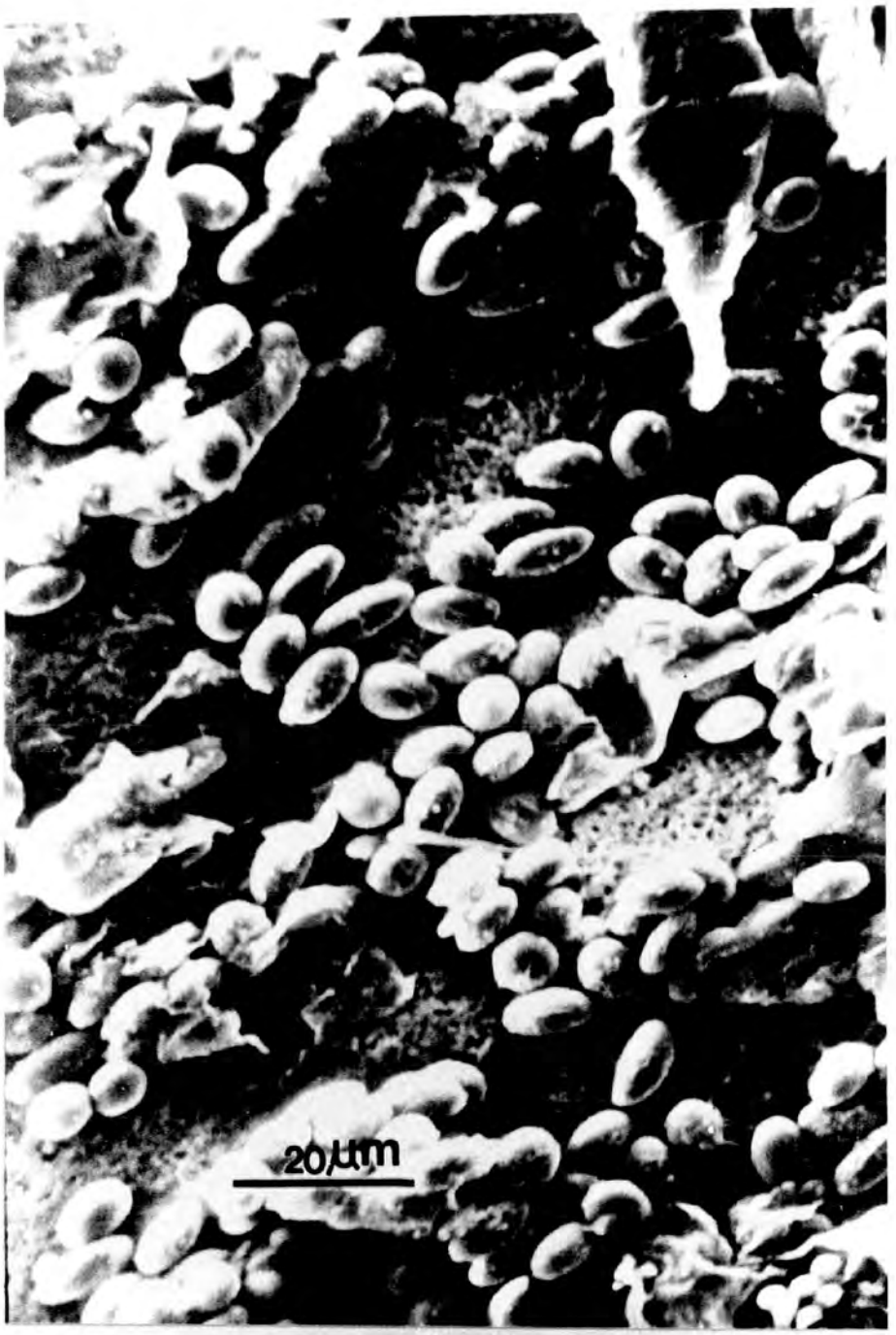


Plate 1. Scanning electron micrograph of permanganate fixed dormant spores.

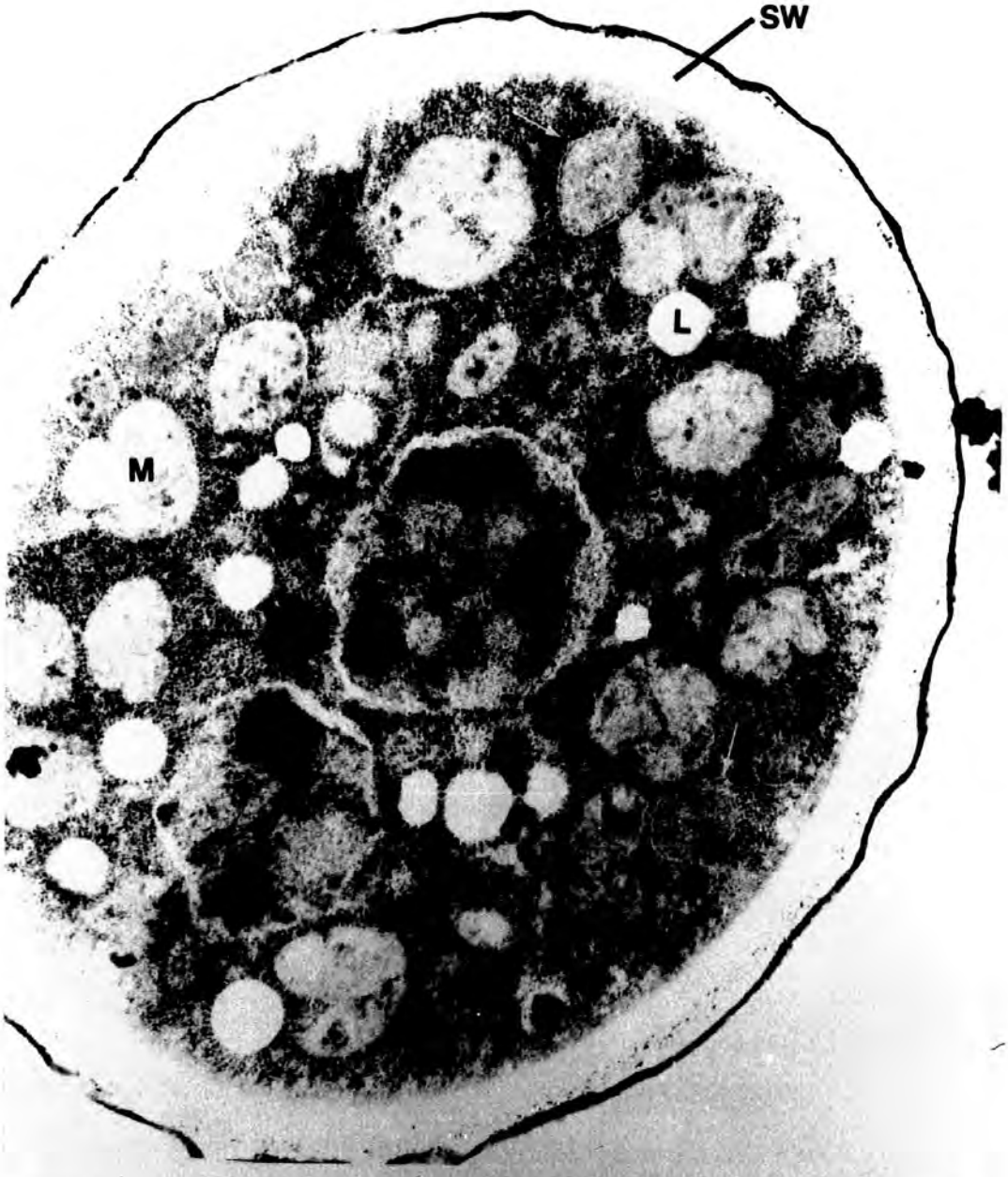


Plate 2a. Dormant spore. Glutaraldehyde/ formaldehyde-osmium fixation. X 11,735.

Plate 2b. Dormant spore. Glutaraldehyde-osmium fixation.
X 28,000.

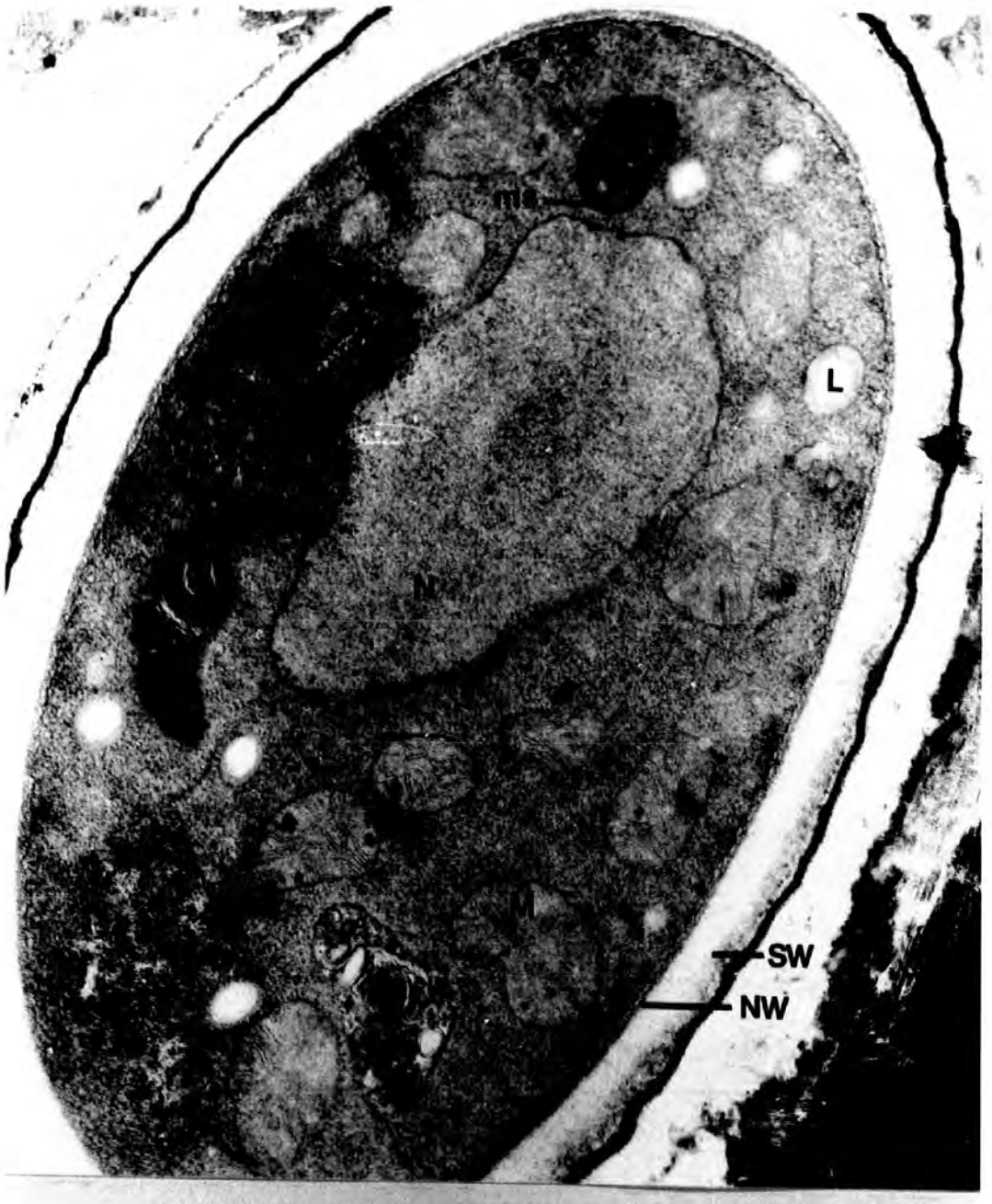


Plate 3. Germinating spore, one hour after heat shock. Glutaraldehyde-osmium fixation. X 28,000.

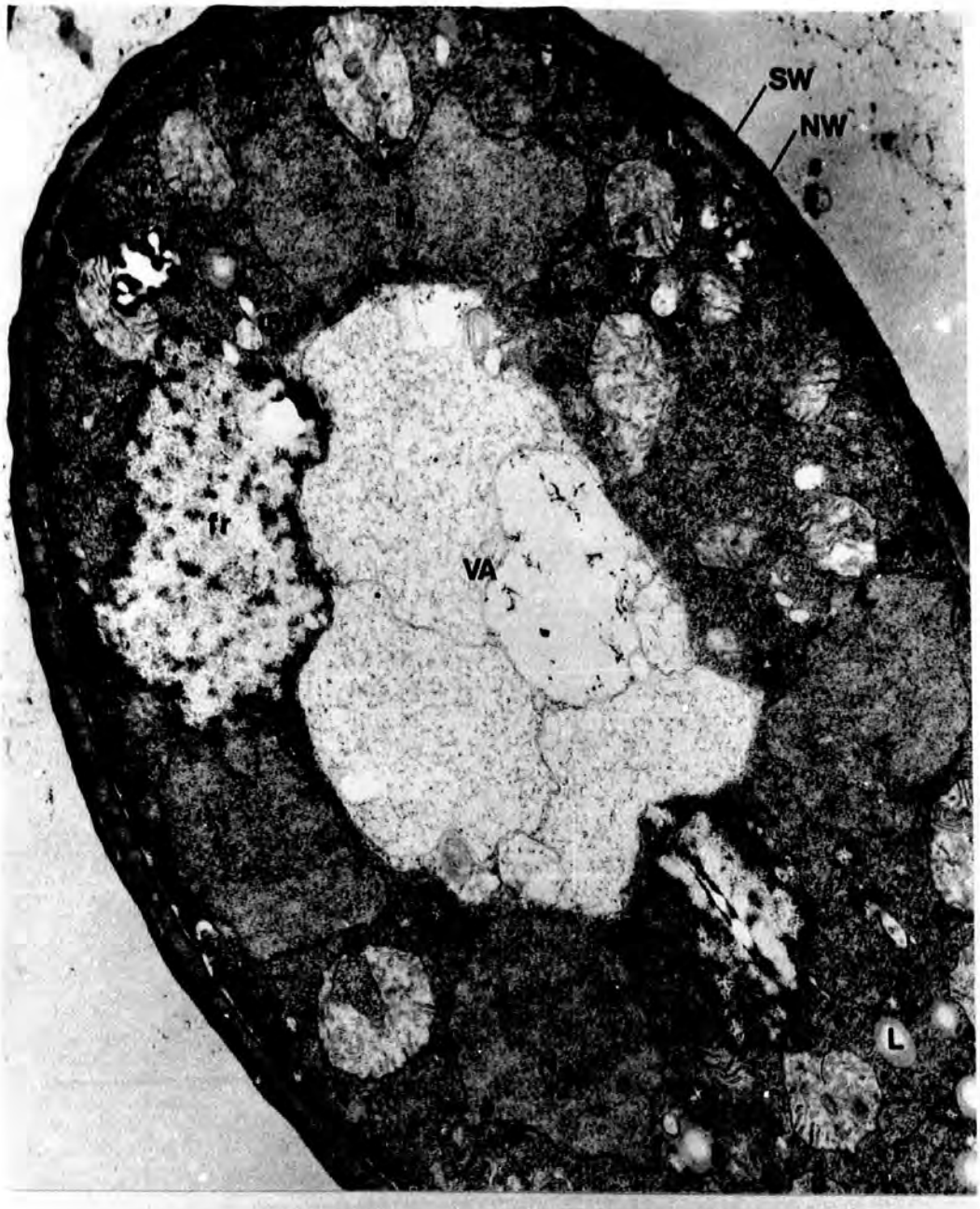


Plate 4. Germinating spore, 4 hours after heat shock. Glutaraldehyde-osmium fixation. X 19,000.

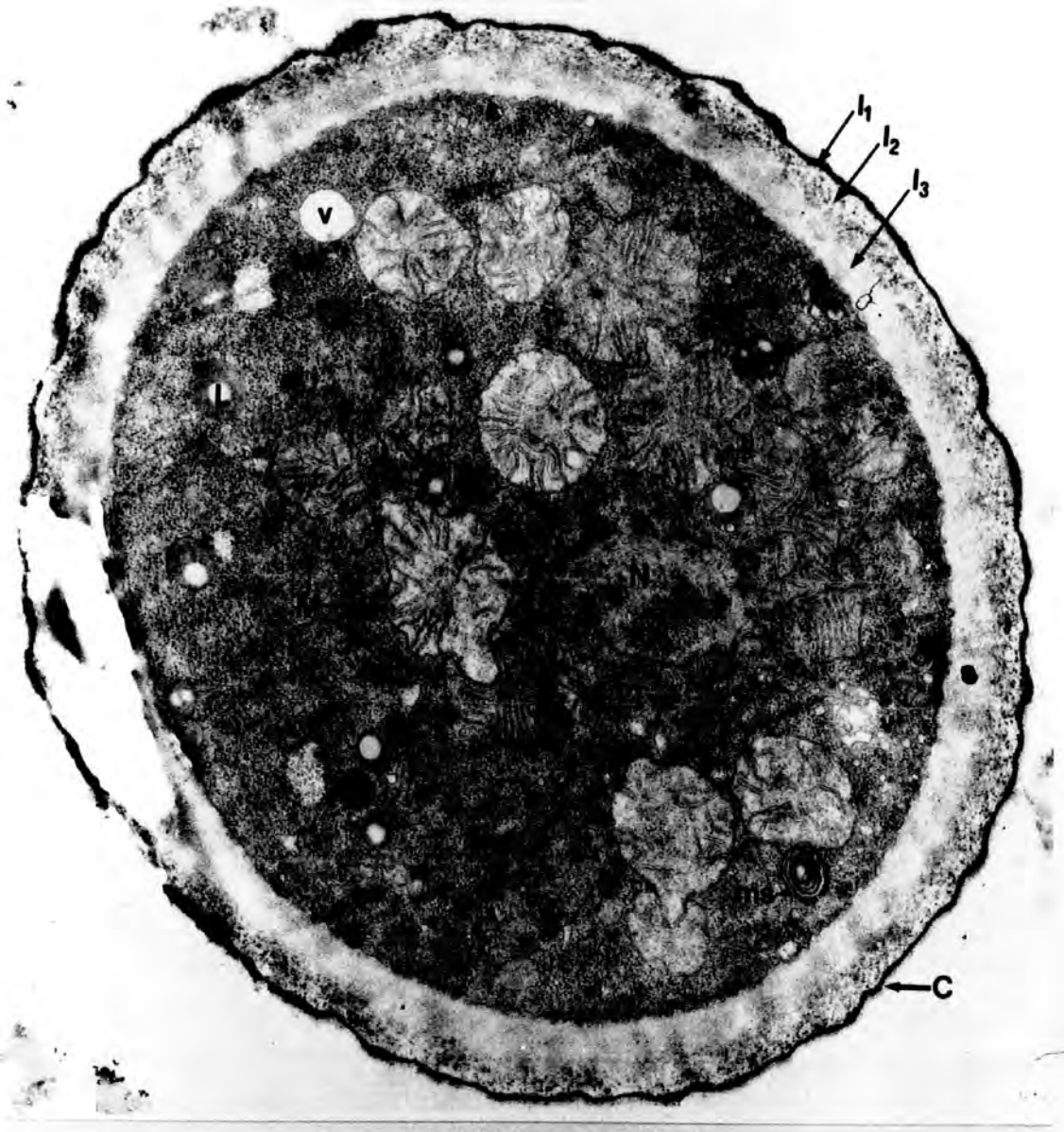


Plate 5. Germinating spore, 6 hours after heat shock. Glutaraldehyde-osmium fixation. X 24,000.

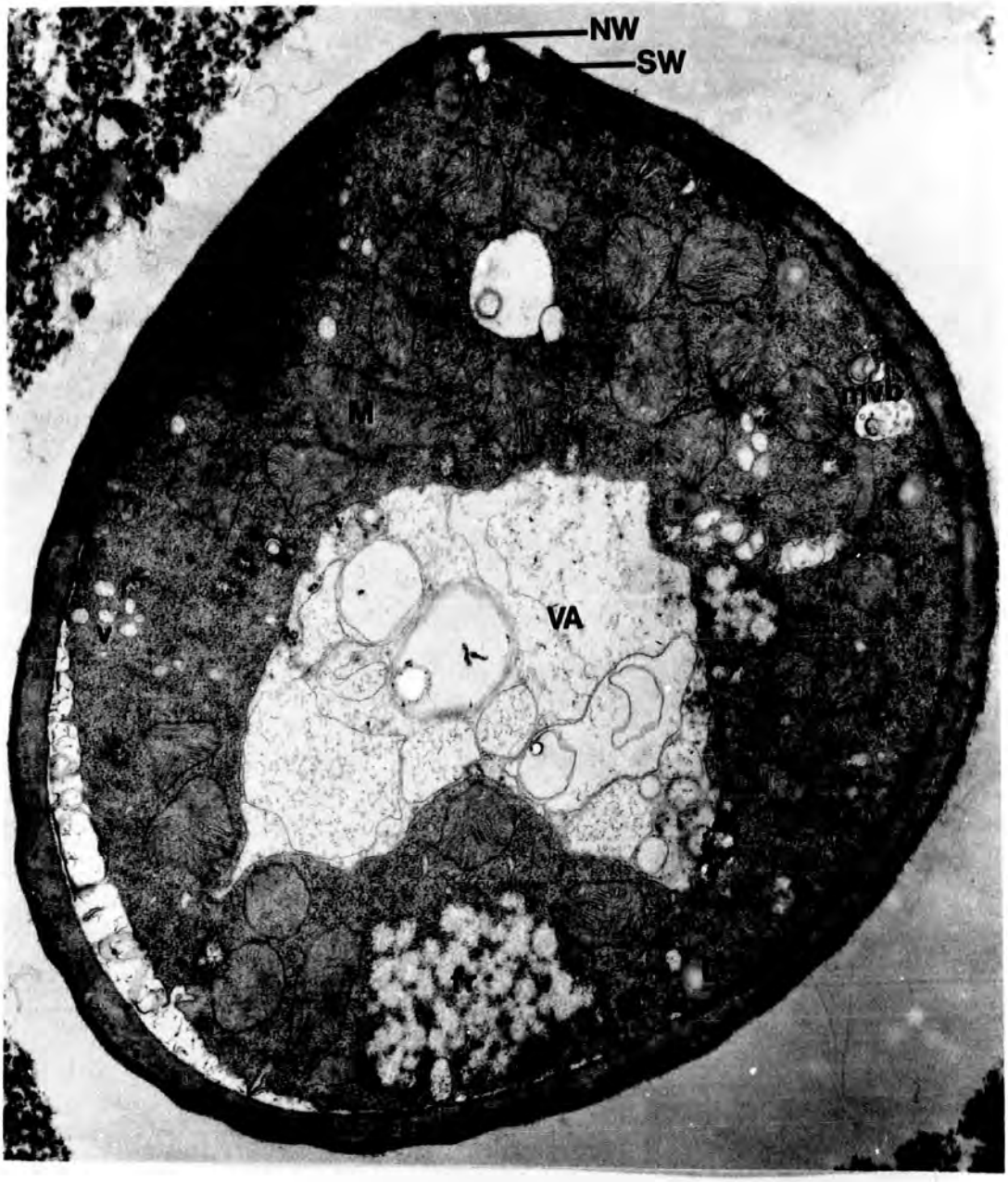


Plate 6. Germinating spore, 8 hours after heat shock. Glutaraldehyde-osmium fixation. X 20,600.

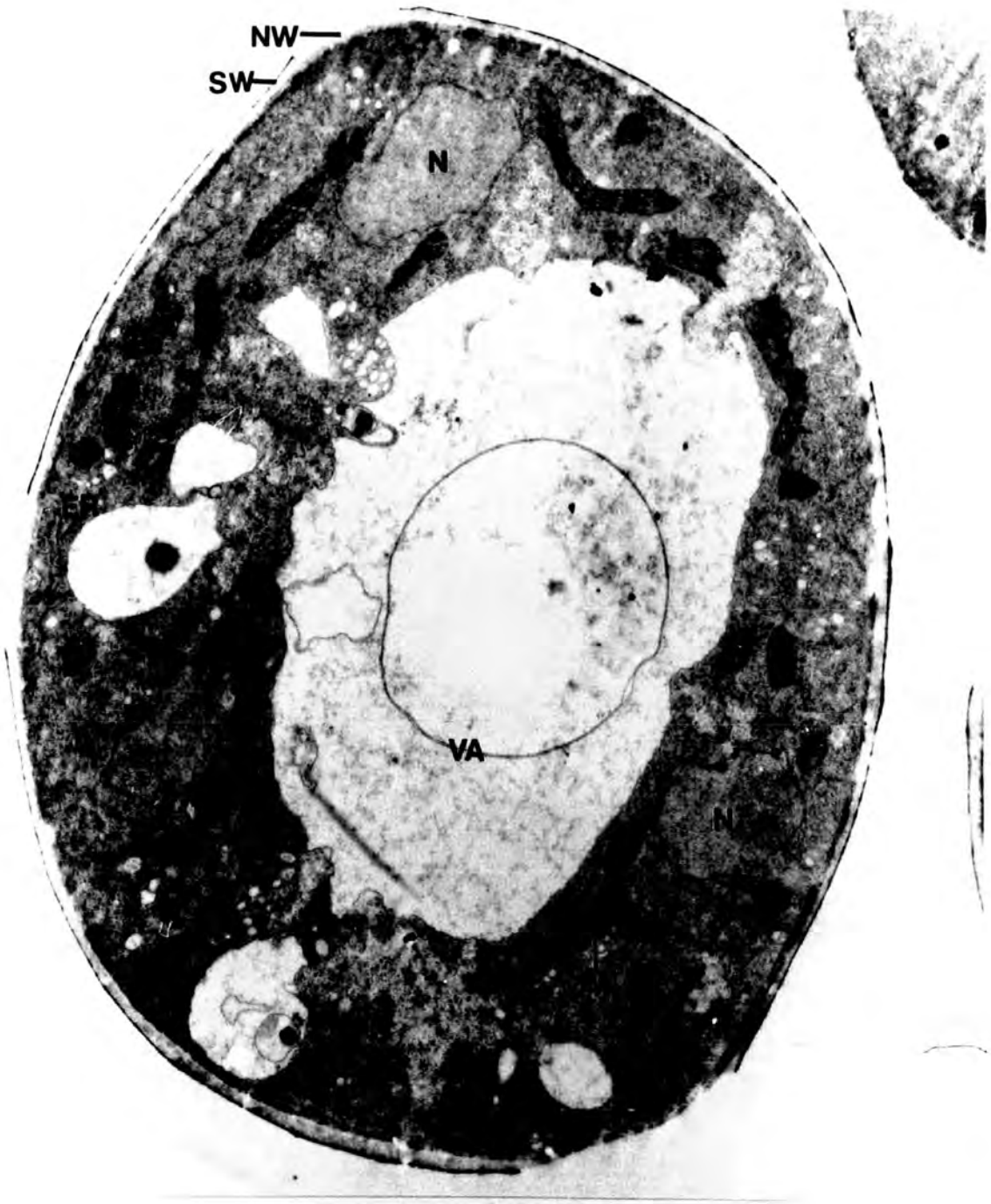


Plate 7. Germinating spore (germling), 12 hours after heat shock. Glutaraldehyde-osmium fixation.
X 11,680.

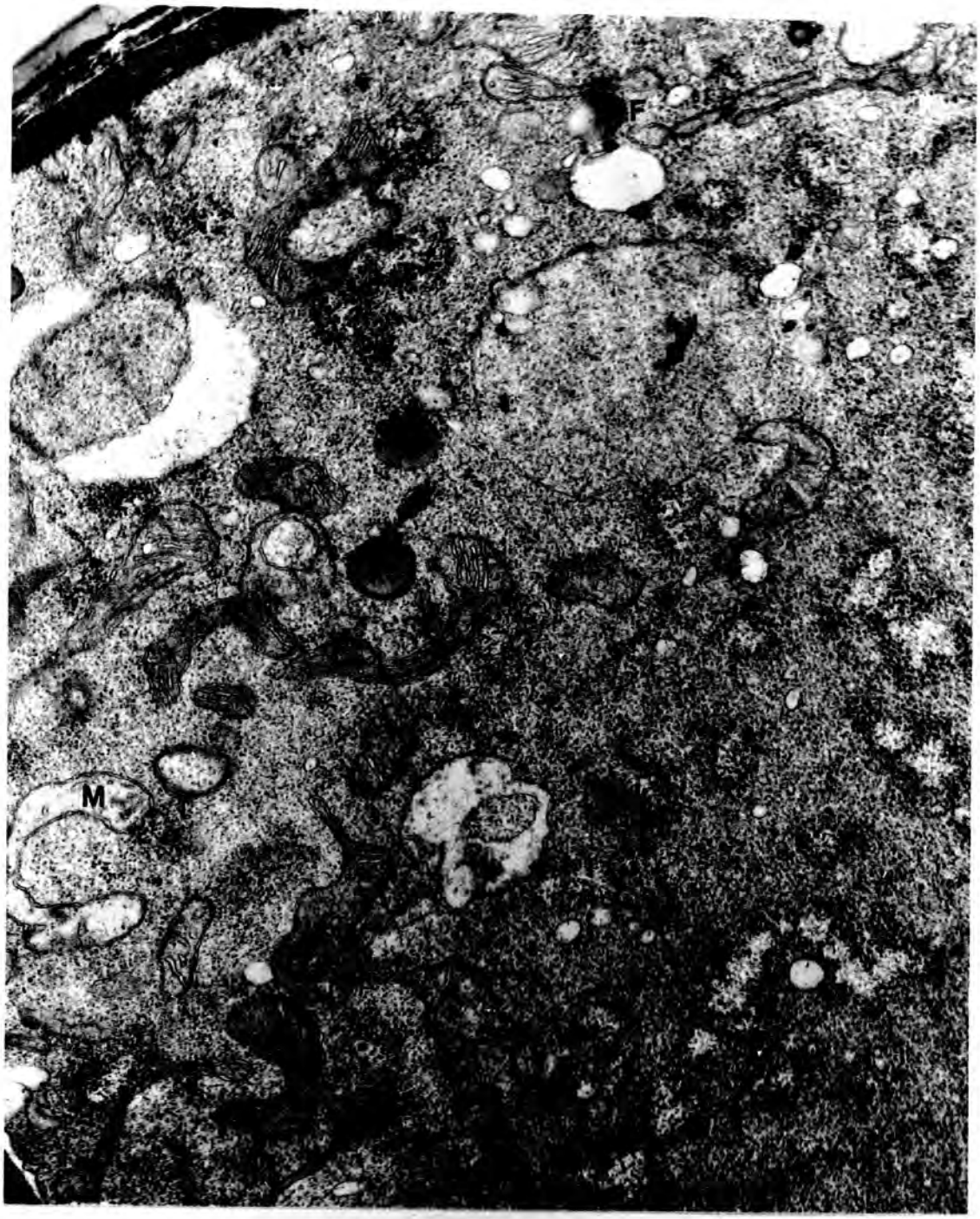


Plate 8. Section through a hypha, 18 hours after heat shock. Glutaraldehyde-osmium fixation.
X 28,780.

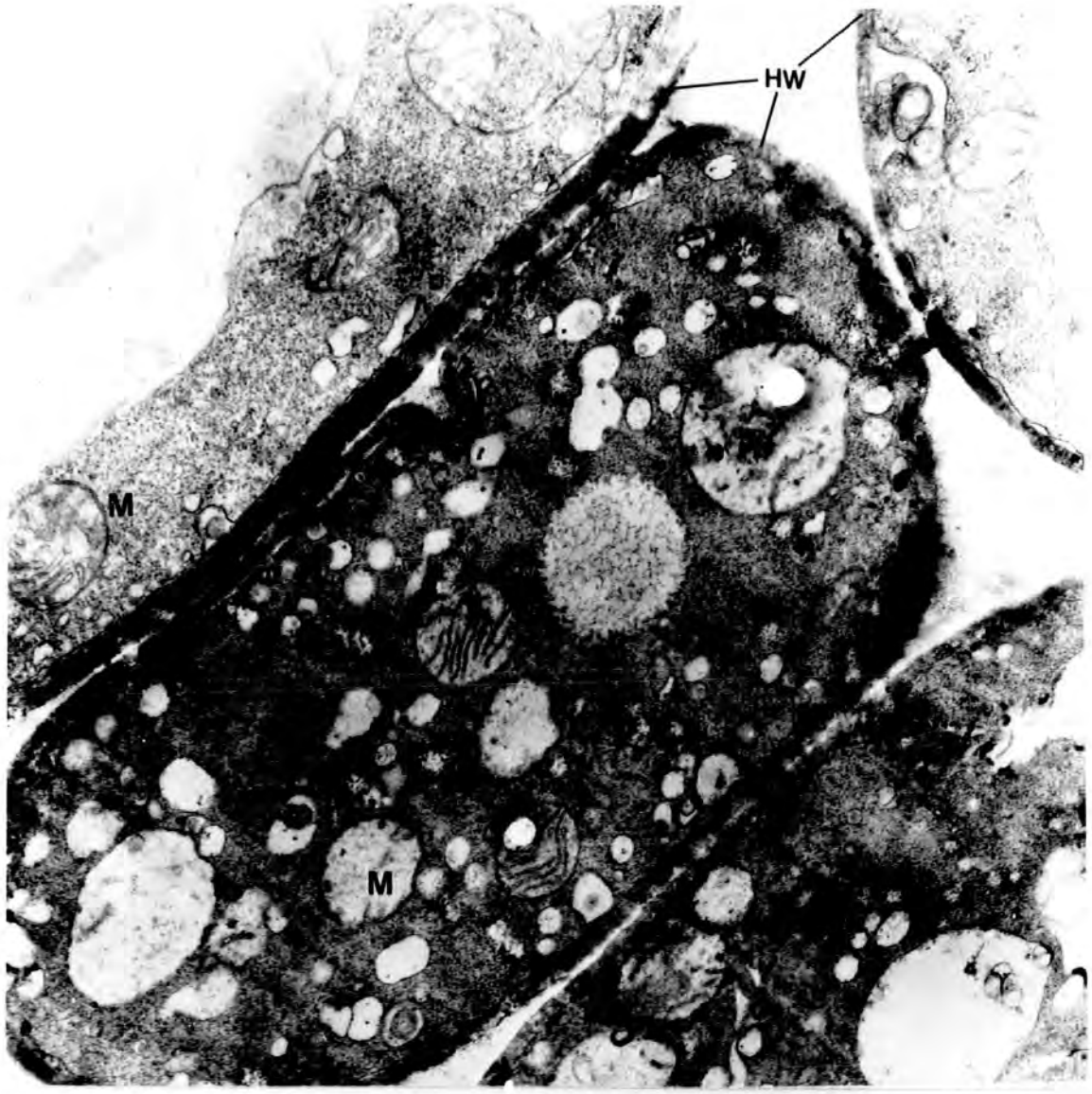


Plate 9a. Section through hyphae, 24 hours after heat shock. Glutaraldehyde/formaldehyde-osmium fixation.
X 22,830.

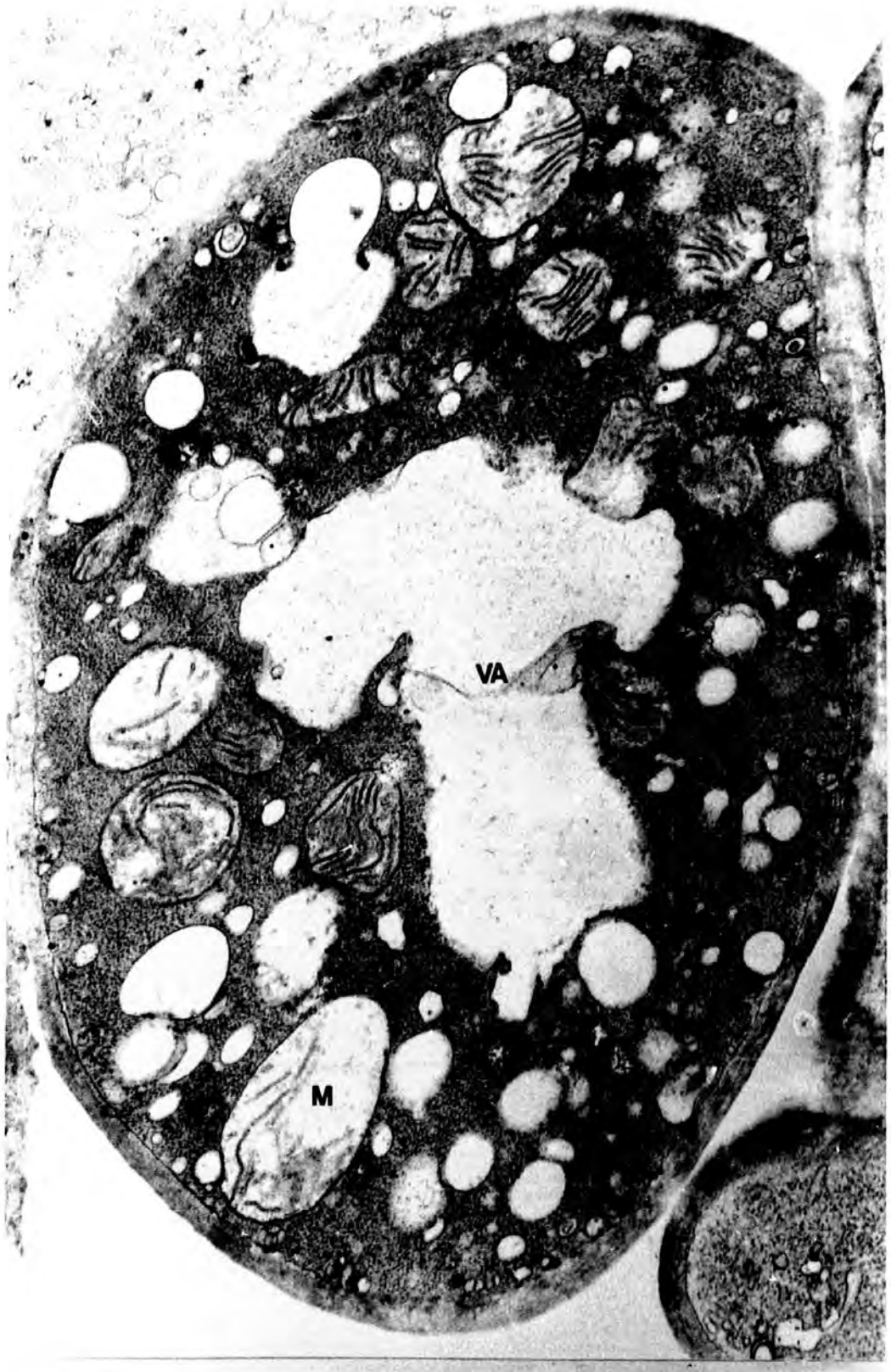


Plate 9b. Section through a hypha, 24 hours after heat shock. Glutaraldehyde-osmium fixation.
X 21,200.

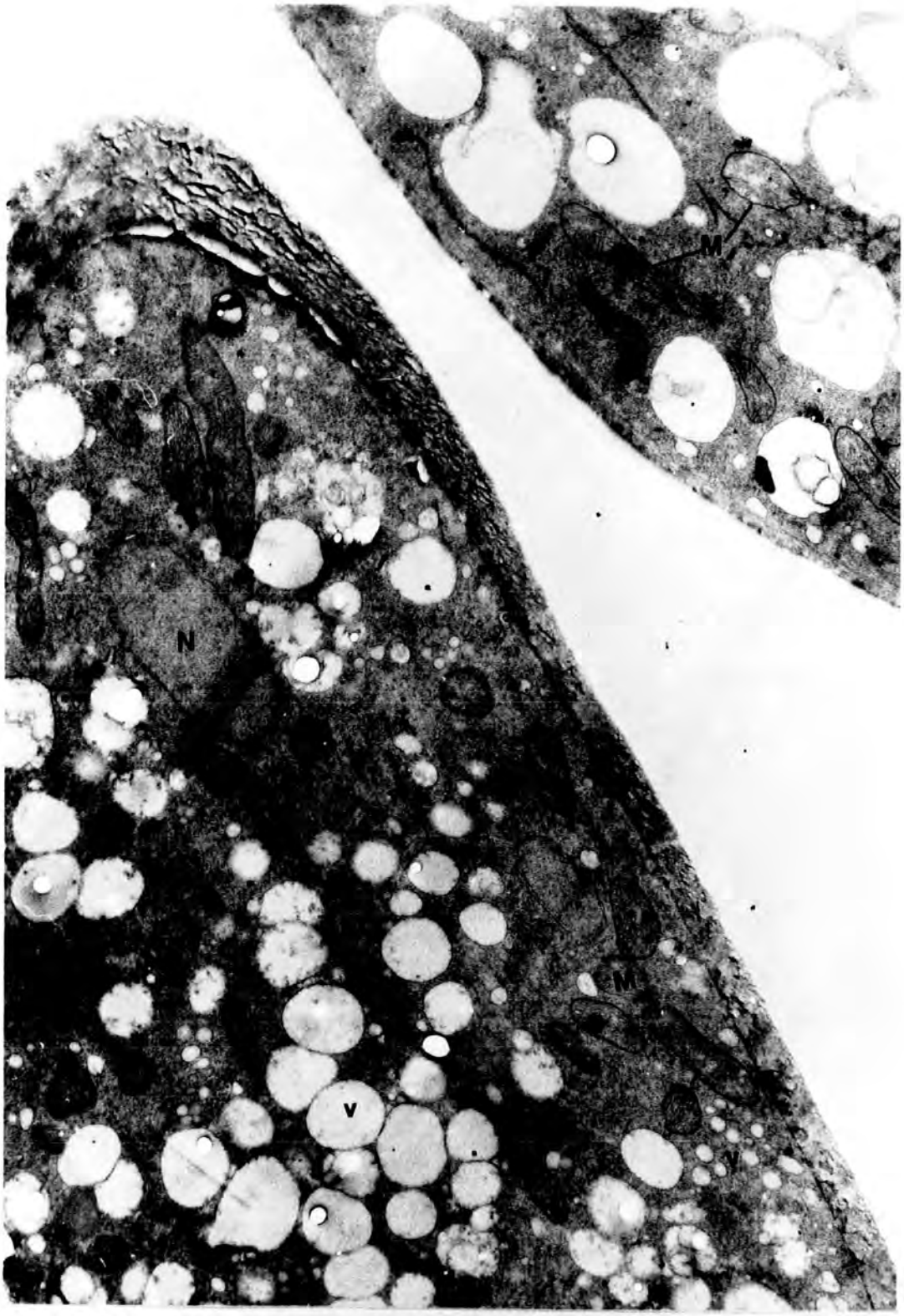


Plate 10. Section through hyphae, 36 hours after heat shock. Glutaraldehyde-osmium fixation.
X 18,000.

Plate 11. Section through a hypha, 48 hours after heat shock. Glutaraldehyde/formaldehyde-osmium fixation.
X 17,500.

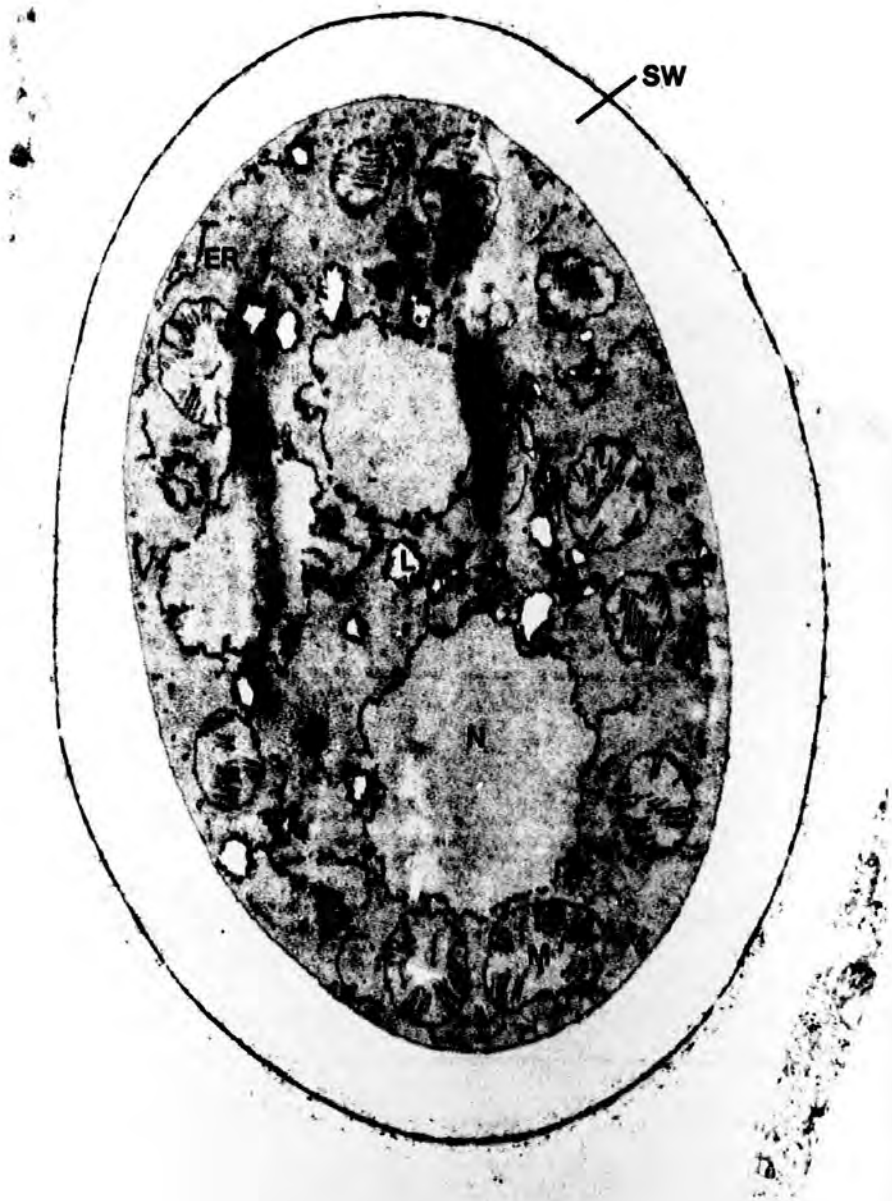


Plate 12. Germinating spore, one hour after heat shock. Permanganate fixation. X 20,675.

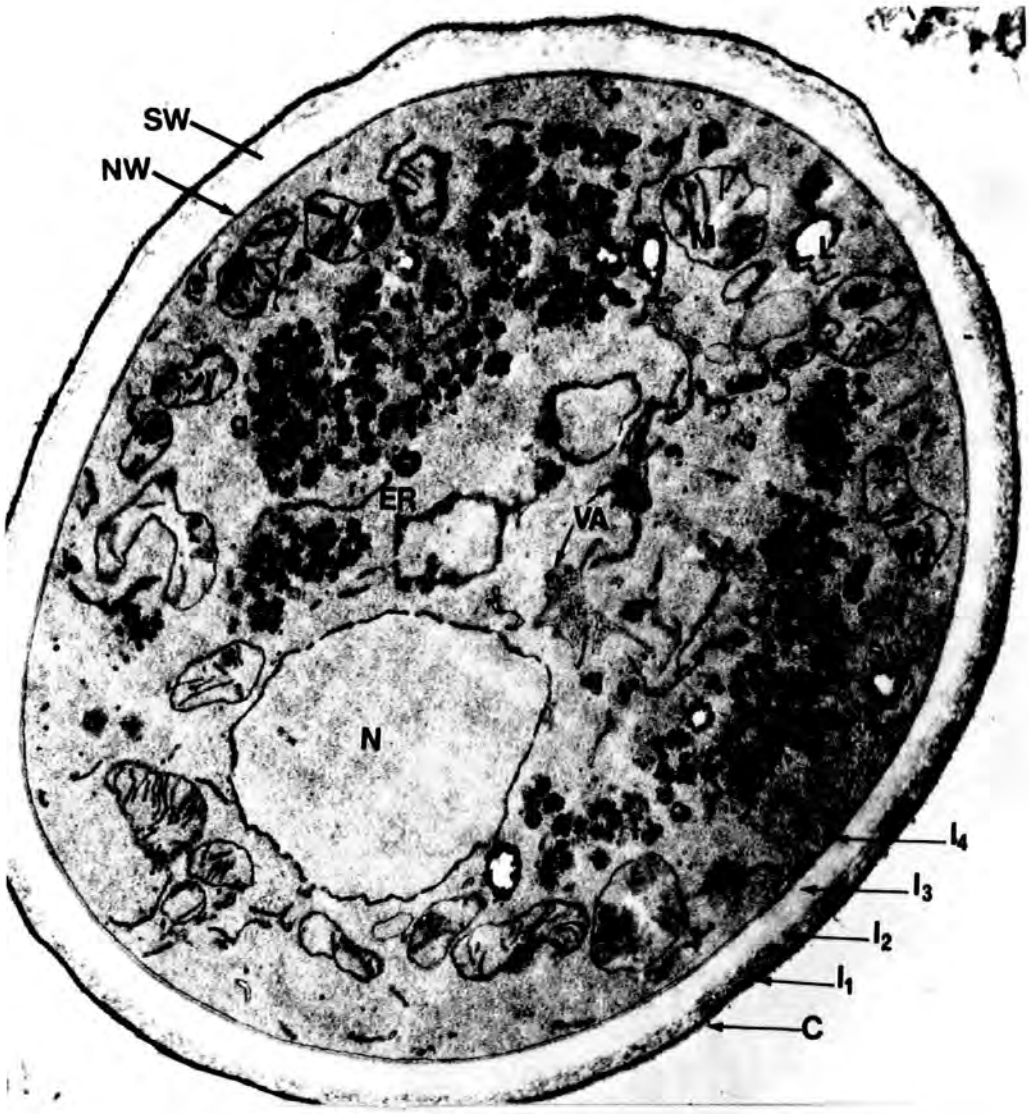


Plate 13. Germinating spore, 4 hours after heat shock. Permanganate fixation. X 17,000.

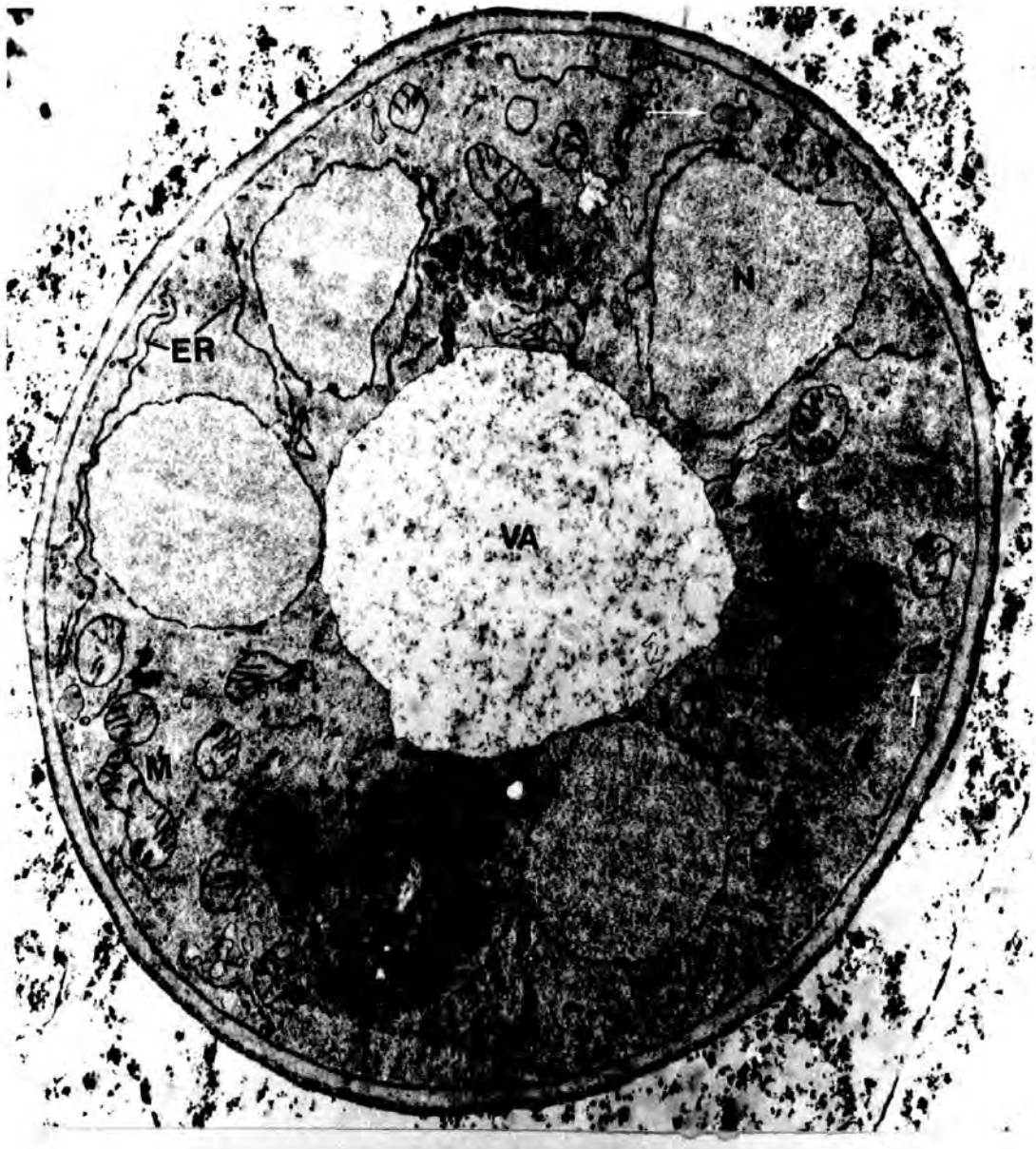


Plate 14. Germinating spore, 6 hours after heat shock. Permanganate fixation. X 14,700.

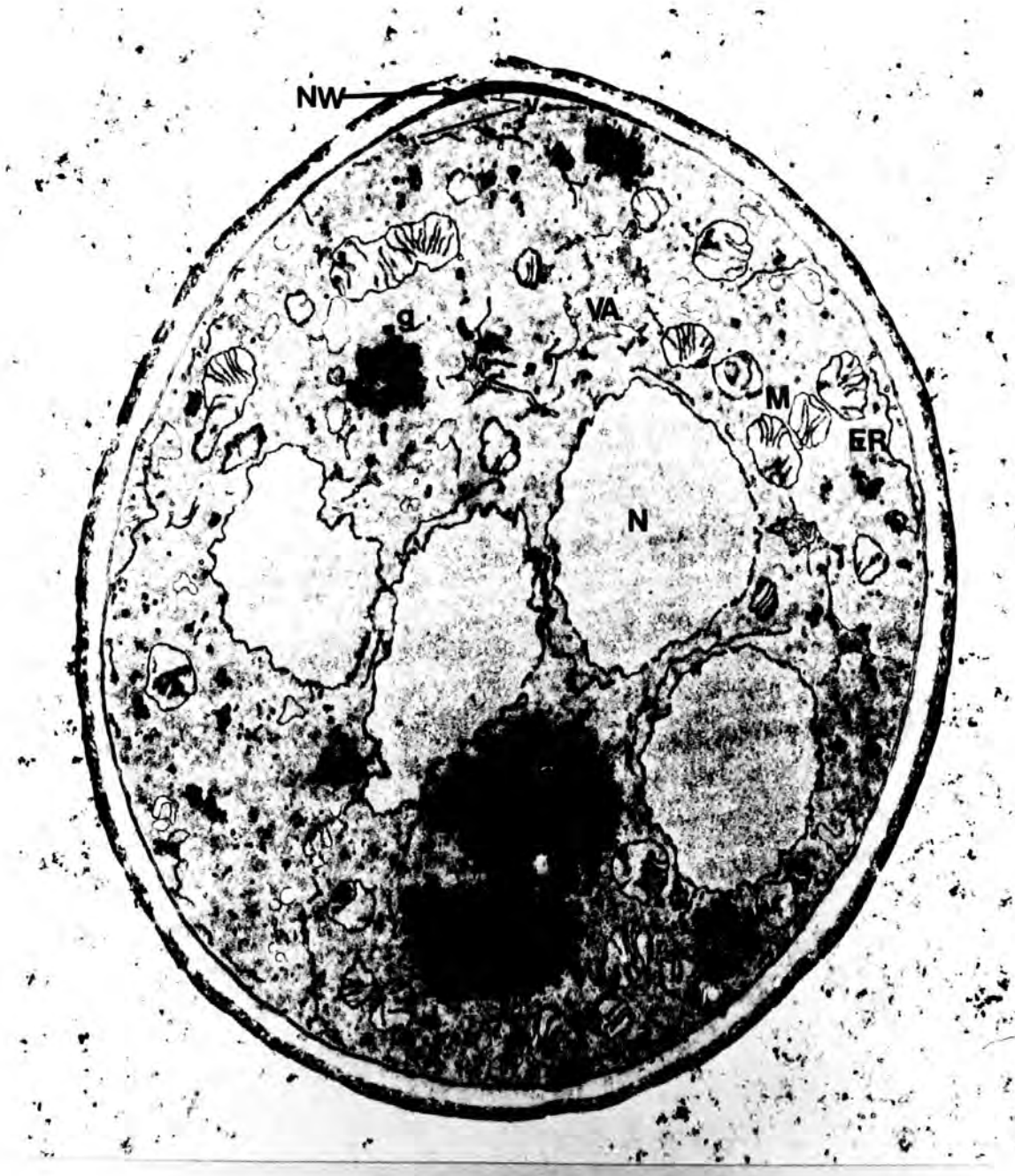


Plate 15. Germinating spore, 8 hours after heat shock. Permanganate fixation. X 15,590.

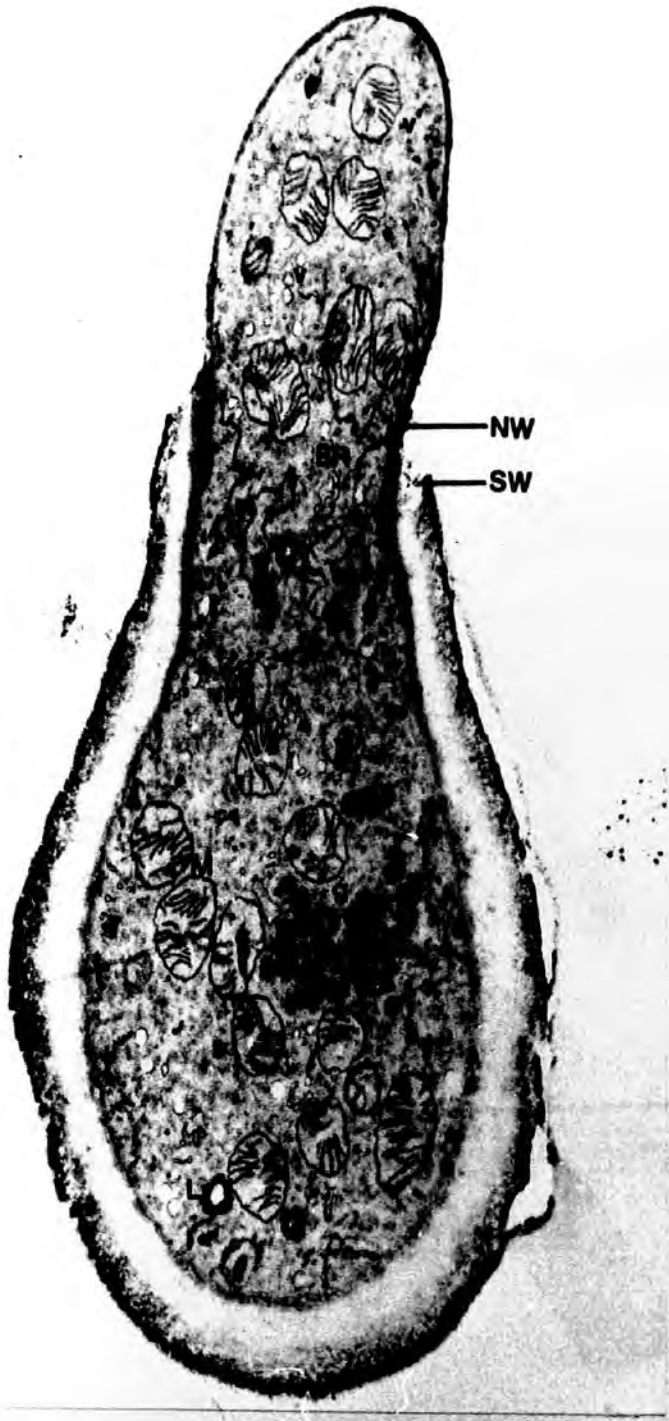


Plate 16. Germinating spore (germling), 8 hours after heat shock. Permanganate fixation.
X 8,680.

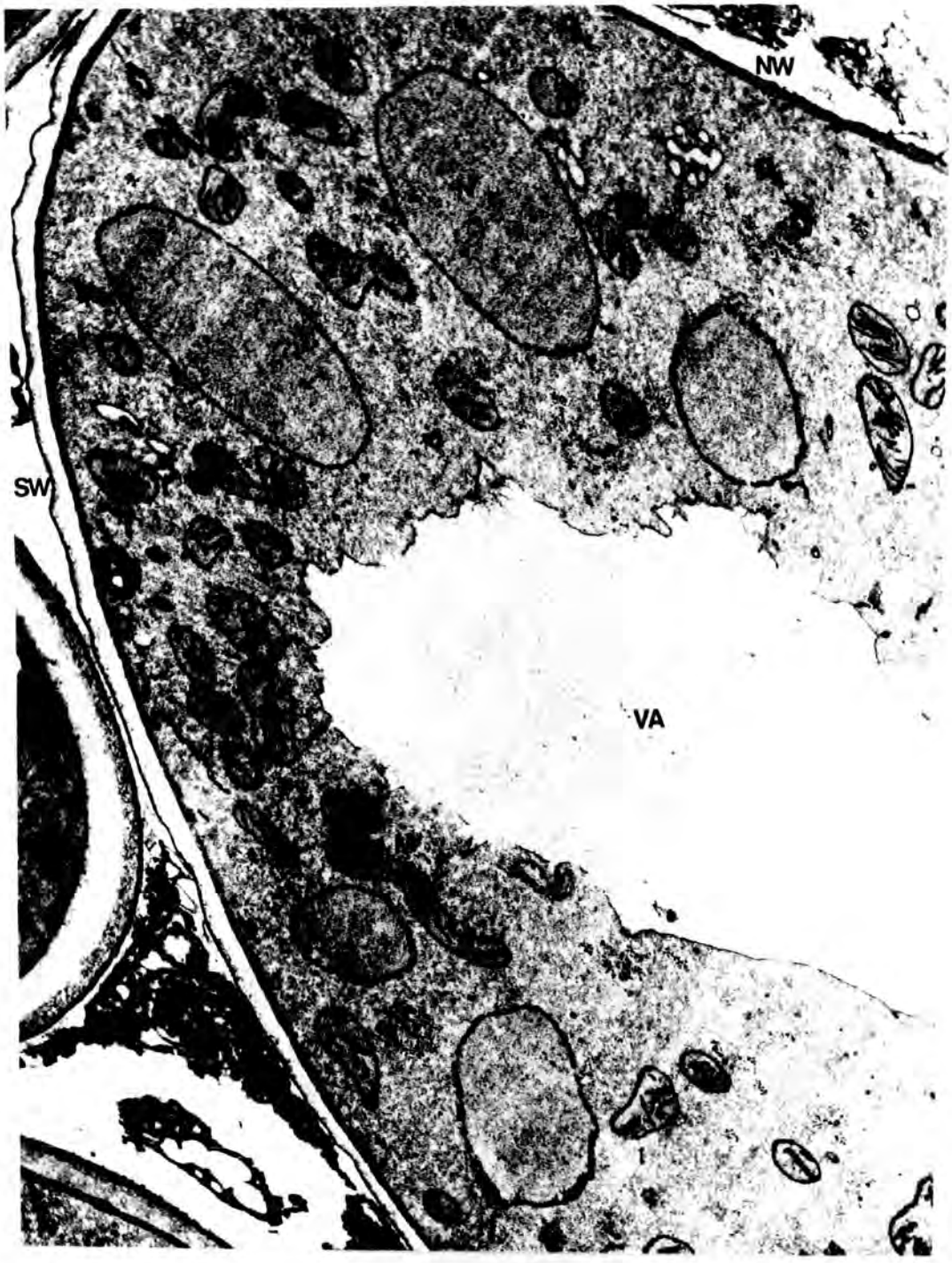


Plate 17. Section through a germling, 12 hours after heat shock. Permanganate fixation.
X 19,775.

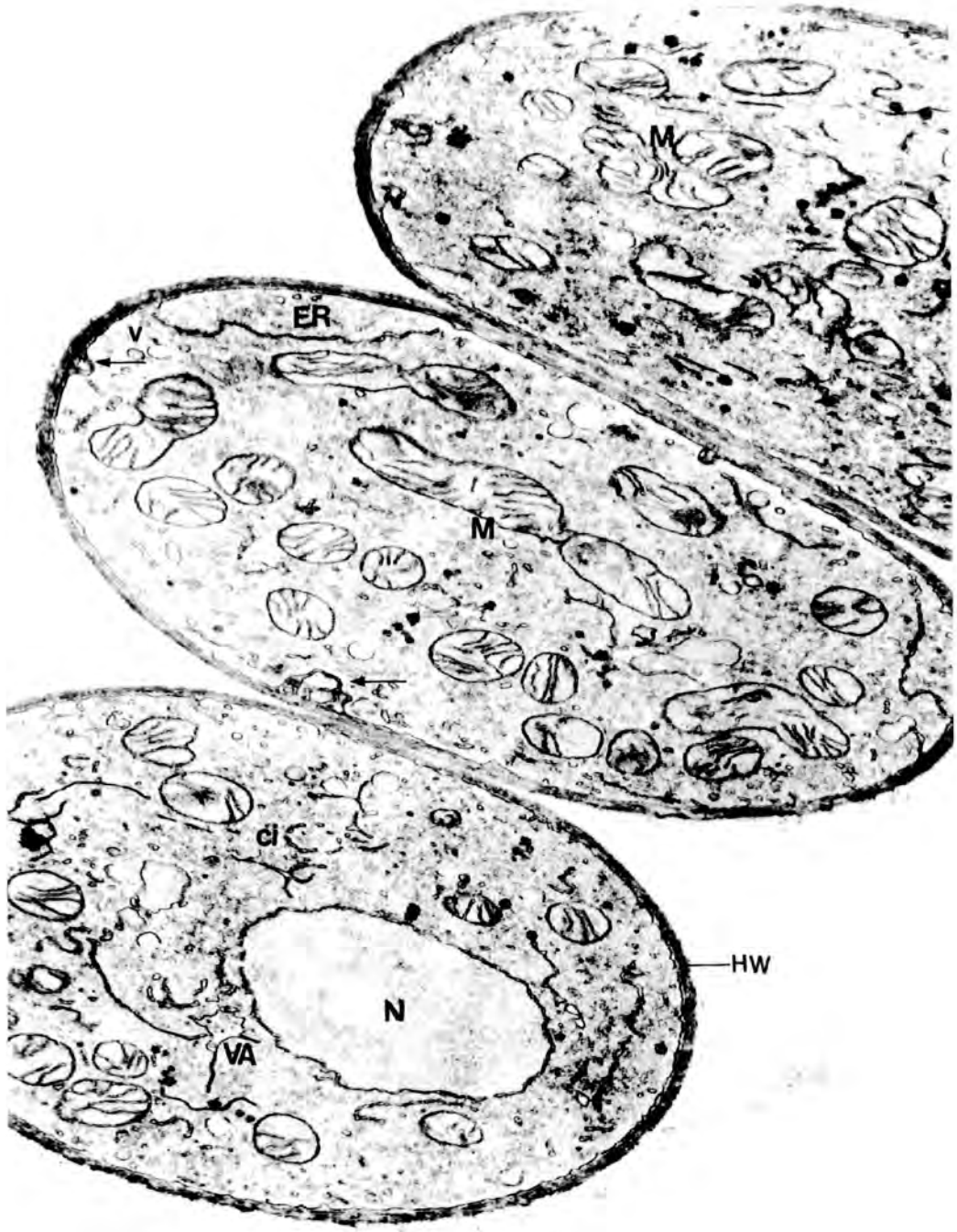


Plate 18. Section of hyphae, 24 hours after heat shock. Permanganate fixation. X 28,000.

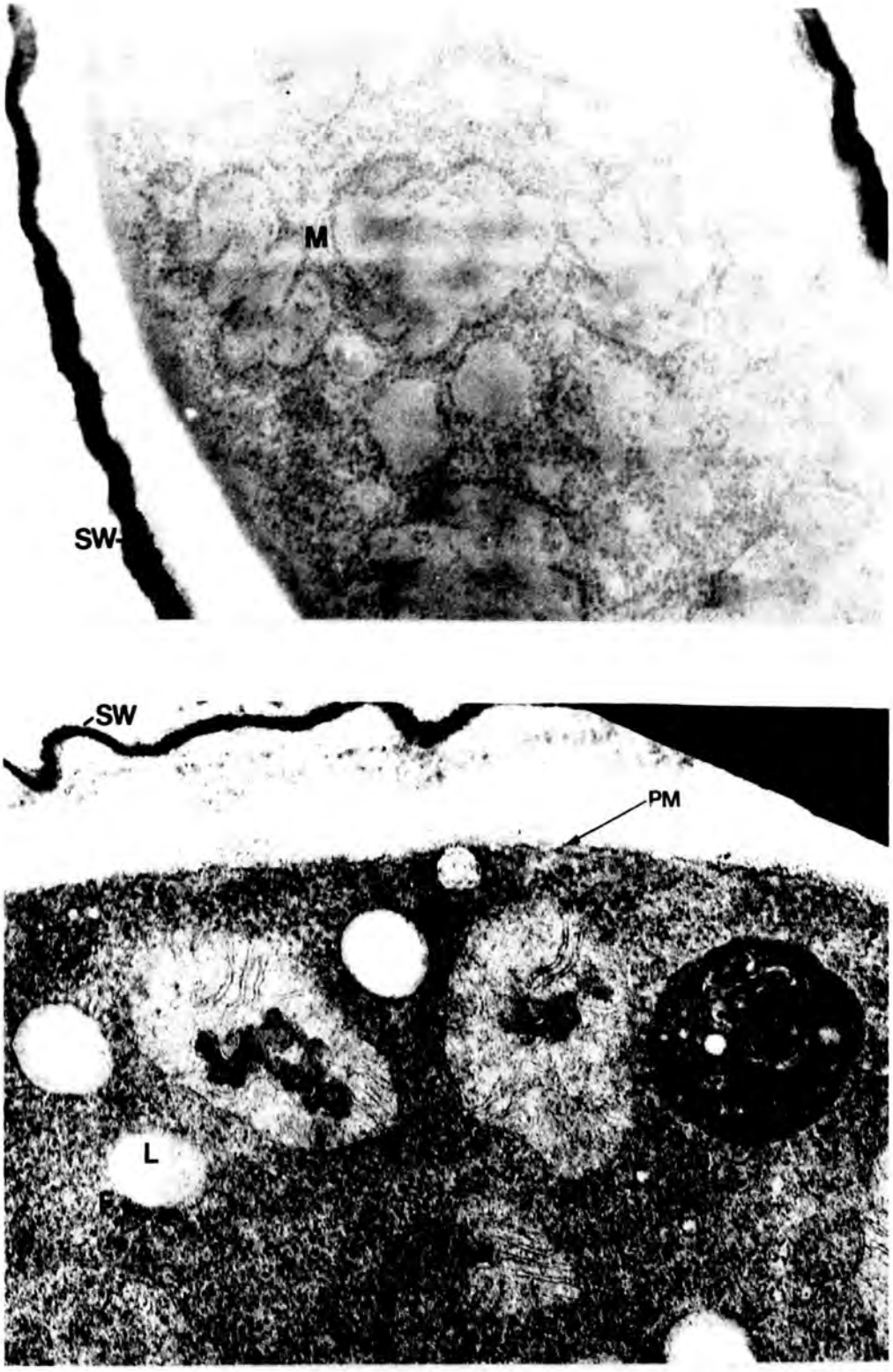


Plate 19.

Fig. A) Higher magnification of a dormant spore. X 45,000.

Fig. B) Higher magnification of germinating spore, one hour after heat shock. X 58,226. Glutaraldehyde-osmium fixation.

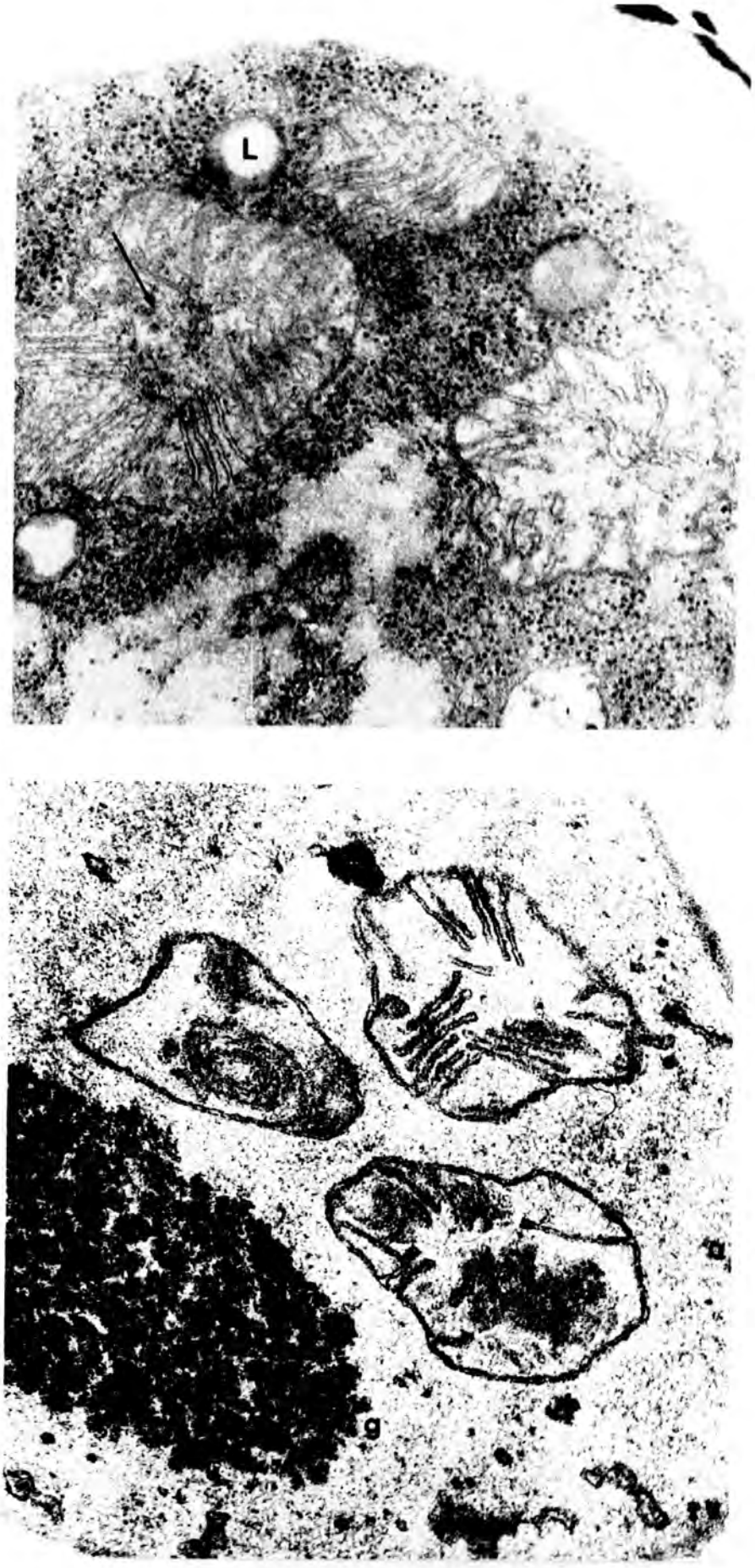


Plate 20. Higher magnification of germinating spores, 4 hours after heat shock.

Fig. A) Glutaraldehyde-osmium fixation. X 48,500.

Fig. B) Permanganate fixation. X 52,930.

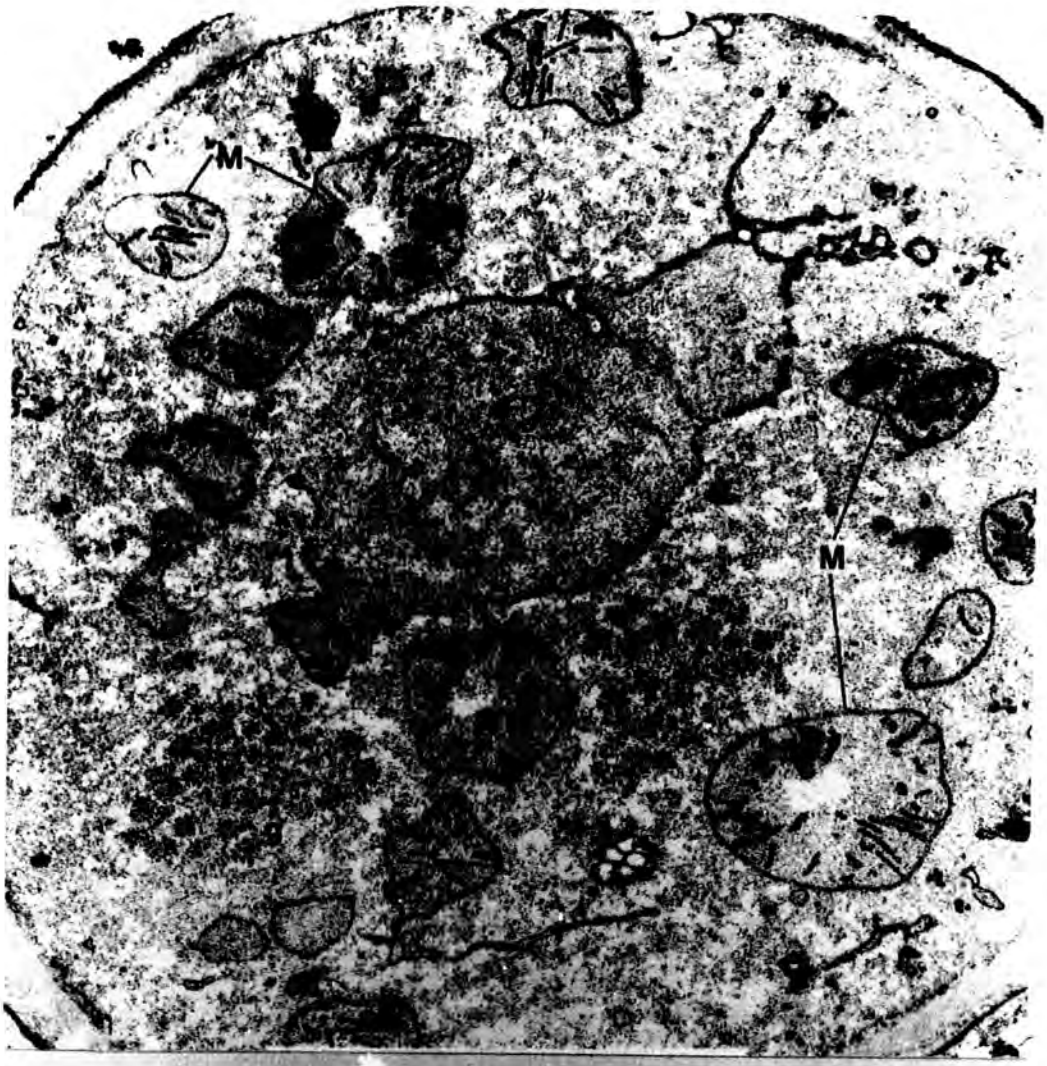


Plate 21. Section through a germinating spore, 6 hours after heat shock. Permanganate fixation.
X 21,850.

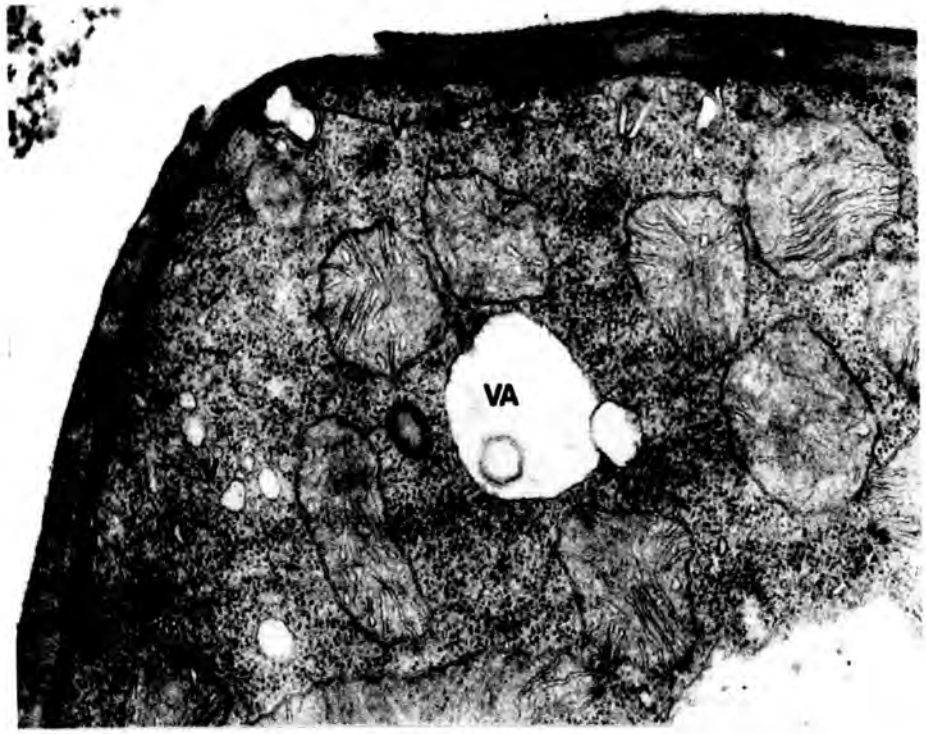


Plate 22. Higher magnification of germinating spores, 8 hours after heat shock.

Fig. A) Glutaraldehyde-osmium fixation. X 35,400.

Fig. B) Permanganate fixation. X 14,930.

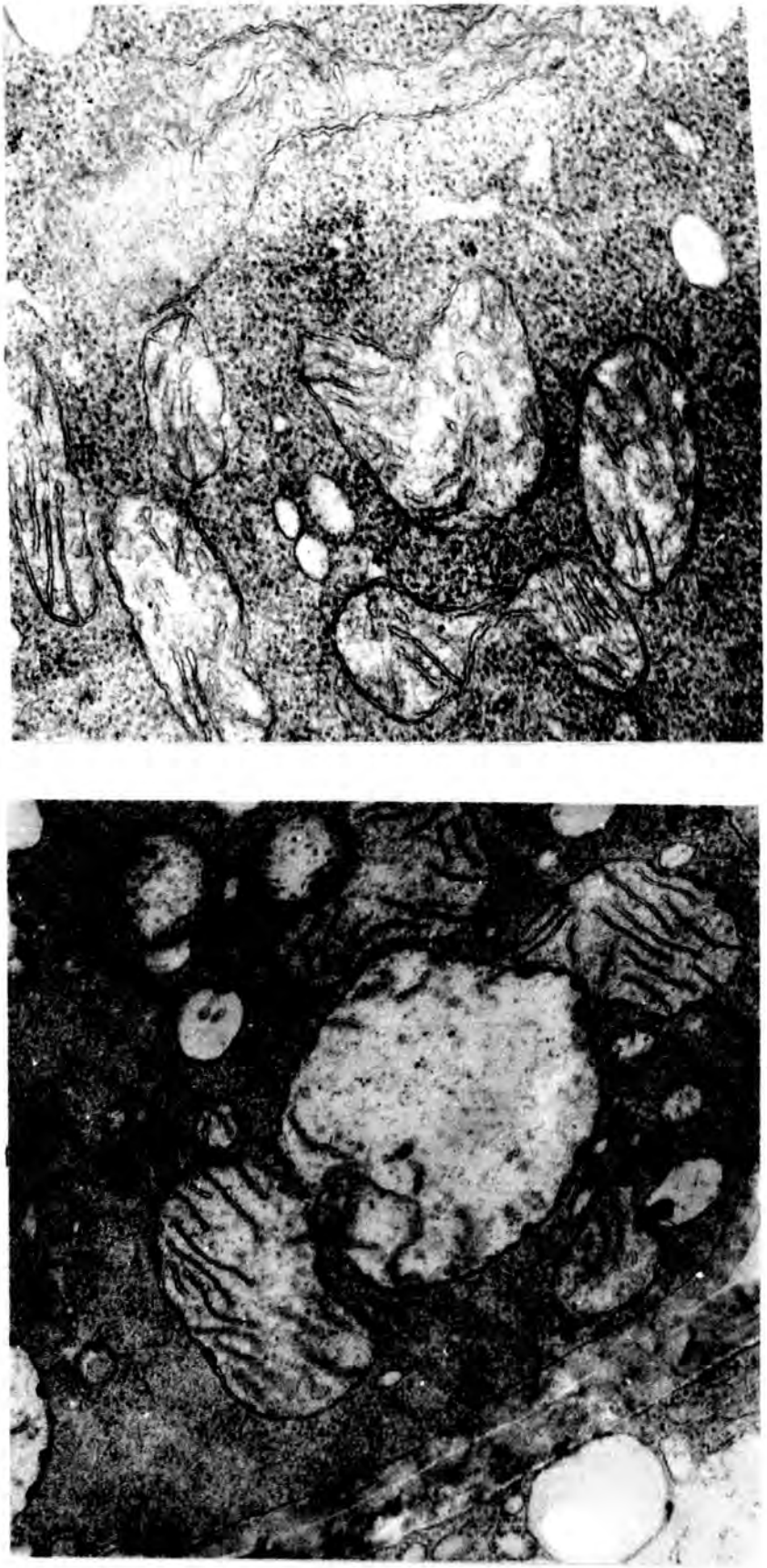
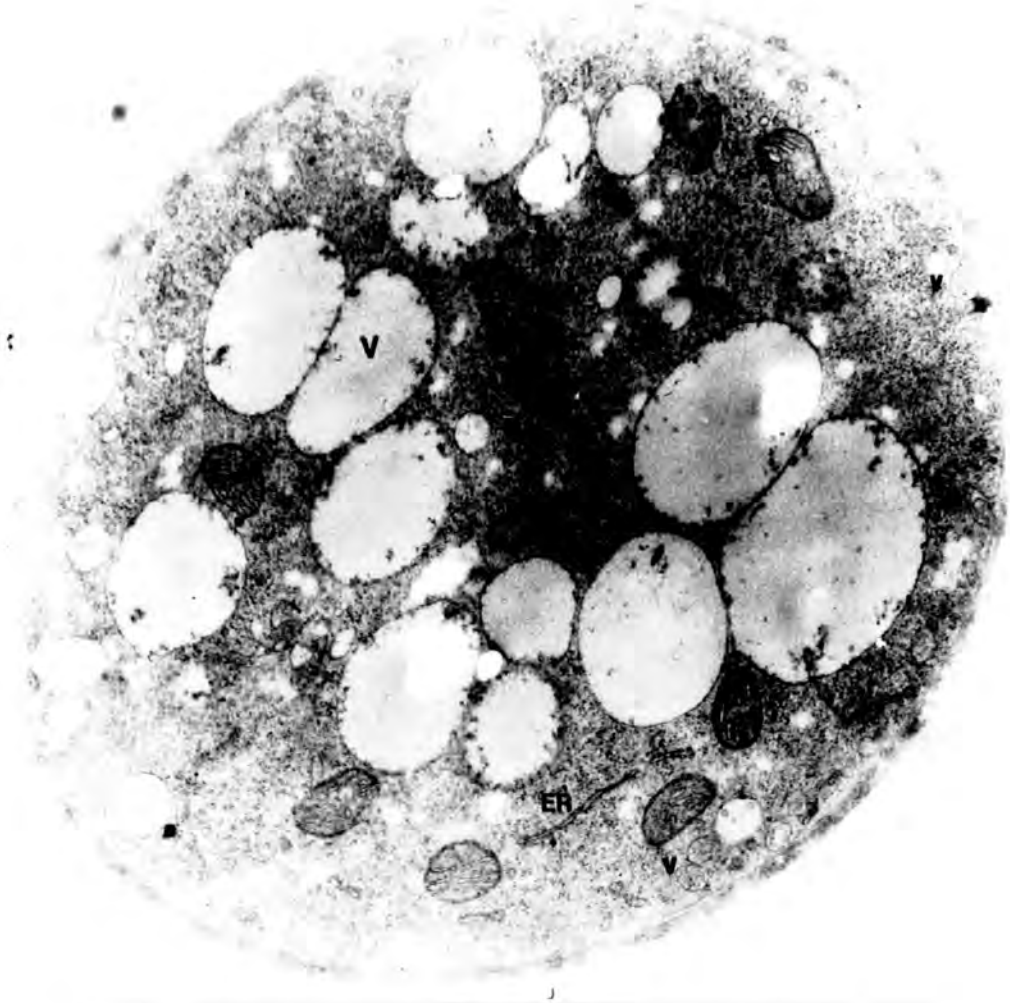


Plate 23. Higher magnification sections of hyphae.

Fig. A) 18 hours after heat shock. X 48,500

Fig. B) 24 hours after heat shock. X 49,600
Glutaraldehyde-osmium fixation.



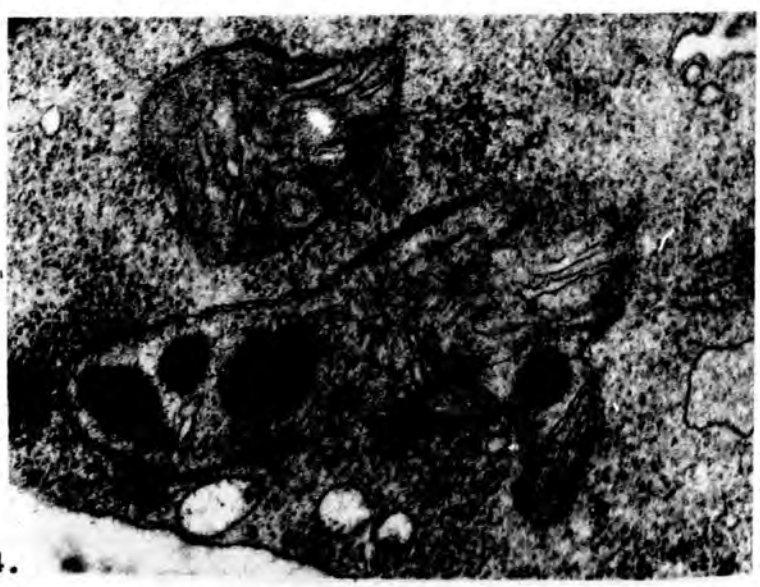


Plate 24.

Fig. A) Section through a hypha, 36 hours after heat shock. Glutaraldehyde/formaldehyde-osmium fixation X 11,400.

Fig. B) Higher magnification of a similar hypha. Glutaraldehyde-osmium fixation. X 50,600.

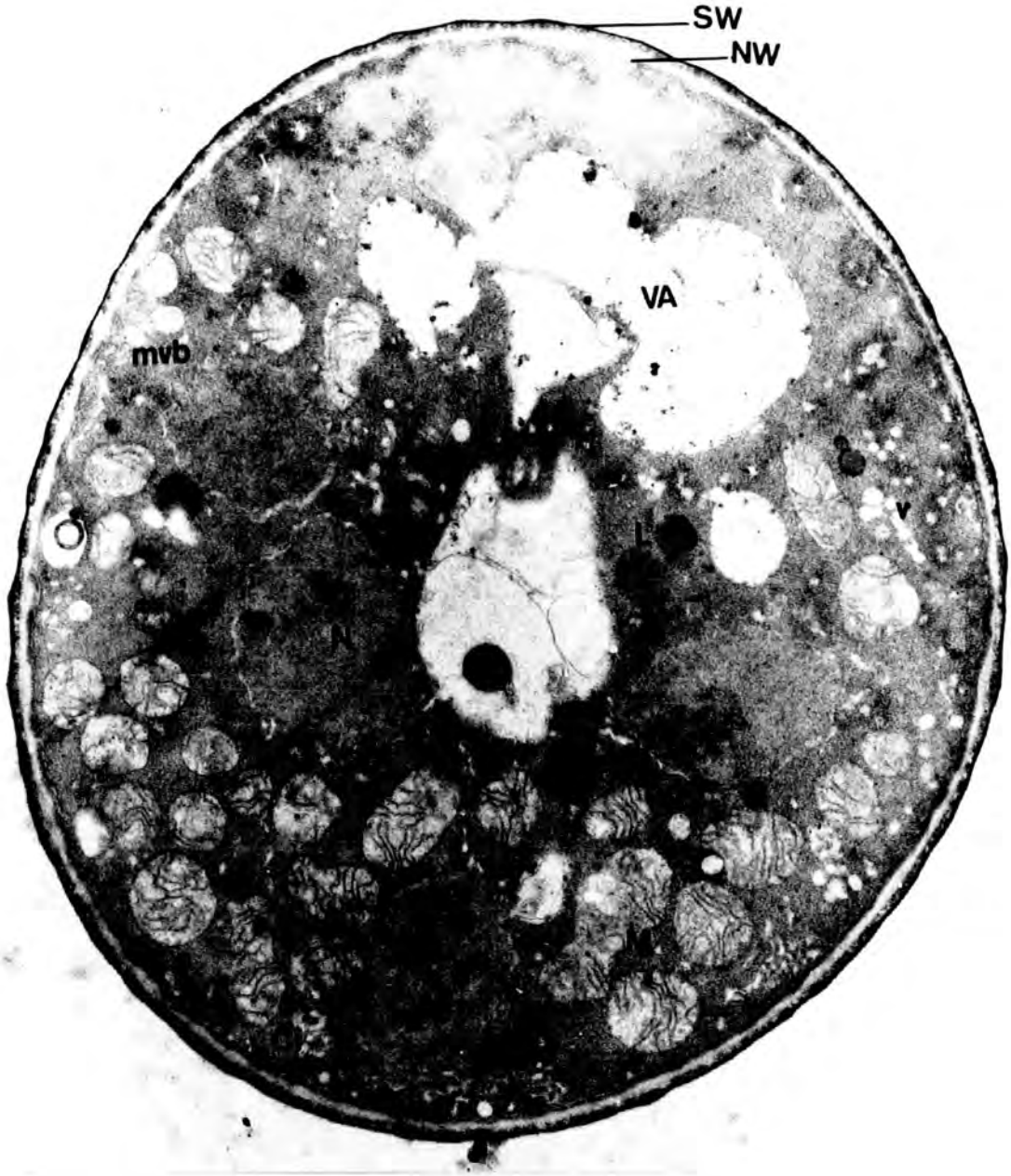
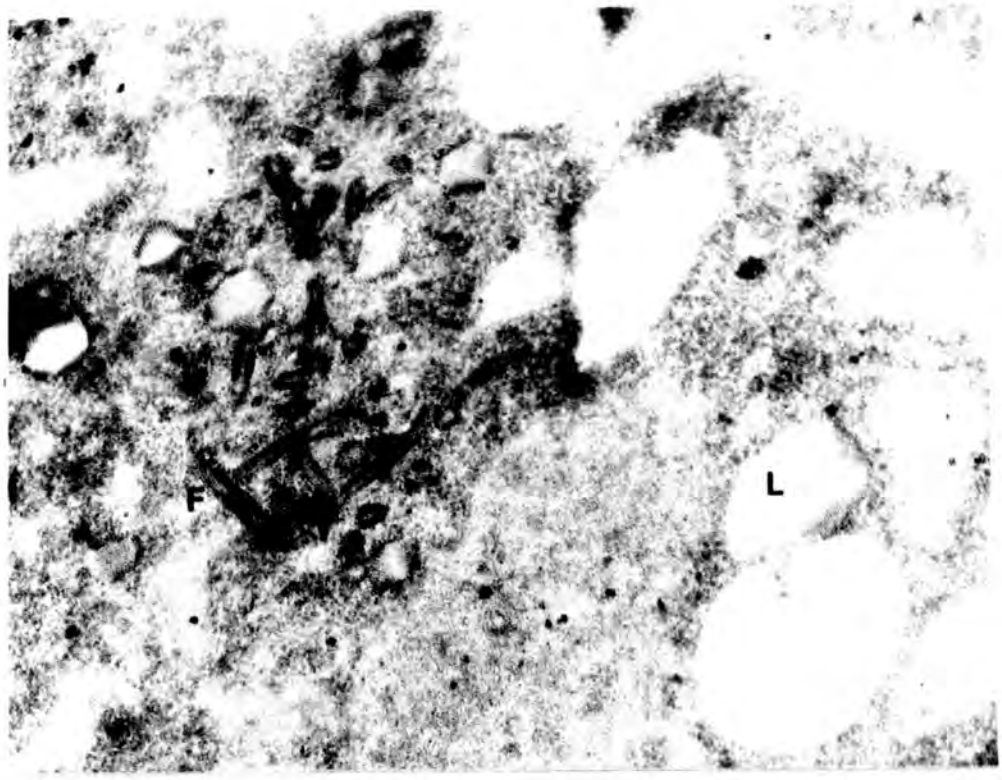


Plate 25. Germinating spore, 4 hours after inoculation in a malt extract liquid medium.
Glutaraldehyde-osmium fixation.
X 7,940.



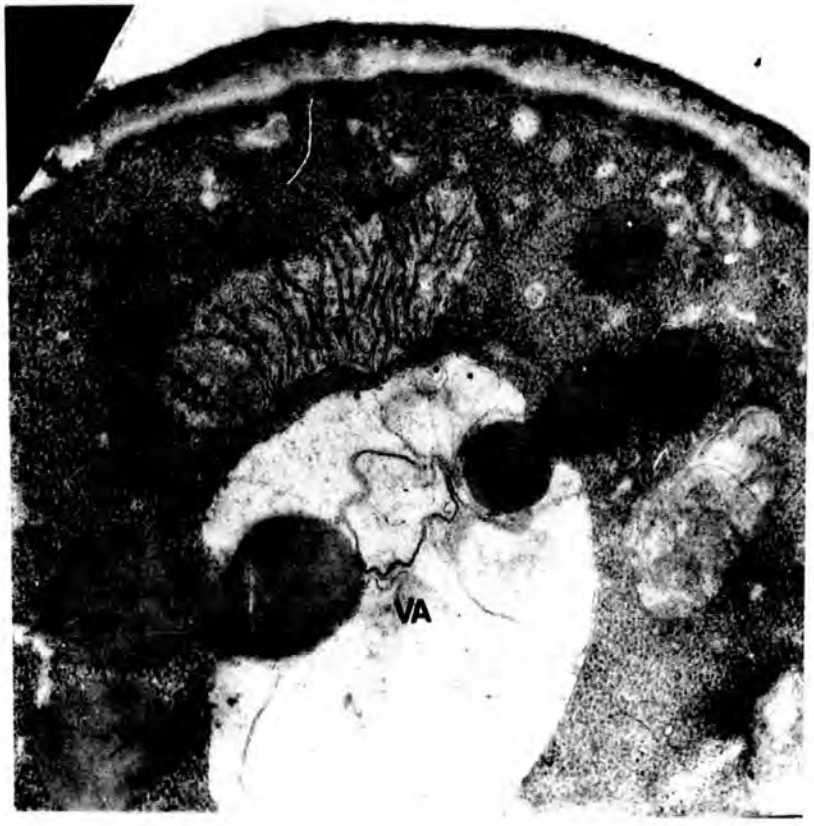


Plate 26. Sections through germinating spores, 4 hours after inoculation in malt extract liquid medium, showing ferritin. Glutaraldehyde-osmium fixation.

Fig. A) X 37,050

Fig. B) X 24,810.

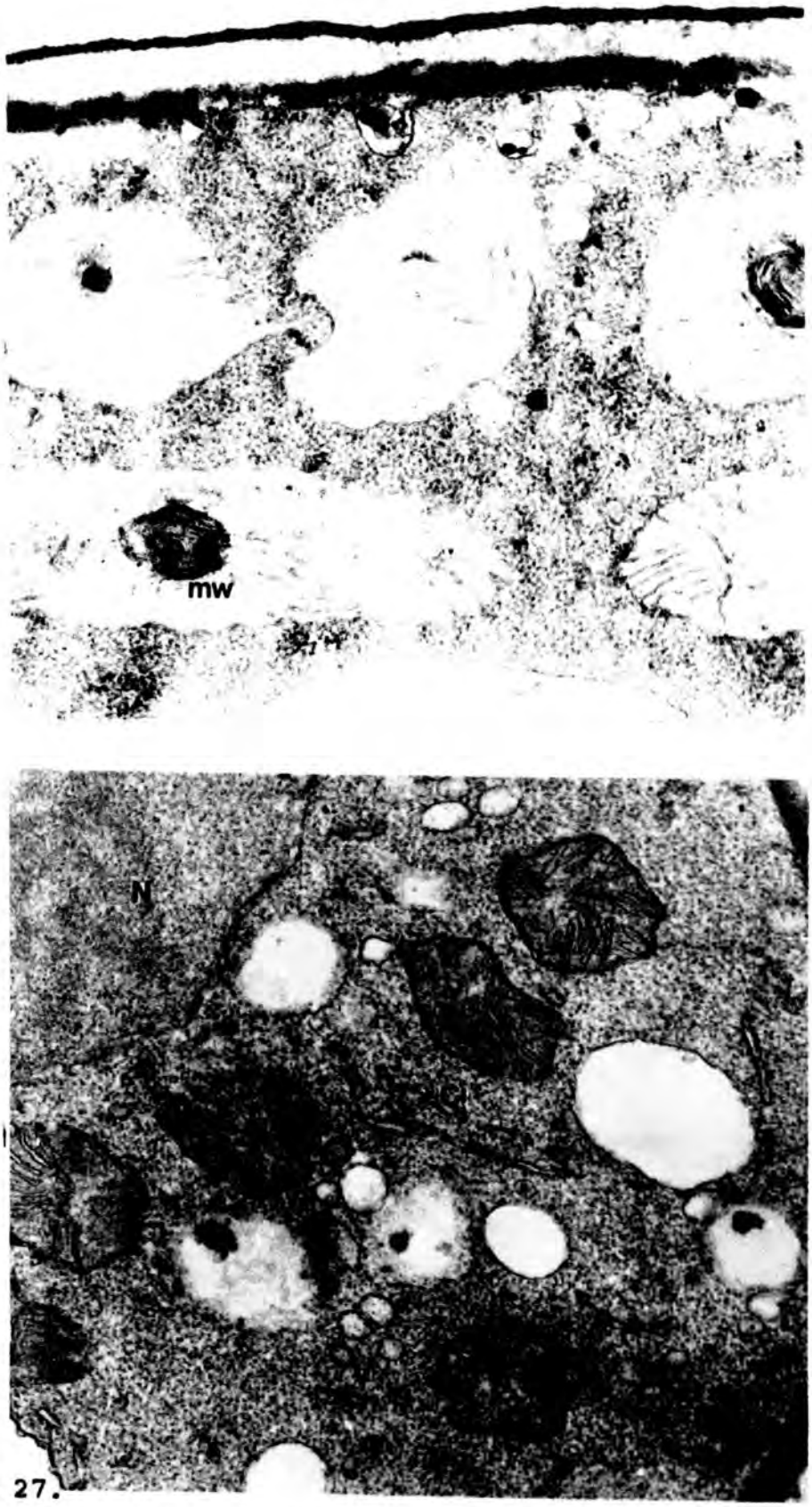
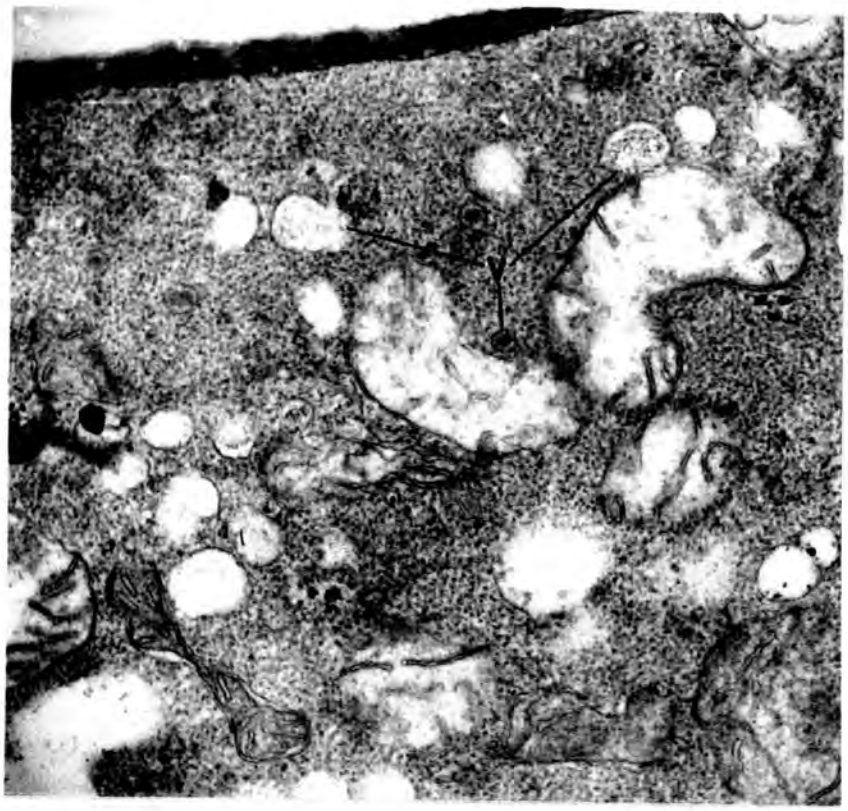


Plate 27.

Fig. A) High magnification section through a germinating spore, 8 hours after inoculation in malt extract medium. X 38,070.

Fig. B) As A but 18 hours after inoculation. X 31,150.
Glutaraldehyde-osmium fixation.



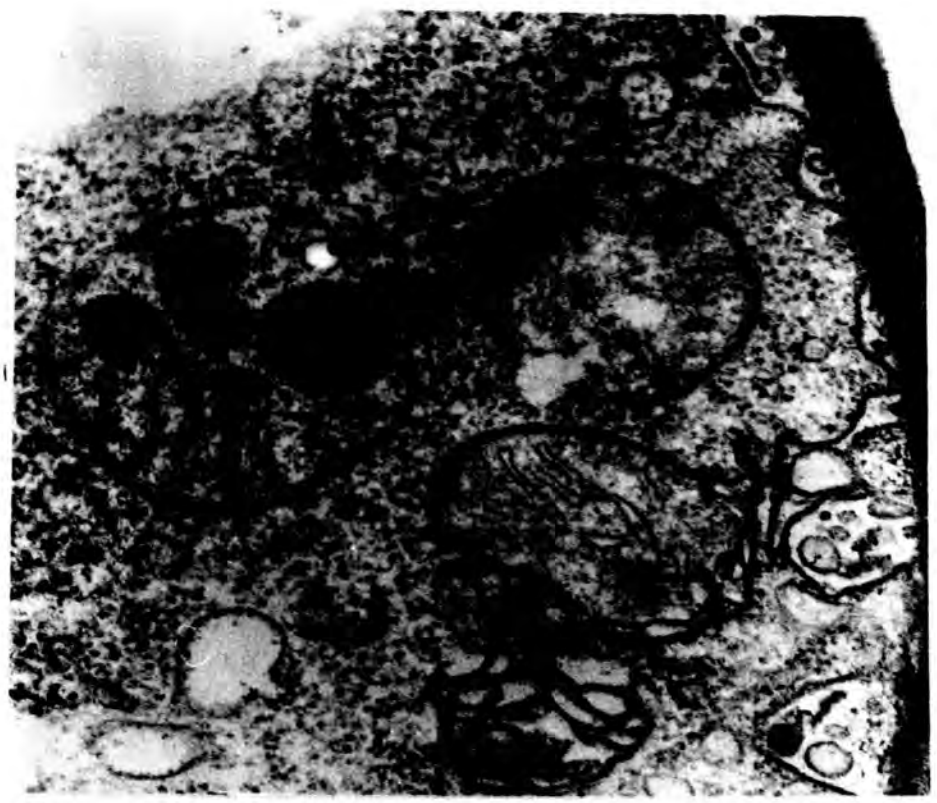


Plate 28.

Fig. A) Section through a hypha, 24 hours after inoculation in malt extract liquid medium. X 35,600.

Fig. B) As A but 36 hours after inoculation. X 63,500.
Glutaraldehyde-osmium fixation.

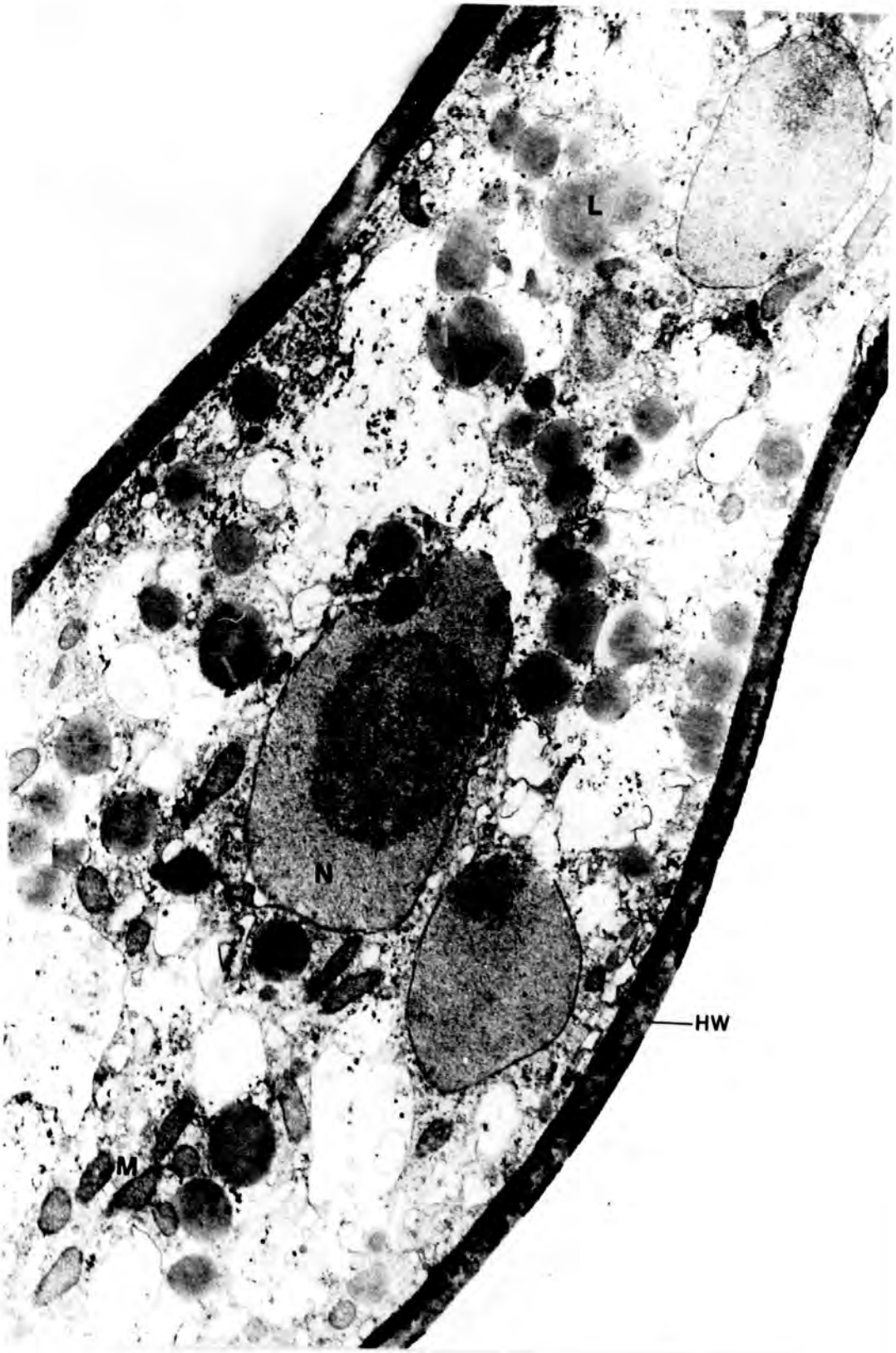
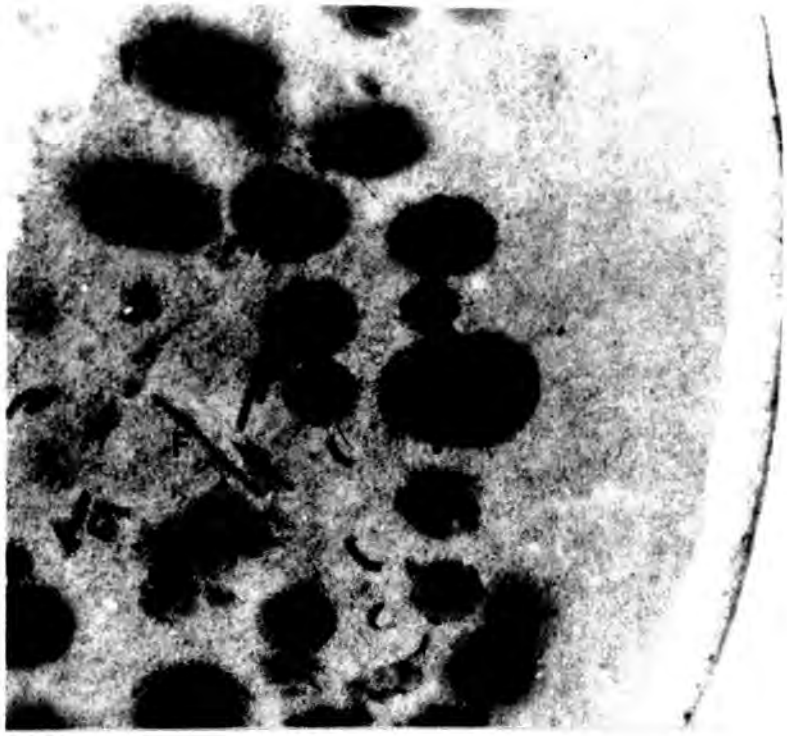


Plate 29. Section through a hypha, 48 hours after inoculation in malt extract liquid medium. X 18,580.
Glutaraldehyde-osmium fixation.



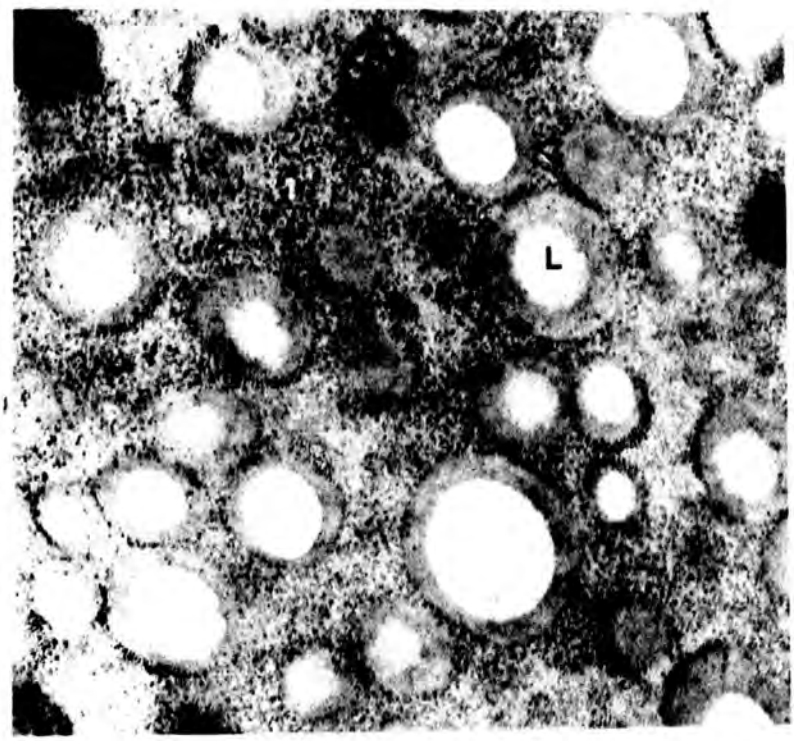


Plate 30.

Fig. A) Ferritin-rich germinating spore, one hour after heat shock. Glutaraldehyde-osmium fixation.
X 31,950.

Fig. B) Ferritin-poor germinating spore, one hour after heat shock. Glutaraldehyde/formaldehyde-osmium fixation.
X 54,000.

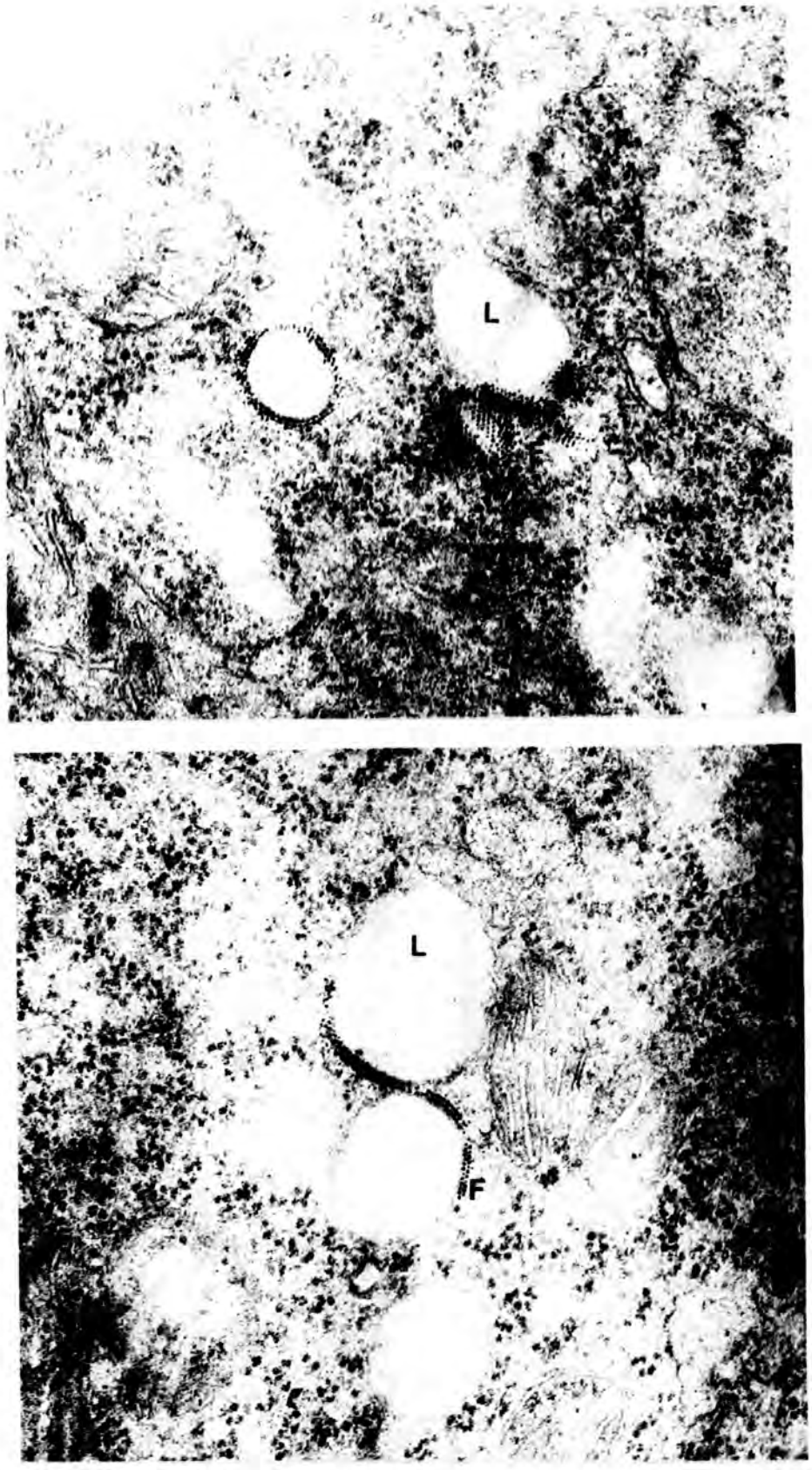


Plate 31. High magnification sections through germinating spores, 8 hours after heat shock, showing different views of ferritin.

Fig. A) X 70,100

Fig. B) X 69,120
Glutaraldehyde-osmium fixation.

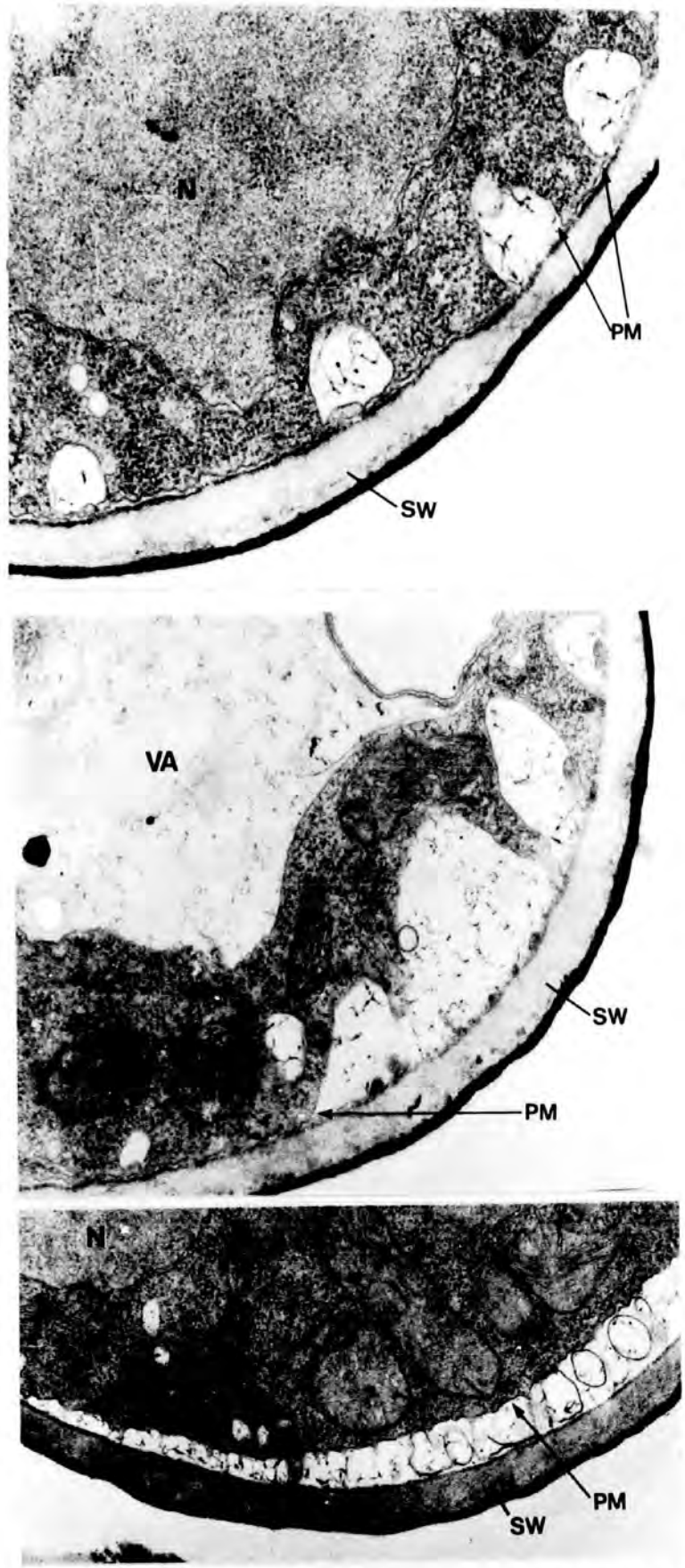


Plate 32. Sections through germinating spores, 8 hours after heat shock.

Fig. A) X 34,600

Fig. B) X 26,080

Fig. C) X 27,050

Glutaraldehyde-osmium fixation.

Plate 33. Section through a germinating spore, 8 hours after heat shock. Permanganate fixation.
X 47,250.

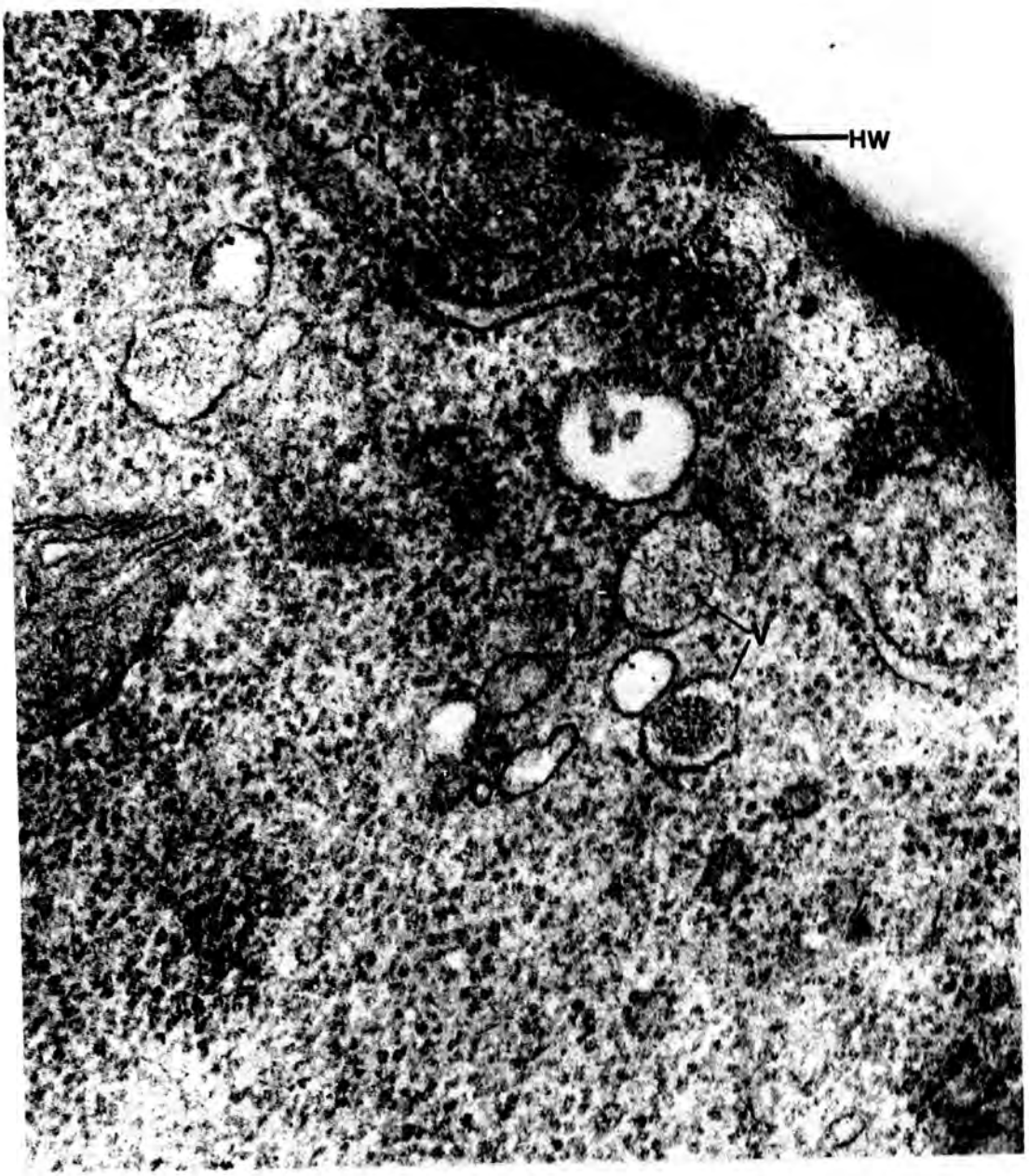
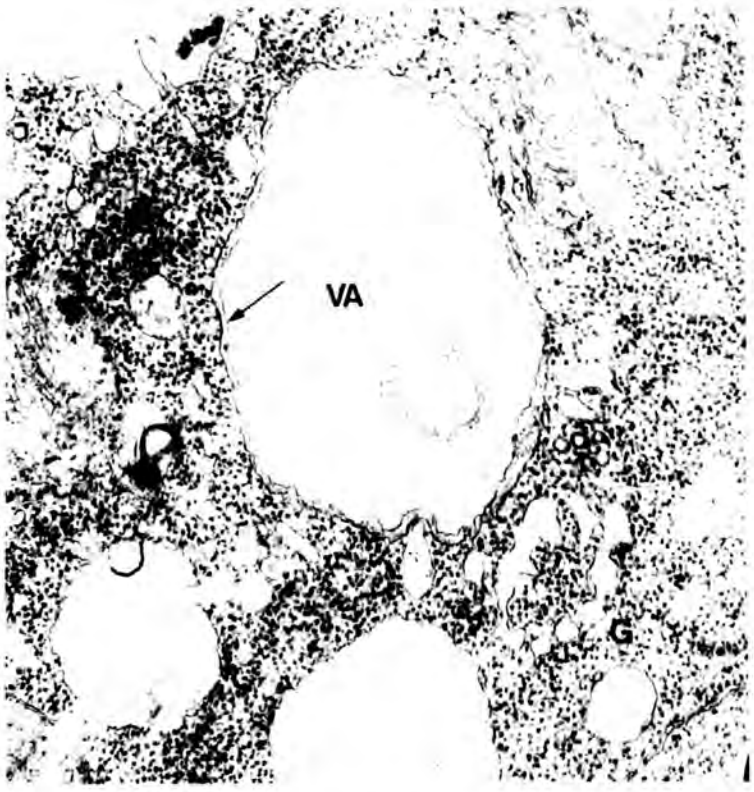


Plate 34. High magnification of a section through a hypha, 36 hours after heat shock.
X 100,700
Glutaraldehyde-osmium fixation.



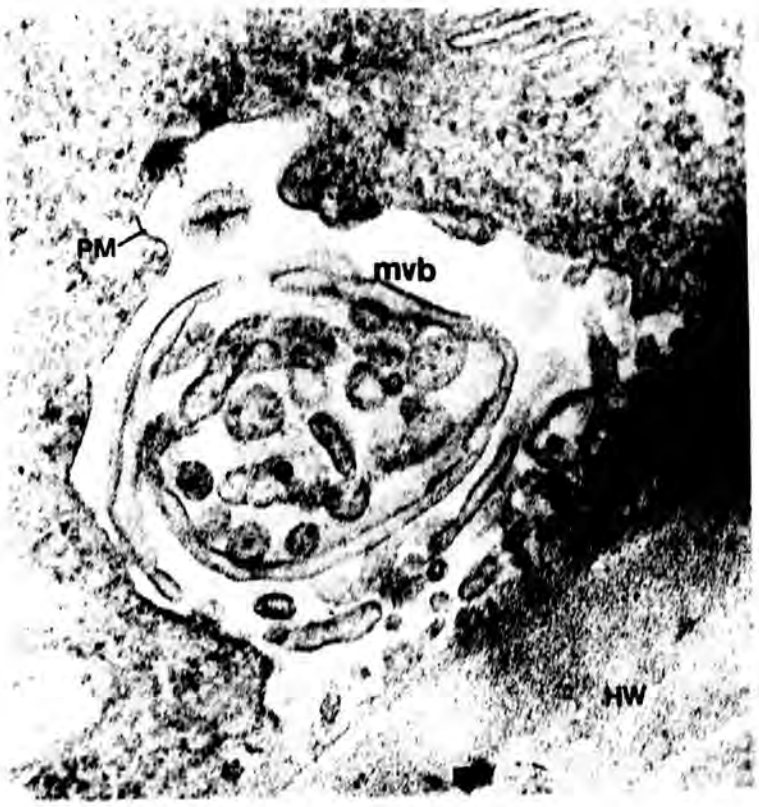


Plate 35

Fig. A) Section through a hypha, 20 hours after heat shock. Glutaraldehyde/formaldehyde-osmium fixation.
X 31,950.

Fig. B) High magnification of a section through a hypha, 48 hours after heat shock. Glutaraldehyde-osmium fixation.
X 92,880.

Discussion

The ultrastructural features of cytoplasmic organelles observed in ungerminated spores of Phycomyces were generally in agreement with those described for many fungal spores reviewed recently by Hawker and Madelin (1976). The three layered wall of ungerminated spores reported here confirms previous observations made in spore formation studies (Hankinson, 1972; Tu & Malhotra, 1976). The outermost electron dense layer was found to form first, followed by the other two. This characteristic of wall layers seems to be ubiquitous in ungerminated spores of the mucroraceous fungi (Grove, 1976).

The curious behaviour of the membranes of ungerminated spores reported here was also revealed in micrographs of Phycomyces spores subjected to aldehyde-osmium double fixation (Grove, 1976, Tu & Malhotra, 1976). The chemistry of the reaction of osmium tetroxide with unsaturated fatty acid chains is known, so is the fact that unsaturated fatty acids are more concentrated in ungerminated spores of Phycomyces (Furch et al., 1976; Marouf & Malhotra, 1976). In an attempt to explain this behaviour one could postulate that unsaturated fatty acids might be confined to most lipid droplets while saturated ones might be specifically incorporated in membranes. The positive appearance of these membranes upon germination could be attributed to an alteration in the fatty acid content of the lipid complement of the membrane as suggested for the

plasma membrane of this fungus (Malhotra et al., 1975). This is supported by the finding of Malhotra & Tewari (1973) using freeze-fractured replicas of plasma membranes of dormant and germinated spores of Phycomyces. They found some alterations to occur in the particle patterns of this membrane as germination proceeded. They suggested that a phase-change in the lipid layer might have occurred and that this behaviour could be related to the controlled dormancy and germination of spores. Similar alterations in the plasma membrane was also reported in Dictyostelium spores as a result of heat activation (Hohl et al., 1978).

The uniform formation of a new wall layer at an early stage of germination and the subsequent growth of this wall confirms similar observations for this fungus and for many other related members of the Zygomycetes (Grove, 1976) and many other fungi (Smith et al., 1976). The discrete electron density of this new layer and its close similarity to hyphal wall sustains the findings of Van Laere et al., (1977) reported earlier (see introduction) of different chemical composition of cell walls during differentiation. A similar situation was described in Mucor rouxii (Bartnicki-Garcia et al., 1968b). Autoradiographic evidence shows that this inner wall is synthesized in an evenly dispersed pattern over the entire spore periphery in Mucor rouxii (Bartnicki-Garcia & Lipman 1969).

Phycomyces, a closely related species, seems to follow a similar pattern as judged by the micrographs presented here. It can thus be included under type III of category IV of the classification scheme devised by Bartnicki-Garcia (1968a).

The transition from the spherical growth phase (Bartnicki-Garcia et al., 1968b) to the more polarized one, in term of wall deposition, is clearly demonstrated in Phycomyces particularly in Plate 15. The direct continuity between germ tube and the innermost layer of spore wall of Phycomyces observed in this study was also reported for this fungus and many other mucoraceous fungi (Grove, 1976). The mechanisms and implications of the transition from the spherical to the polarized growth and the biosynthesis of fungal walls have been extensively discussed (Bartnicki-Garcia 1973; Gooday, 1977).

The occurrence of vesicles at the site of germ tube outgrowth have been reported in many fungi including Phycomyces (Grove, 1976). Such vesicles were also observed in this study though not to a very marked extent, perhaps because of stress caused during manipulations. The involvement of these vesicles, and similar ones, in tip growth of Oomycetous, Zygomycetous and Ascomycetous fungi was recently reviewed (Grove, 1978). Similar vesicles (termed chitosomes) were isolated from the yeast form of Mucor rouxii. They were found to contain chitin

synthetase and were capable of synthesizing microfibrils *in vitro* when provided with substrates and activators (Bracker *et al.*, 1976).

Organelle changes associated with germination of *Phycomyces* described earlier (see results) were analogous in their occurrence to those of other members of the Mucoraceae (see review by Grove, 1976). In respect of mitochondria, however there are differences between the appearance of mitochondria as described in the review by Grove (1976) and the findings reported here. The large lobed mitochondria observed in dormant spores resembled those described for spores of *Rhizopus stolonifer* (Hawker and Madelin, 1976) and *Neurospora* (Lowry & Sussman, 1968). An increase in number and changes in the shape of mitochondria have frequently been observed during germination of many fungal spores (Smith *et al.*, 1976). The ultrastructural changes in mitochondrial profiles described above (see results) were observed during germination and in subsequent differentiation of the mycelium produced. There was a marked change in the affinity of the matrix for the electron dense stain. Whether these configurations were authentic and thus reflected the physiological states of the organism, or whether they just arose as mere artifacts during processing needs discussion.

Firstly, samples were always fixed in suspension thus allowing direct and uniform contact with the fixative. This was believed to eliminate artifacts that might be caused by using large blocks as was reported for liver mitochondria (Butler & Judah, 1970). Secondly, these configurations were observed in samples fixed with different fixatives and when the organism was grown in more than one medium.

Ultrastructure and morphology of mitochondria of fungi and other microorganisms can vary according to the organism, stage of development and external influences (Bracker, 1967; Lloyd, 1974). Such changes were obtained when germinated spores of Rhizopus stolonifer were exposed to low temperature (Matsumoto et al., 1969) or to high temperature for as short as 2-5 minutes (Baker & Smith, 1970). Severe damage and disorganisation of mitochondria occurred in Dictyostelium following suproptimal heating of spores (Cotter & George, 1975). Disorganised mitochondria were shown to occur in developing Penicillium cyclopium during cyclopiazonic acid production (Neethling & McGrath, 1977). Moreover, structural changes in mitochondria, during conidium maturation and germination of Trichoderma viride, were also reported (Rosen et al., 1974). Furthermore, a wide variety of microorganisms have alternative respiratory pathways that may occur during the life cycle of the organism (Linnane et al., 1972). Thus, changes in

respiratory patterns were reported to occur during germination of uredospores of rust fungi (Staples & Wynn, 1965; Maheshwari & Sussman, 1970) and during parasitic development of Claviceps purpurea (Nisbet et al., 1977). In Phycomyces during early germination, glucose was found to be metabolized by more than pathway (Van Laere & Carlier, 1975).

Induction of new pathways is often accompanied by changes in mitochondrial morphology (Linnane et al., 1972). In addition mitochondria of living fungal hyphae were reported to be highly pleomorphic (Armentrout et al., 1968).

Based on the foregoing statements the mitochondrial changes observed during germination and subsequent differentiation could be tentatively interpreted as authentic although certainty must await rigorous biochemical evidence. Indeed mitochondria isolated from mycelia of this organism, in this study using the method of Keyhani et al., (1972) showed different bands in sucrose density gradient but attempts to show intact mitochondria in samples fixed for electron microscopy were not successful.

It may be speculated that mitochondria had undergone division (multiplication) and maturation prior to germ tube production. As growth increased, this was met by further multiplication and the production of typical fungal mitochondria. By the time that mycelia were established, 24 hours after heat shock, the surrounding growth medium

would have acquired new components, as products of metabolism, and lost some of its nutrients. This state of flux could have exerted its influence on the natural metabolism of the organism which in turn necessitated adaptation to this changed situation. Coincidence with this stage was the maximal synthesis of ribosomal RNAs (Pearson, unpublished information) which would suggest active metabolism leading to protein production. Later on, by about 36 hours after heat activation, another major metabolic stage was attained. This was manifested in the appearance of abundant endoplasmic reticulum and more densely stained mitochondria perhaps as preparative steps which eventually resulted in an output of lipid droplets throughout the coenocyte.

An investigation of the metabolism of this organism, under the conditions used in this study, would be required to evaluate the suggestions made above. It must be remarked that mitochondria in the sporangiophore of Phycomyces showed different conformations in different zones studied (Tu et al., 1971). Moreover, mitochondria in the growing zone were shown to respond to light and dark regimes by altering their ultrastructure (Tu & Malhotra, 1975).

Dense inclusion and mitochondrial whorls like those observed during germination have been reported to occur.

Similar inclusions have been observed in mitochondria of animal cells, and were interpreted as transformation of mitochondrial lipoprotein (Sjostrand et al., 1964). Identical whorls were reported to occur in mitochondria of germinating conidia of Neurospora crassa. Their number was related to the level of the metabolizable carbohydrate, and they were found to have lipidic nature (Beck & Greenwalt, 1976).

The mitochondrial granules frequently observed in mycelia, prior to the reappearance of lipid droplets, had clearly shown variation in their content as judged by their electron opacity. Their presence was confirmed by examining serial sections. Similar matrix granules of various sizes were considered to be accumulations of calcium phosphate and/or phospholipids by Bernard & Afzelius (1972).

The behaviour of ferritin, as detected by its electron opacity, during germination of particular spores varied according to the initial ferritin content of those spores. Lipid-free strands of ferritin were found to occur only in germinating spores which had been collected from cultures which contained high iron. These could be ferritin arrays which had lost their specific binding to lipids as a consequence of the dissolution of the latter.

Whether ferritin was membrane-bound could not be clearly demonstrated although evidence in favour of this possibility

exist. The white rings surrounding lipid droplets observed in dormant spores, in which membranes in general exhibited negative appearance, could imply the presence of a membrane. Furthermore, David & Easterbrook (1971) isolated the ferritin-lipid complex and upon analysis found it to consist of triglycerides, ergosterol, β-carotene and contained some protein. Thus, the possibility of a surrounding membrane still exists. Elucidation of this matter requires the use of very elaborate technique such as freeze fracturing.

The disappearance of detectable ferritin, one day after inoculation by which time lipid droplets had vanished, and the reappearance of abundant ferritin one day later (in mycelia grown in presence of iron) upon the actual surface of the newly synthesized lipid droplets is a matter of great importance and significance. It had first indicated the time at which ferritin could be newly synthesized during differentiation. Secondly it showed the need of the presence of lipid droplets for ferritin to be detected, thus strengthened the binding specificity of ferritin to lipid droplets. This suggests an overall coordination between ferritin synthesis and carotenogenesis in the mycelia of Phycomyces.

The above mentioned phenomenon was used as a criterion for studying the iron induction of ferritin synthesis which will be dealt with, in detail, in the next chapter.

Chapter Two: Ferritin

Introduction

Ferritin, the major iron storage protein is a high molecular weight protein found in invertebrates, mammals, plants and fungi (Fagard & Saadi, 1977). It was first obtained from horse spleen in a purified form by Laufberger in 1937. According to Fagard & Saadi (1977) and Crichton et al. (1977), ferritin obtained from horse spleen is composed of a protein shell surrounding a central iron core or micelle. The protein moiety of the molecule (apoferritin) has a molecular weight of about 460,000 daltons and is composed of 24 chemically identical subunits (each 18,500 daltons) arranged as a hollow shell with an external diameter of about 12 nm. The entry and exit of iron occurs through six channels about 1 nm wide that pierce the shell along molecular fourfold symmetry axes (see review by Munro & Linder, 1978). The central iron core occupies a space of a diameter of 7-7.5 nm. It consists of a complex polymer of ferric oxyhydroxyphosphate of a hypothetical formula: $(FeOOH)_8$ $(FeOOPO_3H_2)$. The iron content of the ferritin molecules can vary from approximately zero (apoferritin) to a maximal content of 4500 atoms per molecule, the saturated molecules attaining 900,000 daltons. It is well known, in the animal kingdom, that ferritin has a very important role in the iron metabolism of the cell (Crichton, 1973). The uptake and release of iron by ferritin have been extensively studied and several

hypotheses have been proposed (Harrison et al., 1974). The most remarkable property of ferritin is that its synthesis is stimulated by iron administration (Fineberg & Greenberg, 1955). It is now well established that different tissues in the same species contain different isoferritins which could imply that there are different genes for ferritin (Crichton, 1975). Several reviews on many aspects of ferritin have been published (Granick, 1951; Crichton, 1973; Harrison et al., 1974; Harrison, 1977; Fagard & Saadi, 1977 and Munro & Linder, 1978).

Plant ferritin (phytoferritin) closely resembles animal ferritin when macromolecules of the protein are visualised in the electron microscope (Seckbach, 1972). It was first discovered and isolated from proplastid of non green portions of pea and bean by Hyde et al., (1963). It has since been observed in various tissues of different plants, mainly in cells not associated with active photosynthesis such as: apple fruit, pea and bean root, shoot meristem, conductive cells of Atriplex, Acer pseudoplantanus and willow (Salix fragilis) or excretional tissue of Passiflora and in epithem of hydathodes from Taraxacum and Saxifraga. Phytoferritin was also observed in leaf tissue with photosynthetic capacity reduced or lost through the senescent process, genetic mutation, virus infection or when grown under dim

light or in darkness (reviewed by Seckbach, 1972). It has also been reported in chloroplasts of Nicotiana clevelandi (Sprey et al., 1976) and in the integumentary cells of Oxalis corniculata (Gori, 1977). Recently, Crichton et al., (1978), isolated phytoferritin from the seeds of pea (Pisum sativum) and lentil (Lens esculenta). They determined their molecular weights to be 480,000 for pea apoferritin and 510,000 for lentil apoferritin. They also found that although the quaternary structure of 24 polypeptide chains was observed, the phytoferritins had a larger cavity in the interior than mammalian ferritins and can thus potentially store 1.2 - 1.4 times as much iron. Iron administration to iron-starved plants was found to increase the storage of phytoferritin in chloroplasts of Phaseolus vulgaris (Seckbach, 1968) and Xanthium (Seckbach, 1969).

Fungal ferritin was observed and identified as ferritin by Peat and Banbury (1968) in sections taken from Stage I and Stage IV sporangiophores and in immature sporangia of Phycomyces blakesleeanus. Their identification of 'electron dense particles grouped in short parallel rows forming small 'crystalline' plates lying on the surface of lipoidal globules,' as ferritin was based on the resemblance of such particles to phytoferritin which had been reported

earlier in plastids of Salix fragilis (Robards and Humpherson, 1967). The occurrence of ferritin in Phycomyces sporangiophores was later confirmed by Zaloker (1969) in sections of centrifuged sporangiophore. He reported ferritin to occur as a yellow layer which when sectioned showed particles arranged in two dimensional arrays on the surface of lipid droplets. He also observed that these arrays were irregularly folded, as if they were attached to crumpled patches of membranes although the latter could not be resolved.

Phycomyces ferritin was first isolated from mycelia, sporangiophores and spores of this fungus by David & Easterbrook (1971). They did not mention, however, at which stage of mycelial age this ferritin was isolated. Nevertheless they reported some physical properties of this protein: a sedimentation coefficient of 55 Svedberg (S) (compared to 67 S for animal ferritin) and a buoyant density in CsCl of 1.82 g Cm^{-3}. They also found that in electron micrographs Phycomyces ferritin appeared in unstained preparations as an electron dense iron core (diameter 5 - 6 nm); negative staining revealed a protein shell (diameter 12 nm) surrounding the iron core. With labelled iron (^{59}Fe), they studied the distribution of ferritin in sporangiophores. Of the (^{59}Fe) in sporangiophores,

70 to 80% was found in spores; of this fraction, at least 50% was in ferritin. Also by using labelled iron they concluded that the level of ferritin was regulated by the level of iron in the growth medium. Addition of 15 µg/ml iron to an 'iron-free' medium was found to give a 30 - 50 fold increase in ferritin iron. Whether apoferritin synthesis was regulated by the iron levels was not demonstrated (Bergman et al., 1969).

Fungal ferritin was also isolated and characterized from the mycelium of the mucoraceous fungus Mortierella alpina by Bozarth and Goenaga (1972). They first observed it as a yellowish-brown band in a sucrose density gradient analysis during a study of virus-like particles from this fungus and other fungi. They later termed this protein mycoferritin. Its cellular localization was not reported.

This part of the present investigation, exploiting the findings of the first part, attempts to correlate such findings with tangible biochemical evidences. The question whether iron administration stimulates ferritin synthesis in Phycomyces is dealt with by using radioactive amino acids. Factors and effects proceding to the answer of this question were also studied.

Materials and Methods

1. Ferritin Isolation

At the beginning of this investigation the procedure described by David & Easterbrook (1971) for isolating Phycomyces ferritin was adopted. This involved the use of a blender and a homogeniser for breaking the mycelia open in a cold 0.5M phosphate buffer (pH 6.0). This was followed by a lipid partition with n-butanol in order to dissociate ferritin from lipid so that the former was kept soluble in the aqueous phase. This aqueous phase was then subjected to dialysis against 0.05M phosphate buffer (pH 6.0) followed by lyopholization of this diluted solution. The lyopholized material was suspended in a 10-fold smaller volume with 0.5M phosphate buffer, pH 6.0 and dialyzed against 0.1M acetate buffer, pH 5. At this pH, Phycomyces ferritin was isoelectrically precipitated. It was then collected by low speed centrifugation (2500xg) and kept in solution by resuspending with the isolation buffer and stored in a refrigerator.

After many unsuccessful trials using this procedure it was inevitable to explore the reasons for such a failure. Firstly, it was found out that the Virtis homogeniser ('45') was not efficient in breaking most of the mycelia open even when different speeds, various containers and/or longer times were used. David & Easterbrook (1971) used however a different homogeniser which had been originally designed to

create high pressure. Secondly, the use of the Virtis homogeniser required large volumes of the buffer especially when large amount of mycelia was used (e.g.: one litre buffer/100 g mycelia). This rendered dialysis of large volumes risky because of the salt effect that may occur during the subsequent lyopholization which would denature the protein if present. So finally the principles of the fore mentioned procedure were maintained with some modifications.

The following procedure was routinely used:-

1) Mycelia were collected by filtration through either Whatman No. 1 filter paper or Miracloth and weighed before processing as wet weight.

2) Acid-washed sand was washed with several changes of distilled water and finally with the isolation buffer (0.5M phosphate buffer pH 6.0).

3) Batches of mycelia were mixed with equal volumes of sand and were ground in a mortar containing a few millilitres (ml) of cold isolation buffer until microscopic examination showed satisfactory disruption.

4) The final volume of the homogenate was adjusted by adding more buffer to a ratio of 3 ml buffer/1g mycelium.

5) To this homogenate a similar volume of n-butanol was added and the container was vigorously shaken and incubated

in a water bath at 40°C until contents acquired similar temperature (warming proved to be important).

6) This was followed by a manual continuous and vigorous shaking for 10 minutes.

7) The thick homogenate was filtered through Miracloth in a Buchner funnel to remove sand and mycelial debris.

8) Filtrate was centrifuged at10,000 g for 10 min. to separate butanol and aqueous phases.

9) The aqueous phase was aspirated by immersing a plastic tube attached to a suction flask thus avoiding butanol contamination.

10) The butanol phase and the interphase layer of denatured proteins were discarded.

11) The appearance of the aqueous phase varied according to the age of mycelium and was generally milky for older mycelia. This milky material was later identified as glycogen and gave positive reaction (brown colour) with potassium iodide solution.

12) The aqueous phase was dialyzed against 5 litre (L) of 0.05M phosphate buffer, pH 6.0 in a cold room for one day in order to remove any dissolved butanol.

13) After dialysis the volume of the solution was measured and brought to 70% saturation with ammonium sulphate (536.9 g/l) by adding portions of the salt with continuous mild stirring.

The container was then kept in a cold room (4°C) overnight.

14) The next day this solution was centrifuged at 10,000xg for 20 minutes and the pale yellow supernatant discarded.

15) The protein pellet was dissolved in minimal volume (not exceeding 10-15 ml) of 0.5M phosphate buffer, pH 6.0. This was then centrifuged in a conical test tube at low speed (2500xg) for 10 min. to remove any remaining debris.

16) The clear solution, whether it contained glycogen or not, was dialyzed against 2 L of 0.1M phosphate buffer pH 5 in a cold room for 2 days with a fresh buffer each day.

17) Alternatively, glycogen could be removed by centrifugation at 38,000 g for one hour to give a jelly-like pellet which redissolved upon warming or in hot water. This step was later abandoned since it caused a reduction in protein yield.

18) The isoelectrically precipitated ferritin was then collected by centrifugation at 10,000xg for 10 min. and the pellet was washed with fresh cold acetate buffer (pH 5).

19) Washing was continued until the supernatant did not give the characteristic RNA-like u.v. profiles.

20) The washed pellet was suspended with a small amount (5-15 ml) of distilled water in a polycarbonate bottle or a pyrex test tube and was lyopholyzed for one day.

21) The dry lyopholyzed protein was collected and weighed in a dry clean vial and stored in a refrigerator.

22) Purity of each ferritin preparation was primarily checked by making a 1 mg/ml solution in phosphate buffer (preferably 0.5M pH 6.0) and a u.v. absorption spectrum (250-450 nm) was recorded. A further purity criterion was achieved by using gel electrophoresis.

23) Phycomyces ferritin prepared in this way was found to be sparingly soluble in distilled water. When in solution, ferritin could be precipitated within 6 hours by dialysis against distilled water.

2. Acrylamide Gel Electrophoresis

The recipe for making polyacrylamide gels was a modification of that which was described by David & Easterbrook (1971). Plexiglas tubes (100 x 6 mm internal diameter) were used for casting gel mixture to give a final gel length of 8 Cm. For native ferritin, 5% gels were made by combining the following ingredients for making 24 ml gel mixture:-

6 ml of 0.2M sodium phosphate buffer, pH 7.5
6 " " 20% aqueous acrylamide (0.53% methylenebisacrylamide)
12 " " distilled water
20 µl of N,N,N,N-tetramethylethylenediamine (TEMED)
200 µl of 10% ammonium persulphate (freshly prepared)

Gel tubes were filled to the 8 Cm mark and a thin layer of water was overlayered on top of each column, using a micro-syringe, to give an even surface. Gels set within 5-10 min. at room temperature and were of good quality. The electrode

buffer was 0.05M sodium phosphate (pH 7.5). A few crystals of sucrose were added to ferritin solution (1 mg/ml) and different volumes (50-100µl) were loaded onto individual gels. Electrophoresis was carried out at a constant current for about 12 hours at 5 mA/gel. After completion of electrophoresis, gels were allowed to wash for overnight in 7% acetic acid solution before being scanned in a Joyce Loebel Scan 400 gel scanner operating at 265 nm. Gels were stained for ferritin iron in a diluted solution (0.1 - 1%) of potassium ferrocyanide in 7% acetic acid. Coomassie brilliant blue (CBB) was used for staining the protein. This stain was prepared according to Weber & Osborn (1969) by dissolving 1.25 g of CBB in a mixture of 454 ml of 50% methanol and 46 ml of acetic acid. Gels were stained for 2-3 hours and were destained by using several changes of the destaining solution (75 ml of acetic acid, 50 ml of methanol and 875 ml of water) either at room or elevated temperature (40 - 60^{o}C).

Sodium dodecylsulphate (SDS)-acrylamide gel electrophoresis was performed according to the method of Weber *et al.*, (1972) with slight modifications. 10% gels (8 Cm long) were obtained by mixing the following combinations:-

12 ml of 0.2M sodium phosphate buffer containing 0.2% SDS (pH 7.5)

12 ml of 20% aqueous acrylamide (0.53% methylenebisacrylamide)
20 µl of TEMED
200 µl of 10% ammonium persulphate (freshly prepared)

Samples were prepared by dissolving one milligram (mg) of the protein in one ml of 0.1M phosphate buffer (pH 7.5) containing 1% SDS (w/v) and 1% β-mercaptoethanol (v/v) in a tightly capped small vial. This was incubated in an oven at 100°C until the solution turned colourless and the formation of a greenish colloidal material ceased (20-40 min). The vial was left to cool down and the colloidal material was removed by centrifugation in conical test tubes. The supernatant was transferred to a clean vial containing few crystals of sucrose (10% w/v). Samples (50-100µl) were loaded as such by using a microsyringe. For each gel, 3-5µl of 0.05% aqueous Bromophenol blue in sucrose was applied as a tracking dye. Electrophoresis was performed at a constant current of 8 mA/gel until the marker dye was about 2 cm from the end of the gel. The electrode buffer was 0.05M phosphate buffer containing 0.1% SDS (pH 7.5).

3. Molecular Weight Determination

Two different systems of SDS polyacrylamide gel electrophoresis were employed for this purpose, the method of Weber et al., (1972) representing a continuous system and the method of Laemmli (1970) as an example of a discontinuous one. Both were subjected to slight modifications.

For the first method, gel preparations were as described earlier except that pH of both the gel and the electrode buffer were raised to 7.9. because better separation of protein mixture was observed, though bands were not sharp. For the second method, 8 cm of 10% separation gel was obtained by mixing the following combinations for each preparation:-

12 ml of 0.75M Tris/HCl buffer containing 0.2% SDS (pH 8.8)
12 " " 20% aqueous arcylamide (containing 0.53% cross linker)
20 µl of TEMED
200 µl of 10% ammonium persulphate (freshly prepared)

After the gels had set (5-10 min. from casting) they were allowed to stand at room temperature overnight, with a layer of water on each gel, to ensure complete and even polymerization. A stacking 5% gel (1-1.5 cm long) was prepared in a similar fashion to give a final concentration of 0.125M Tris/HCl plus 0.1% SDS (pH 6.8).

Dissociation of each protein with SDS was individually performed by dissolving one mg of each in one ml of 0.0623M Tris/HCl buffer (pH 6.8) containing 1% SDS and 1% β-mercaptoethanol followed by heating for 2 min. in boiling water with the exception of horse ferritin which required over 20 min. Mixtures of various combinations of protein markers were prepared, and different loading volumes

(10-15µl) were applied in sucrose. Electrophoresis was carried out with a current of 3 mA/gel for approximately 3½ hours. Bromophenol blue was used as a tracking dye as before. The electrode buffer (pH 8.3) contained 0.05M Tris and 0.38M glycine and 0.1% SDS.

Principles and equipments for disc electrophoresis were explained in detail by Davis (1964). The following proteins were used as markers:-

pepsin (MW 35,000), carbonic anhydrase (MW 29,000), chymotrypsinogen (MW 25,700), β-lactoglobulin (MW 18,400), myoglobin (MW 17,200) and ribonuclease (MW 13,700).

These molecular weights and the method by which Phycomyces ferritin was estimated were according to Weber & Osborn (1969).

4. Radioactivity incorporation and measurement

4.1 Radioactivity incorporation

The following radioactive materials were used:-

a) (4,5 ^{3}H-L-Leucine) specific activity 58 Ci/mM Concentrations used: 100, 125 µCi/100ml MM

b) (4,5 ^{3}H-L-Leucine) specific activity 40 Ci/mM Concentrations used: 120 µCi/100ml MM

c) (U-^{14}C) protein hydrolysate specific activity 56 mCi/mAtom Concentrations used: 2, 2.4 and 10 µCi/100 MM

Radioactive material was added to mycelia growing at various stages of growth (24, 36, 43 and 48 hours) to a final concentration as stated above. The mycelia were allowed different times (24, 2 and one hour or 20 minutes) to take up the label before

the addition of $FeCl_3$ to a final concentration of 15 µg/ml MM. Various volumes of MM (100, 200 and 500 ml) in appropriate sized flasks were used in order to obtain larger yield of labelled mycelia. The latter was proportionally mixed (w/w) with unlabelled mycelia (carrier) and were processed as one batch for protein isolation as described earlier. Controls were treated similarly except in the addition of iron (details will be described in Results).

4.2 Radioactivity measurement:-

Preliminary test of a likely incorporation of labelled material was performed by making a one mg/0.1 ml solution of the lyopholised protein mixture (labelled and carrier) in 0.5M phosphate buffer (pH 6). To this 5 or 10 ml of the scintillation fluid, described below, were added and contents of the glass vial were swirled. Counts were read in a Beckman LS-200B liquid scintillation counter.

Scintillation fluid was composed of the following components:-

1000 ml of toluene containing 4g of PPO (2,5 Diphenyloxazole)
500 ml of Triton (type X-100)

Further test and measurement of radioactivity were achieved by using electrophoretically purified protein both in the native and SDS-dissociated form. Gels and electrophoresis were performed as previously described except in the amount of sample each gel received. The maximal loading which gave a

reasonable band, though diffused, was 300µg for native protein mixture and up to 500µg for the SDS-dissociated one. Such loads were necessary in order to obtain usable counts. Gels were individually scanned and a u.v. profile was obtained for each. The required gel was then frozen in foil "boats" with solid CO_2 and sliced to a 1mm thick slice with a gel slicer (The Mickle Laboratory Engineering Co., Surrey). Each slice was transferred, sequentially, to the bottom of a glass vial containing 0.1 ml of 30% hydrogen peroxide (gel depolymeriser). The vials were tightly capped and incubated in an oven for 2-3 hours at an angle position so that each slice was surrounded by the agent. When depolymerisation was complete, caps were left loosened for a further hour in order to reduce volume. This gave a clear solution when 5 ml of the scintillation fluid were showered on the contents using a Zip Pette (Jencons). Radioactivity was counted as before.

5. Application of Antibiotics

Actinomycin D and cycloheximide were used as potential protein synthesis inhibitors. The effective concentration of cycloheximide was determined by setting up an experiment where a concentration gradient was created. The final concentrations of the antibiotic were:- 0, 10, 20, 30, 40, 50, 100 and 200 µg/ml MM respectively. Appropriate amounts of

the inhibitor (stock solution 20 mg/ml) were added to cultures which had been growing for 48 hours. Mycelia (2 replicas) were filtered off a day later, dried and weighed to a constant weight. The minimal concentration found to reduce weight was 20 µg/ml MM and thus was used for inhibition experiment. The observed reduction of weight caused by using higher concentration than 20 µg/ml MM was associated with what seemed to be a lysis or disintegration of mycelia as judged by the yellowish turbid colouration of filtrates of such mycelia. This was confirmed to be the case since nearly all the yellowish colouration could be extracted with n-butanol indicating the escape of lipid material and carotenes. Actinomycin D was recommended to be effective at equal concentration (20 µg/ml MM) and thus was used in inhibition experiment.

6. Protein and Iron Determination

Protein was determined according to the method of Lowry et al., (1951). The Folin-phenol reagent was diluted 1:10 with distilled water before it produced a colour. Bovine serum albumin was used as a standard. Optical densities were read at 750 nm in a Uvispeck spectrophotometer (Hilger & Watts, London).

Iron was determined with the colour reagent tripyridyl-s-triazine according to the procedure of Fischer & Price (1964).

Ferrous ammonium sulphate and ferric chloride were used as standards. Optical densities were recorded at 590 nm by using Unicam SP 800A spectrophotometer.

7. Amino Acid Composition.

This was performed on a single sample of Phycomyces ferritin only. One milligram of the protein was hydrolysed in 1.5 ml of 6M HCl at 105°C in an evacu ated (0.05 torr), sealed Pyrex tube for 22 hours according to the method of Evans & Boulter (1974). To determine cyst(e)ine a sample was oxidized with performic acid to cysteic acid according to Moore (1963) prior to the hydrolysis. Single amino acid analyses were performed on Locarte Autoanalyser, accommodating a single column of Permutit Zeocarb 225 ion-exchange resin, and linked to an automatic digital peak-area integrator (Infotronics, model CRS-210). Nor-leucine was used as an internal standard. Tryptophan was not determined.

8. Antisera and Immunodiffusion

Antiserum to Phycomyces ferritin was raised in New Zealand White rabbits by single subcutaneous injections of 2-4 mg of ferritin in Freunds complete adjuvant (Freund, 1947). This was followed 4 weeks later by 3 injections (1 subcutaneous, 2 into the ear vein) at daily intervals. After 14 days the rabbits were bled from the marginal ear vein into 60 ml glass tubes. The blood was clotted by incubation at 37°C

for 2 hours and the serum expelled by clot retraction at 4°C for 2 hours. The antiserum was decanted from the clot and centrifuged briefly to remove any red blood cells. The clarified antiserum was filter sterilized (0.22 Millipore filters) and stored at 4°C in the presence of 0.05% azide.

Agarose slabs (2mm thick) were prepared by casting in plastic immunodiffusion plates (Miles Research Products). 10 ml aliquots of a 1% (w/w) agarose solution in Tris EDTA Borate buffer pH 8.6, at 60°C was pipetted into the plastic plates arranged on a levelling table and allowed to set at room temperature. The gels were cooled in the cold room (4°C) prior to cutting the sample wells using a cutter/template kit (Miles research products).

Up to 30 µl of samples and antiserum (Anti-Phycomyces ferritin) were introduced in the wells and allowed to diffuse for 24-48 hours at 4°C in a humidity box. After immunodiffusion was complete, the gels were washed briefly in distilled water and then pressed with paper towelling and heavy weights. The pressed gels were washed with dilute borate buffer saline pH 8, distilled water and finally dried down in a hot air stream and stained briefly in 0.025% CBB in 50% methanol, 7% acetic acid, destained and dried.

9. Chemical and Reagents

All protein markers, acrylamide and antibiotics were

purchased from Sigma. Anti-horse ferritin serum was obtained from Miles-Yeda Ltd (Rehovoth). Radioactive materials were obtained from the Radiochemical Centre (Amersham). Materials used for making buffers for protein isolation were of analytical grades. Reagents for protein and iron determination were purchased from BDH. Materials used for electrophoresis were of Analar grade.

10. Cultures, Medium and Inoculum

Preparation of media and inoculum and cultures were as described in the previous chapter unless otherwise stated.

Results

1. Some Characterizations of Phycomyces Ferritin (Phycoferritin)

1.1 Purity

The isolated protein (see Plate 43) was primarily checked for the presence of RNA or nucleotide-like material by recording the u.v. absorption spectrum. In the absence of such contaminants the spectrum of phycoferritin would be similar to that shown in Fig. 1 (traced from original spectrum). This closely resembled the spectrum obtained for horse spleen ferritin (Fig. 2) except that phycoferritin showed slightly higher absorption near 280 nm. This could reflect the higher amount of protein present in phycoferritin (P/Fe ratio of 8-10) compared to horse ferritin having a P/Fe ratio of 5 (Fischbach & Anderegg, 1965).

Gel electrophoresis of native phycoferritin showed one major band, a minor band and aggregates which did not enter the gel (Plate 36 gel a). All components indicated the presence of iron and protein and corresponded to more than one peak when scanned (Fig. 3A). Whether the minor band represented a dimer could not be confirmed since it mostly appeared when a high load was applied onto gels. The major band of phycoferritin corresponded, in migration, to the middle band of native horse ferritin shown in Fig. 3B.

Gel electrophoresis of phycoferritin subunit SDS complex revealed one single band (Plate 36 gel b) corresponding to one peak (Fig. 4A). No fast moving polypeptides was observed when a time course experiment was performed. A comparison between phycoferritin and horse ferritin polypeptides-SDS complex was obtainable as visible discrete bands (Plate 37 gel b) when small loadings were used (5 µg or less). A control gel showed horse ferritin subunits to move ahead of phycoferritin. A mixture of both gave two discrete peaks (Fig 4B) indicating different molecular weights.

Because of the high insolubility of phycoferritin in water, attempts to crystallise it with cadmium sulphate were not successful. Irregularily-shaped crystals were only obtained when buffered solutions were used. No further attempts were undertaken to explore this problem since crystallisation of animal ferritin is comparatively easier because of its high solubility.

1.2 Molecular Weight of Phycoferritin Subunit

The selection of several protein markers of a narrow range of subunit molecular weight was aimed at assessing the contradictory data reported for this protein by David & Easterbrook (1971), David (1974) and Crichton (1975) to be 25,000, 18,500 and 18,000 daltons respectively. Gel electrophoresis was performed on markers either by loading

two markers onto each gel (Plate 37 gels c,d and e) or as a mixture of phycoferritin and markers (Plate 37 gel a). The gel of the latter was scanned and traced to give a better resolution (Fig. 5A). It must be said, however, that although the discontinuous system was expected to give good separation of the protein bands, nonebetter than that shown in Fig 5B could be obtained. This could perhaps be due to the relatively low molecular weight of polypeptides of proteins used (no separation between myoglobin and ribonuclease could be observed under the conditions employed). By plotting the relative migration of the bands in gel a (Plate 37) a subunit molecular weight was estimated to be 20,200 daltons (Fig. 6). Several other gels gave a similar value but it was never found to be less than 20,000 or more than 21,000 daltons.

1.3 Immunological Specificity

It was interesting to explore if phycoferritin would exhibit any serological property comparable to animal ferritins. This was primarily tested against anti-horse ferritin serum by employing the principles of Ouchterlomy technique (cited in Boyd, 1966). As shown in Plate 38 Fig. A, no precipitin band was formed against phycoferritin despite the use of serial dilutions (1:1 - 1:16). However when antiserum to phycoferritin was raised and isolated from rabbits, it formed

precipitin bands whether the reaction was with either the native or the denatured form of the protein (Plate 38 Fig. B). This might indicate specificity of phycoferritin although a wider range of anti-ferritins should have been used before drawing any conclusion.

2. Effect of Mycelia Age on Protein Yield

Time course experiments were set up to isolate phycoferritin from one, two and three day-old mycelia grown in iron supplemented MM (15 µg/ml MM $FeCl_3$). Such experiments were necessary to assess EM observations and to determine the suitable growth stage at which ferritin could be specifically studied.

The one day-old mycelia (maximally 5-8g) had a whitish to pale yellow appearance. This was shown later in the butanol phase as pale yellowish colouration which indicated the presence of small amounts of carotenoids at this stage. The isoelectric precipitation step did not, eventually, give a visible protein pellet. Although the supernatant showed slight yellowish colouration, longer dialysis time did not result in any visi ble protein sedimentation. Subsequent gel electrophoresis showed no obvious peak. The two and three day-old mycelia had a bright distinct yellow colour which corresponded to a yellow-orange colouration in the butanol phase. The presence of ferritin in these two

samples was observed as a brownish pellet after the isoelectric precipitation step.

3. Effect of Different iron Concentrations on Protein Yield

It was interesting to learn whether a concentration of iron lower and higher than 15 µg/ml MM would effect the yield of mycelial phycoferritin. The range of iron concentrations chosen was: 7.5, 30 and 60 µg/ml MM $FeCl_3$. Independent successive experiments were performed on each concentration respectively. The protein yields of the three day-old mycelia of these experiments were compared with the previously known yield of similar mycelia grown on 15 µg/ml MM iron.

It was found that no remarkable difference existed and protein yield was in the range of 12-14% (w/w). Growth of mycelia growing in the presence of 60 µg/ml iron seemed not to be affected by such a high concentration since mycelial yield was similar to previous experiments. Nevertheless it was preferable to use the 15 µg/ml concentration as a standard for subsequent experiments.

4. Effect of the Presence and Absence of Iron in the Medium

4.1 On Growth

An experiment was performed to observe the effect of iron presence or absence on the growth of mycelia. This was done on three sets of cultures by adding iron (15 µg/ml MM) before inoculation to the first set, and after 48 hours from

inoculation, to the second set, while the third set had no iron added apart from impurity.

250 ml flasks each having 100 ml of MM were inoculated with one ml of a spore suspension each. The flasks were incubated at 20°C in a Gallenkamp horizontal shaker tank running at about 90 cycles/min. The flasks were illuminated from underneath with warm white fluorescent tubes (about 1500 Lux). Although each flask was inoculated with the same volume of the spore suspension, the pattern of mycelial pellet growth of each set varied. Flasks which received iron before inoculation showed a denser growth and more uniformity in their tiny pellets, 24 hours after inoculation. Cultures which did not receive iron gave mycelia of spherical or radial appearance and the addition of iron, 48 hours after inoculation, did not change this pattern. Another noticeable observation was in the colour of 2, 3 and 4 day-old filtered mycelia of each set. Those grown in presence of iron from inoculation had a greenish tint while the other two gave a strong yellow colour despite the addition of iron to one of them. It was suggested ~~(Banbury, personal communication)~~ that perhaps because of the dense mycelial growth, in samples which received iron from the beginning, these mycelia might have been in direct contact with air which could have led to the stimulation of melanin production thus causing the greenish tinge.

The results of this experiment were illustrated in growth curves as shown in Fig. 7. Each reading was plotted as the mean of a constant dry weight of two replicas.

4.2 On Protein Yield

This was a comparison to determine whether the addition of iron (15 µg/ml MM) to cultures growing for 48 hours after inoculation would yield phycoferritin the next day similar to cultures which received iron before inoculation (i.e. testing iron induction of ferritin synthesis).

It was found that both types produced ferritin to about an equal yield percentage. The colour of the lyopholized protein produced after the addition of iron to two day-old cultures was comparatively more brownish (12% w/w iron) compared to ferritin produced by mycelia which initially received iron (8% w/w iron).

Samples were collected before and after the addition of iron (every two hours for 12 hours) and processsd for electron microscopy to detect the earliest appearance of ferritin. It was found that abundant lipid-bound and some dispersed ferritin was detectable in stained sections not earlier than six hours after the addition of iron. Mycelia from cultures which did not receive iron were used as control. They showed no or very little ferritin present.

5. Effect of Zinc Sulphate

It was shown from studies on Zn-fed rats that zinc interfered with the uptake of iron thus giving rise to the production of 'iron poor ferritin' and a condition resembling iron deficiency anemia. It was also found that zinc did not interfere with the amount of protein moiety formed during iron-induced ferritin synthesis (Matrone et al., 1975). It was interesting to know if such findings would occur in Phycomyces ferritin synthesis system.

5.1 Determination of effective concentration of Zinc

An experiment was set up to determine the effective concentration of zinc sulphate in the medium which would be intolerable to the fungus. The range of concentrations chosen was: 0.4, 4, 8, 12, 16 and 20 µg/ml MM zinc sulphate in the presence of 15 µg/ml $FeCl_3$ in the medium. All flasks were inoculated with equal volume of the spore suspension and cultures allowed to grow for three days. Mycelia were filtered off and weighed as a wet weight. It was found that mycelial yield of each flask of the series was closely similar. The initial and final pH of cultures was found to lie between 4.5 - 4 and between 6.5 - 4.3 respectively (readings of 0.4 and 20 µg/ml MM zinc sulphate containing cultures respectively). Mycelial colour was distinctly different from that of the control mycelia (0.4 µg/ml) in being pale yellow instead of a strong clear one. The filtrate of the flask which had

20 µg/ml showed some turbidity which upon centrifugation sedimented as a white material. This was not a contaminant but rather like a mineral material which dissolved in hydrochloric acid. The concentration of 16 µg/ml was chosen for subsequent experiments.

5.2 Effect of high Zn/Fe concentration on ferritin

Ferritin was isolated from three day-old mycelia which had been grown in MM in the presence of zinc sulphate (16 µg/ml) and $FeCl_3$ (15 µg/ml). Gel electrophoresis of this protein revealed one major band which was stainable with both CBB and potassium ferrocyanide. The colour of the lyopholized protein was noticeably fainter than ferritin isolated in standard conditions (15 µg/ml iron only). This suggested the presence of either less iron or an artifact as a result of inadequate lyopholization (salt effect). Ferritin iron determination of this protein was found to be 5.8% (w/w) compared to about 10% (w/w) for standard ferritin. Protein yield percentage was 14% (w/w).

5.3 Effect on Iron induction of ferritin synthesis

This was a comparative study in order to observe whether the addition of iron (and thus ferritin synthesis stimulation) to high Zn-fed mycelia would lead to a similar finding as shown earlier. Cultures were left to grow in MM for two days after inoculation in the presence of 16 µg/ml MM zinc sulphate. Ferric chloride was then added to a final

concentration of 15 µg/ml MM and mycelia were allowed to grow for another day before they were filtered off and weighed.

Ferritin was then isolated as usual and found to give a major band when gel electrophoresed. The lyopholized ferritin looked darker in colour (more brownish) than ferritin of earlier preparation although the yield percentage did not differ significantly. Iron determination of this protein, unlike the earlier one, was found to be 11% (w/w) similar to iron level in ferritin obtained by the conventional way (section 4.2).

6. Radioactivity Incorporation into Ferritin

6.1 Induction of ferritin synthesis

It was shown earlier that the addition of iron (15 µg/ml MM) to growing mycelia led eventually to the isolation of ferritin in increased amounts. The increase, however, could have been due to either an iron-induced stimulation of total apoferritin synthesis or to an incorporation of iron as a complex into preformed apoferritin shells. To resolve this point, in six experiments, use was made of radiochemically labelled amino acids added to experimental cultures in the presence or absence of iron in the following manner. Three cultures were designated as:

Flask A. Iron present from time of spore inoculation

Flask B. Iron added during growth of mycelia

Flask C. No iron addition

After 48 hours growth as above ^{3}H-leucine, to a final concentration of 100μCi/ml was added to each flask and then after one hour (to facilitate label uptake) iron was added to the flask B only. All three flasks were then incubated further for 24 hours before the mycelia were harvested. They were then, proportionally, mixed with unlabelled mycelia (received iron two days after inoculation) and processed for ferritin isolation. A maximum of 300μg of each protein sample was loaded onto gels and the latter were individually sliced and counted. It was found, where the protein band was expected, that counts were in the range of 100-300 CPM (counts per minute).

The specific activities (CPM/mg radioactive protein) of ferritin samples obtained from the second experiment were: 4030, 3360 and 700 for ferritin obtained from cultures A, B and C above respectively.

Although such findings demonstrated, to a certain extent, an induction of new protein synthesis, the low counts of gel slices rendered any further studies encompassed with uncertainty. Several possibilities were attributed for such low counts and a suggestion to use ^{14}C labelled amino acid was made. A uniformally labelled ^{14}C protein hydrolysate

(specific activity 56 mCi/m Atom) was used in a similar fashion as above in three successive independent experiments. The main difference was in the concentration of label to be used. It was thought, due to the high specific activity and counting efficiency of ^{14}C, that a final concentration of as little as 2μCi/100ml MM would, theoretically, be sufficient. This was proved wrong since gel slices gave counts similar to background even when label's concentration was raised to 10μCi/100ml MM, longer times for label uptake allowed and/or larger amounts of labelled mycelia were used. Nevertheless higher counts were obtained either by counting the whole protein yield (up to 12 mg) or by cutting and digesting the whole length of the protein band (2-3 Cm).

It was found that specific activities calculated for each ferritin sample of these three experiments, though tentative, were indicative of a de novo synthesis of ferritin. The specific activities of these experiments were as shown below and in Fig. 8.

Specific Activity (CPM/mg radioactive protein)			Flask
Exp 3	Exp 4	Exp 5	
2205	3890	4447	A
3111	3866	4327	B
700	802	897	C

During the second experiment, employing ^{14}C protein hydrolysate, a follow up (tracing) of radioactivity uptake and radioactivity counts through the ferritin isolation procedure, was achieved on a control culture. It was found that approximately one third of the initial counts were obtainable two hours after the addition of label indicating an effective uptake by mycelia. During the isolation procedure, counts after the first dialysis step were about eight times less than initial ones. After isoelectric precipitation, the supernatant gave a reading sixteen times less than the initial one (before butanol extraction of lipids). This emphasized the need for applying much higher concentration of the label. For safety and economic reasons, the use of higher concentration of ^{14}C was not recommended. Instead ^{3}H-leucine (specific activity 40Ci/mM) was used in the sixth and last experiment. In this particular experiment conditions of obtaining larger mycelial yield from a minimal volume of the medium were achieved by inoculating 200mlMM (one litre flask) with a heavy inoculum (3-4 ml of spore suspension). The radioactivity concentration applied was 120μCi/100ml MM and allowed 30 min for uptake before the addition of iron to one flask. It was found that gel slices of native ferritin did not give higher counts than 300 CPM presumably because of the diffused band. Counts of gel slices of SDS-dissociated protein were much improved by using

discontinuous gel electrophoresis (Tris/Glycine system) which allowed the application of higher sample load (up to 500 µg protein). Attempts to use such a system for native, lyopholized phycoferritin were unsuccessful although ideal results were obtained when high concentrations of either horse spleen ferritin (liquid) or myoglobin (lyopholized) were used.

Counts produced by SDS-protein gel slices were used to calculate the specific activities of different protein samples. These were: 30196, 29807 and 5200 for ferritins obtained from flasks A, B and C as above. These specific activities were illustrated in Fig. 8 for a comparative purpose. These findings clearly supported previous lower incorporation data and were indicative of a stimulant action of iron on phycoferritin synthesis.

6.2 Effect of Protein Synthesis Inhibitors

To gain an idea on the mechanism by which iron led to the synthesis of ferritin, and to support this finding, actinomycin D and cycloheximide were used as potential mRNA and protein synthesis inhibitors respectively. ^{3}H-leucine (specific activity 58Ci/mM) was added to a final concentration of 125µCi/100ml MM to four flasks (A, B, C & D) of mycelial cultures grown for 43 hours, after inoculation, in MM which did not receive iron. The latter was added 30 min later to

three flasks only (A, B, C). Actinomycin D and cycloheximide were added 40 min later to the second and third flasks only (B & C). After 20 hours mycelia were individually collected and processed for ferritin isolation with proportional amount of unlabelled mycelia. Gel slices of native ferritin showed slight improvement in counting (see Fig. 9A). Nevertheless, they were indicative of different levels of radioactivity incorporation in the four samples of protein studied (Fig. 9). Similarly, radiochemical profiles of the SDS-dissociated protein (Fig. 10) showed clearly the level of radioactivity incorporation. They revealed first that the addition of iron to cultures of growing mycelia did cause a de novo synthesis of apoferritin (Fig. 10A) compared to the level of synthesis in the absence of added iron (Fig 10D). Secondly, this induced synthesis was not affected by the presence of actinomycin D in the medium but was drastically reduced by the presence of cycloheximide under conditions of this experiment. Specific activities of such phycoferritin samples were found to be:

40906 (CPM/mg radioactive protein) for Flask A as above
37990 (" " ") " " B " "
6592 (" " ") " " C " "
4380 (" " ") " " D " "

The significance of these findings will be further discussed later.

Electron microscopic observations of this experiment supported the above mentioned findings. Abundant amount of ferritin like that shown in Plate 40 could be detected in sections of mycelia sampled 10 hours after the addition of iron to mycelia grown either in the presence or absence of Actinomycin D. Before the addition of iron very little or no ferritin could be detected (Plate 39). Scarce or very little amount of ferritin could be detected in sections of iron/cycloheximide treated mycelia. Sections of mycelia sampled 20 hours after the addition of cycloheximide were characterized by the numerous dense granules present (Plate 41) compared to Actinomycin D treated mycelia (Plate 42). The nature of such deposits was not investigated but they could represent insoluble phenolic material.

Plate 36. Polyacrylamide gel electrophoresis of phycoferritin:-

a) native form. 5% gel was electrophoresed in phosphate buffer (pH 7.5) for 12 hours at 5 mA/gel.

b) SDS-subunit complex. 10% gel was electrophoresed in phosphate-SDS buffer (pH 7.5) for 3½ hours at 8 mA/gel.

Bands were stained with CBB.

Plate 37. Polyacrylamide gels of phycoferritin SDS-subunit complex and different protein markers. 10% gels were electrophoresed in phosphate-SDS buffer (pH 7.9) for 3½ hours at 8 mA/gel. Bands corresponding to the following proteins reading from the cathode to the anode. They were stained with CBB.

a) pepsin, carbonic anhydrase, chymotrypsinogen, phycoferritin, β-lactoglobulin and myoglobin.

b) phycoferritin and horse spleen ferritin.

c) chymotrypsinogen and β-lactoglobulin.

d) carbonic anhydrase and myoglobin.

e) pepsin and ribonuclease

Arrow shows a band of undissociated protein.

Fig. 1

UV absorption spectrum of *Phycomyces* ferritin

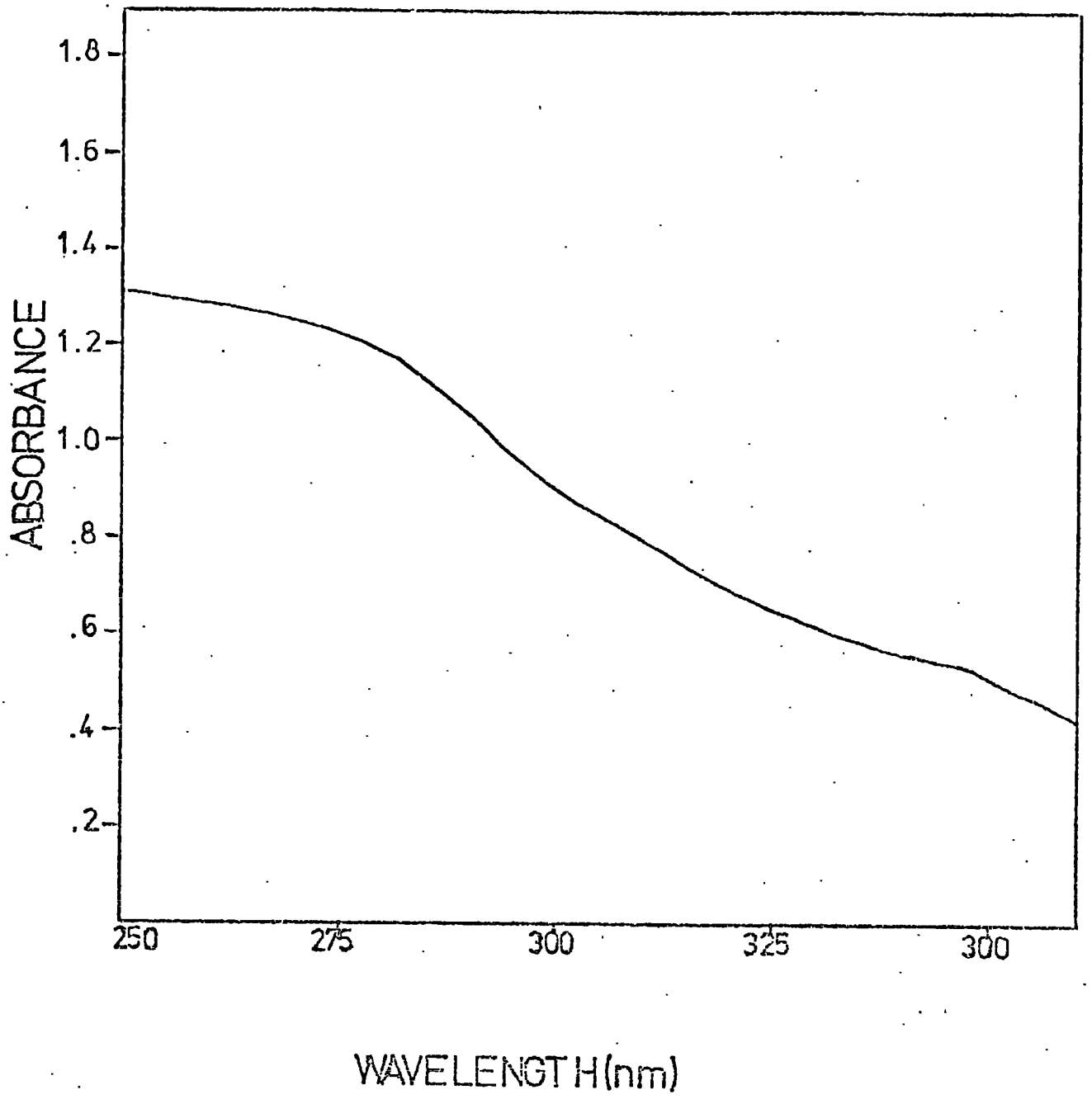

1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
ABSORBANCE
250
275
300
325
300
WAVELENGTH(nm)

Fig. 2

UV absorption spectrum of horse spleen ferritin

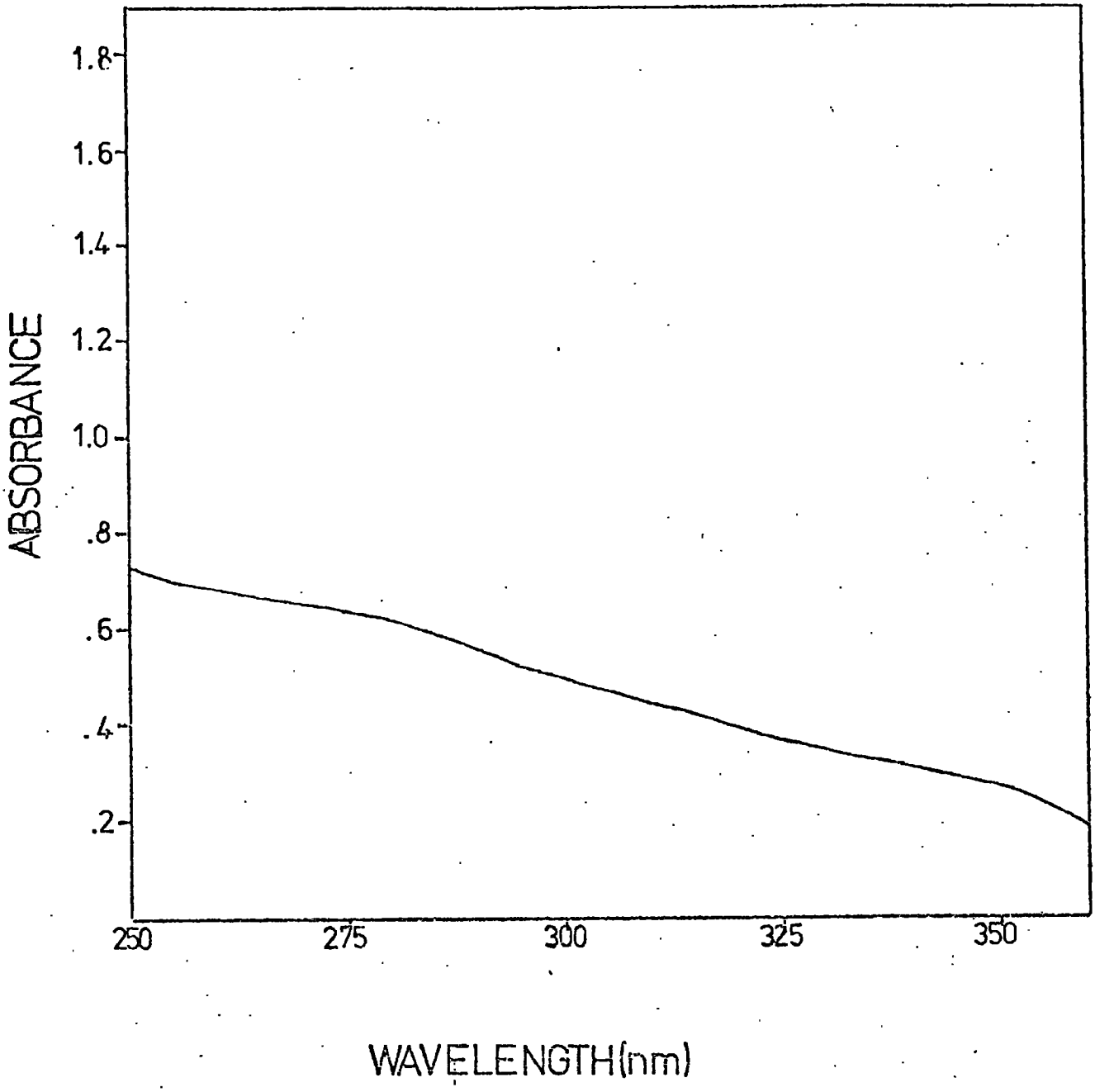

ABSORBANCE
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
250
275
300
325
350
WAVELENGTH(nm)

Fig. 3

Polyacrylamide gel u.v. profiles. 5% gels were electrophoresed in phosphate buffer (pH 7.5) for 12 hours at 5 mA/gel

A) Native phycoferritin

B) Native horse spleen ferritin

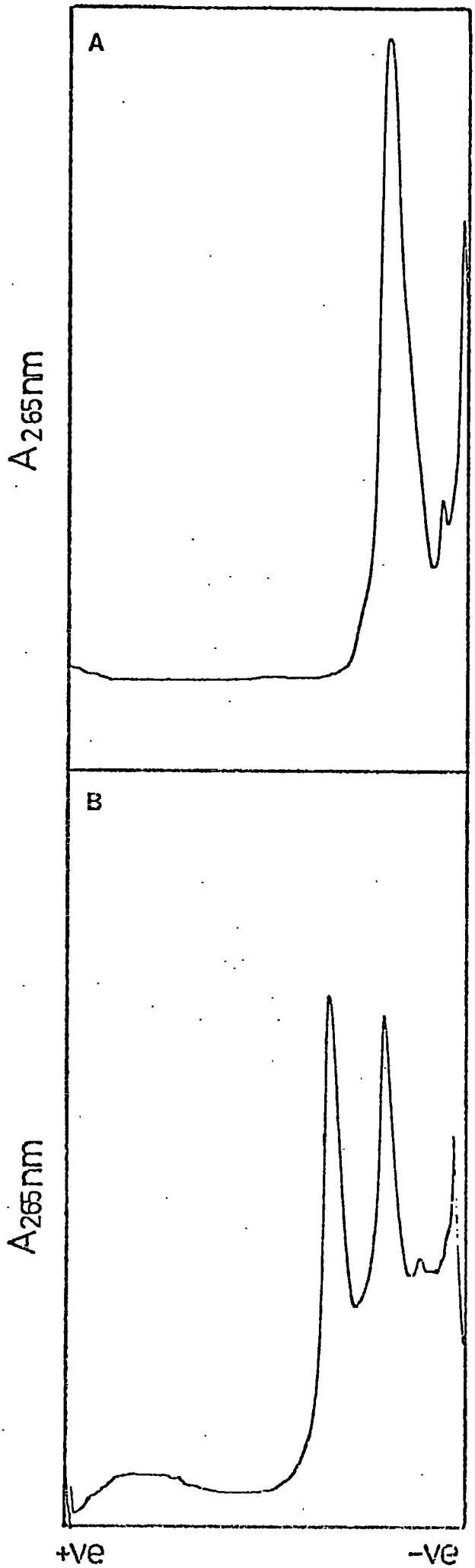

A
A_{265nm}
B
A_{265nm}
+ve
-ve

Fig. 4

Polyacrylamide gels u.v. profiles. 10% gels were electrophoresed in phosphate-SDS buffer (pH 7.5) for about 3.5 hours at 8 mA/gel

A) SDS-subunits complex of phycoferritin

B) SDS-subunits complex of phycoferritin (peak 1)

SDS-subunits complex of horse ferritin (peak 2)

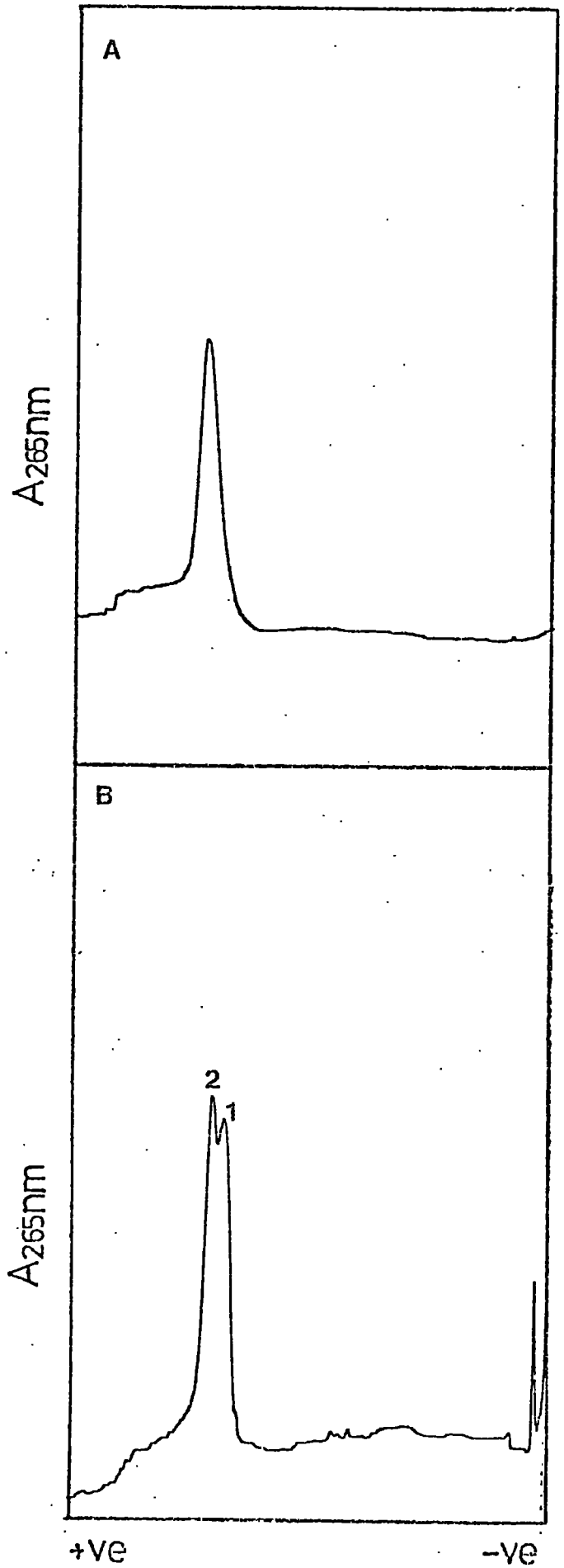

A
A_{265nm}
B
2
1
A_{265nm}
+ve
-ve

Fig. 5A

Polyacrylamide gel u.v. profiles. 10% gels were electrophoresed in phosphate-SDS buffer (pH 7.9) for about 3.5 hours at 8 mA/gel

peak 1) pepsin

peak 2) carbonic anhydrase

peak 3) chymotrypsinogen

peak 4) phycoferritin

peak 5) β-lactoglobulin

peak 6) myoglobin

Fig. 5B

Polyacrylamide gel u.v. profiles. 10% running gel (pH 8.8), 5% stacking gel (pH 6.8). Electrophoresis carried out in Tris-glycine SDS buffer, pH 8.3 for about 3.5 hours at 3 mA/gel

peak 1) pepsin

peak 2) carbonic anhydrase

peak 3) chymotrypsinogen

peak 4) phycoferritin

peak 5) β-lactoglobulin

peak 6) myoglobin

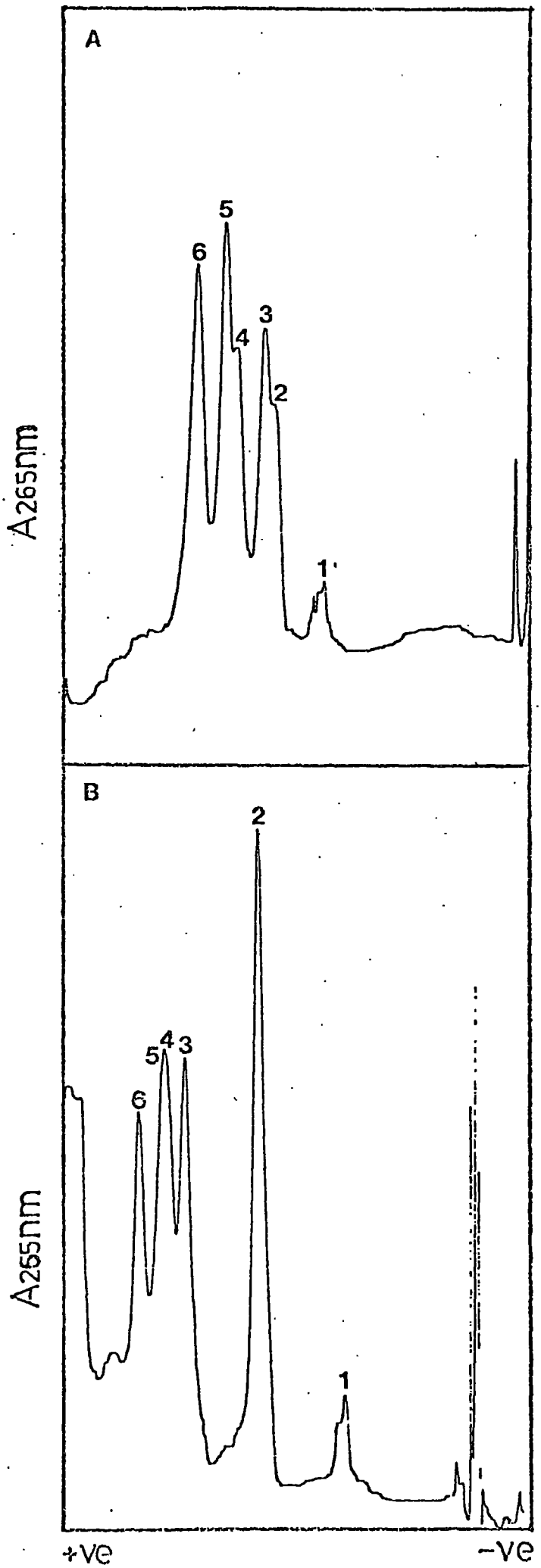

A
6
5
4
3
2
1
A265nm
B
2
6
5
4
3
1
A265nm
+ve
-ve

Fig. 6

Mobility/molecular weight calibration curve for phycoferritin

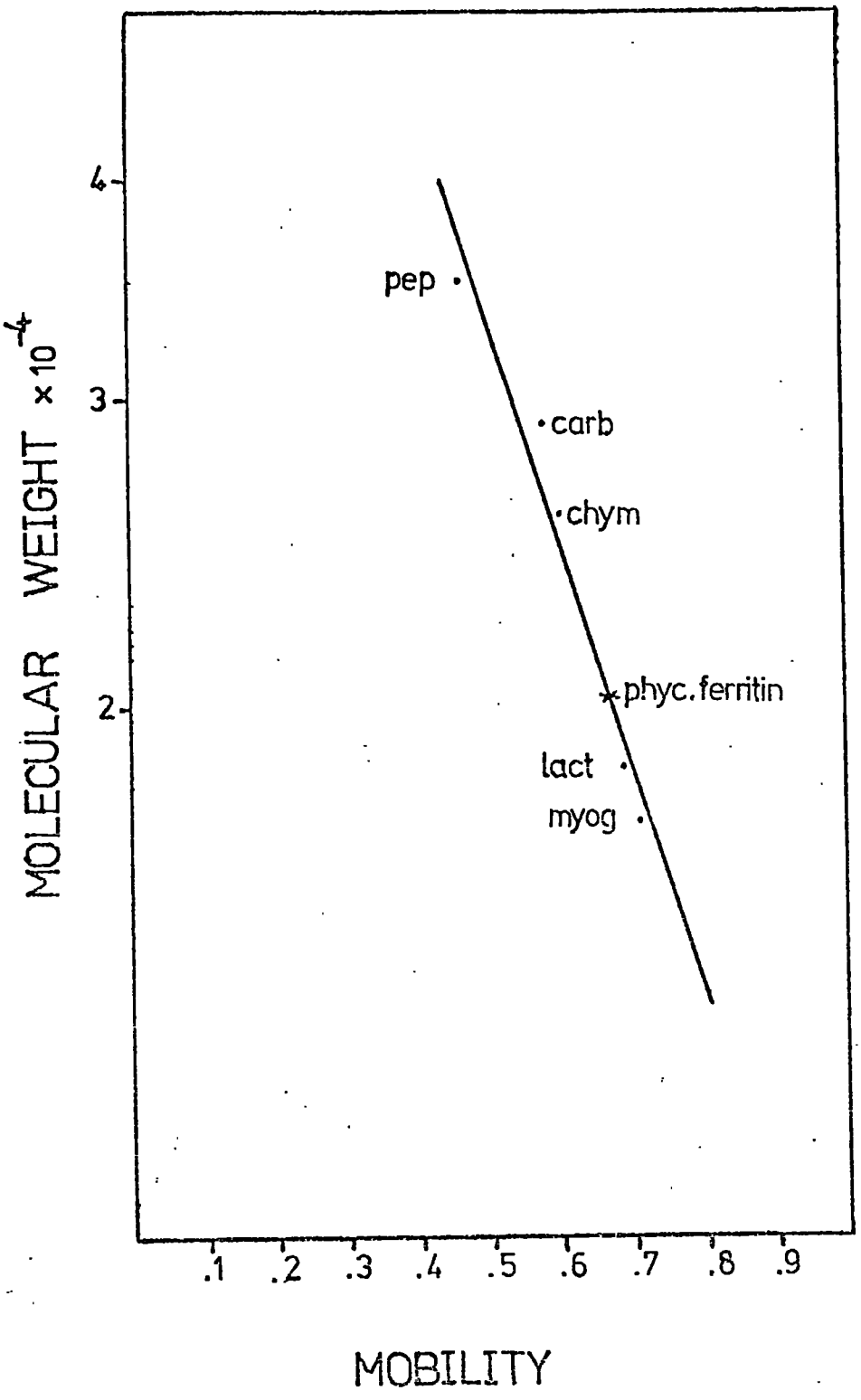

MOLECULAR WEIGHT ×10⁻⁴
4
3
2
pep
carb
chym
phyc.
ferritin
lact
myog
.1
.2
.3
.4
.5
.6
.7
.8
.9
MOBILITY

Fig. 7

Growth curves of Phycomyces in presence and absence of iron.

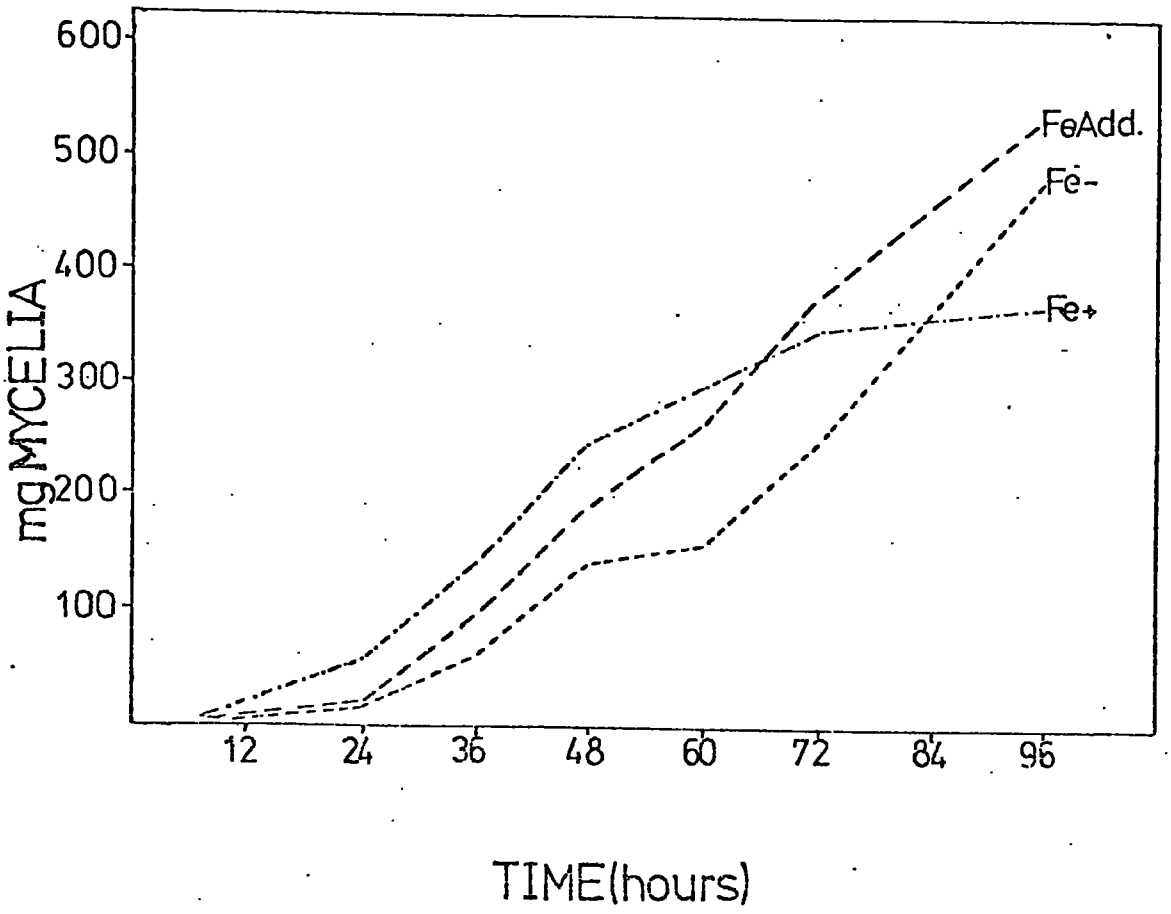

600
500
400
300
200
100
mg MYCELIA
FeAdd.
Fe-
Fe+
12
24
36
48
60
72
84
96
TIME(hours)

Fig. 8

Specific activities of phycoferritin following feeding of mycelial cultures with ^{14}C-protein hydrolysate (▨) or ^{3}H-leucine (▩) in the presence and absence of iron and when iron was added to growing mycelia.

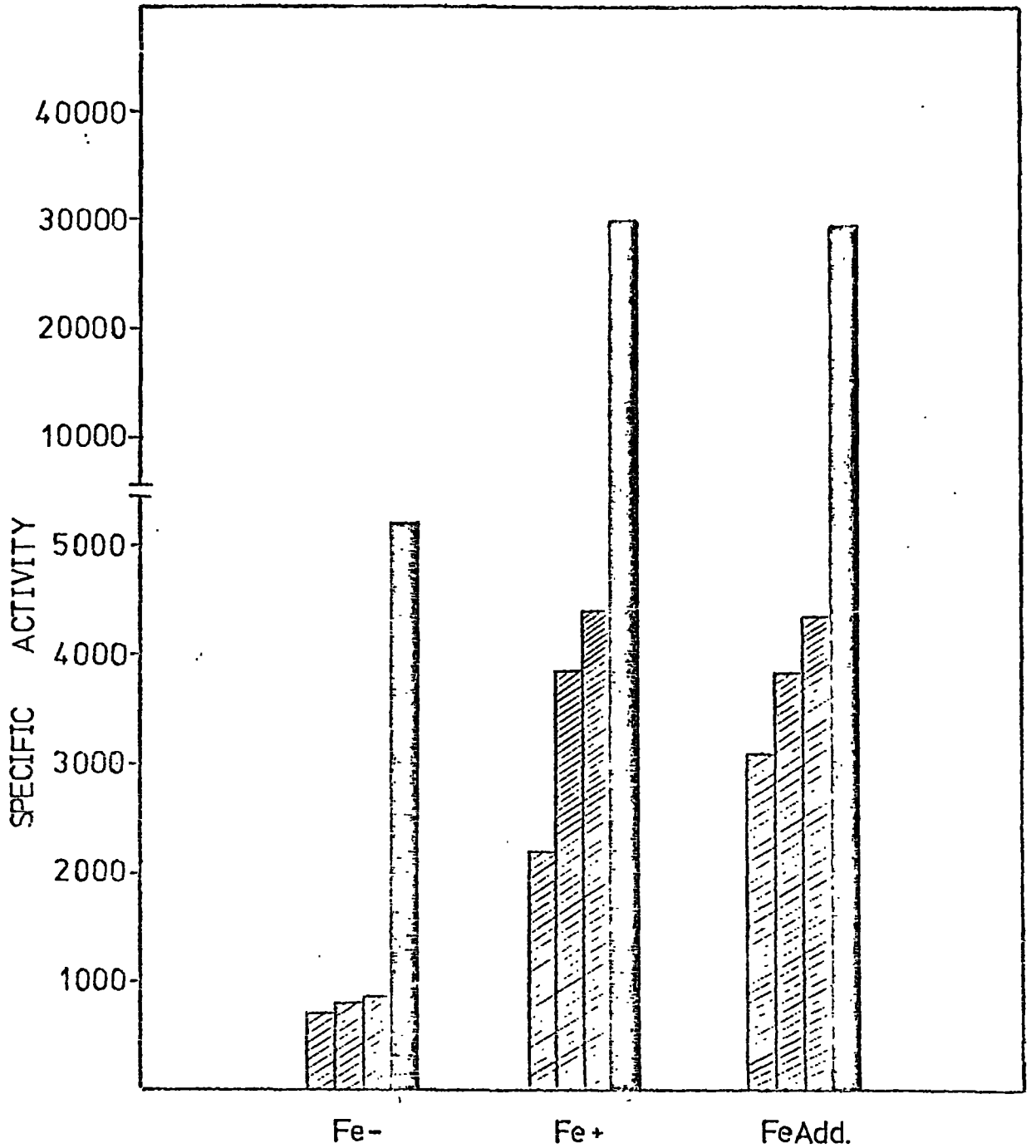

40000
30000
20000
10000
5000
4000
3000
2000
1000
SPECIFIC ACTIVITY
Fe-
Fe+
FeAdd.

Fig. 9

Radiochemical profiles of polyacrylamide 5% gels. Gel buffer was 0.05 M phosphate (pH 7.5) and electrophoresis was carried out at 5 mA/gel for 12 hours. Top of the gel at left

A) Iron (15µg/ml MM) added 43 hours after inoculation

B) Iron (15µg/ml MM) added 43 hours after inoculation but in presence of actinomycin D (20µg/ml MM)

C) As B but in presence of cycloheximide (20µg/ml MM)

D) As A but without iron added

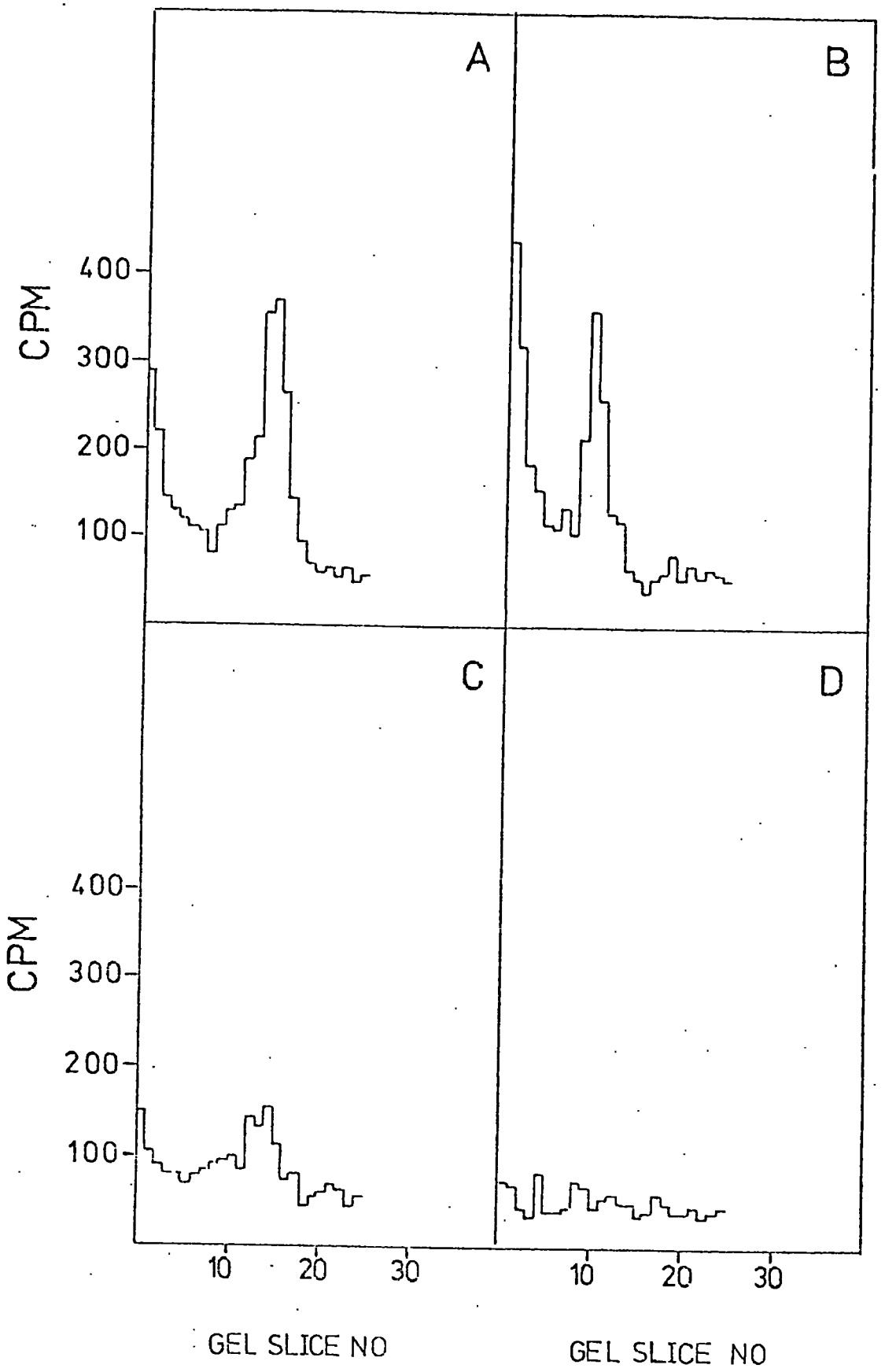

A
B
C
D
CPM
400
300
200
100
10
20
30
GEL SLICE NO

Fig. 10

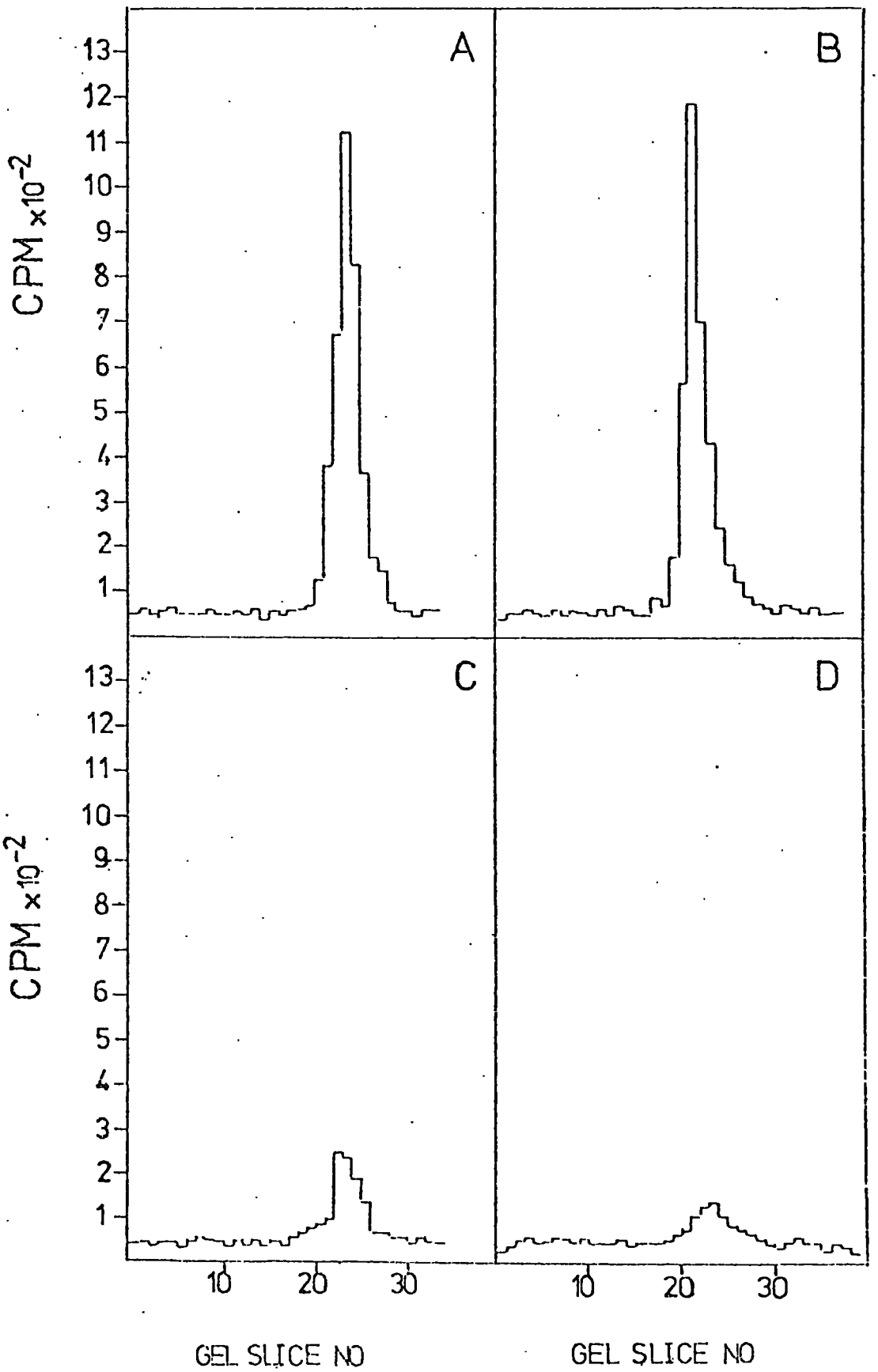
Radiochemical profiles of polyacrylamide 10% gels.
Running gel buffer was Tris-HCl (pH 8.8) and electrophoresis was carried out in a tris/glycine-SDS buffer (pH 8.3) for about 3 mA/gel. Top of gels at the left.

A) Iron (15µg/ml MM) added 43 hours after inoculation

B) Iron (15µg/ml MM) added 43 hours after inoculation but in presence of actinomycin D (20µg/ml MM)

C) As B but in presence of cycloheximide (20µg/ml MM)

D) As A but without iron added.


A
B
C
D
CPM x10-2
CPM x10-2
13
12
11
10
9
8
7
6
5
4
3
2
1
10
20
30
GEL SLICE NO
GEL SLICE NO

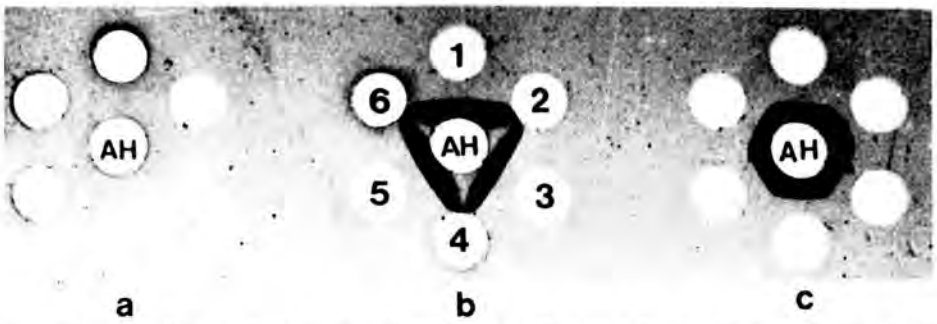


Fig. A) Immunodiffusion reactions of anti-horse ferritin (AH) against:-

a) serial dilutions of phycoferritin

b) phycoferritin (wells 2, 4 & 6) and horse spleen ferritin (wells 1, 3 & 5)

c) serial dilutions of horse spleen ferritin

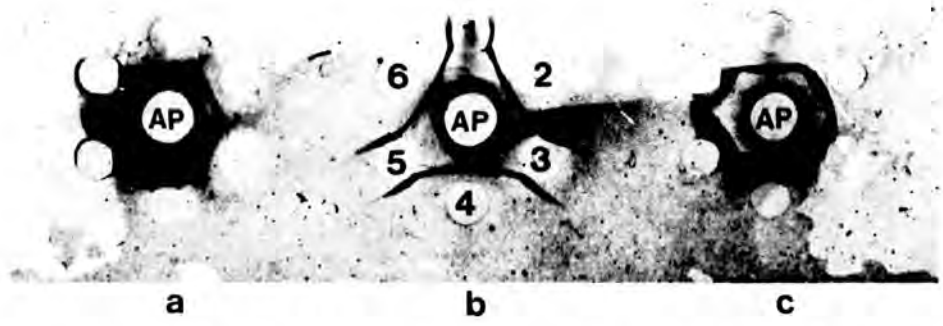


Fig. B) Immunodiffusion reactions of anti-phycoferritin (AP) against:-

a) serial dilutions of native phycoferritin

b) native form (wells 2, 4 & 6) and SDS-subunit complex (wells 1, 3 & 5)

c) As a).

Plate 38.

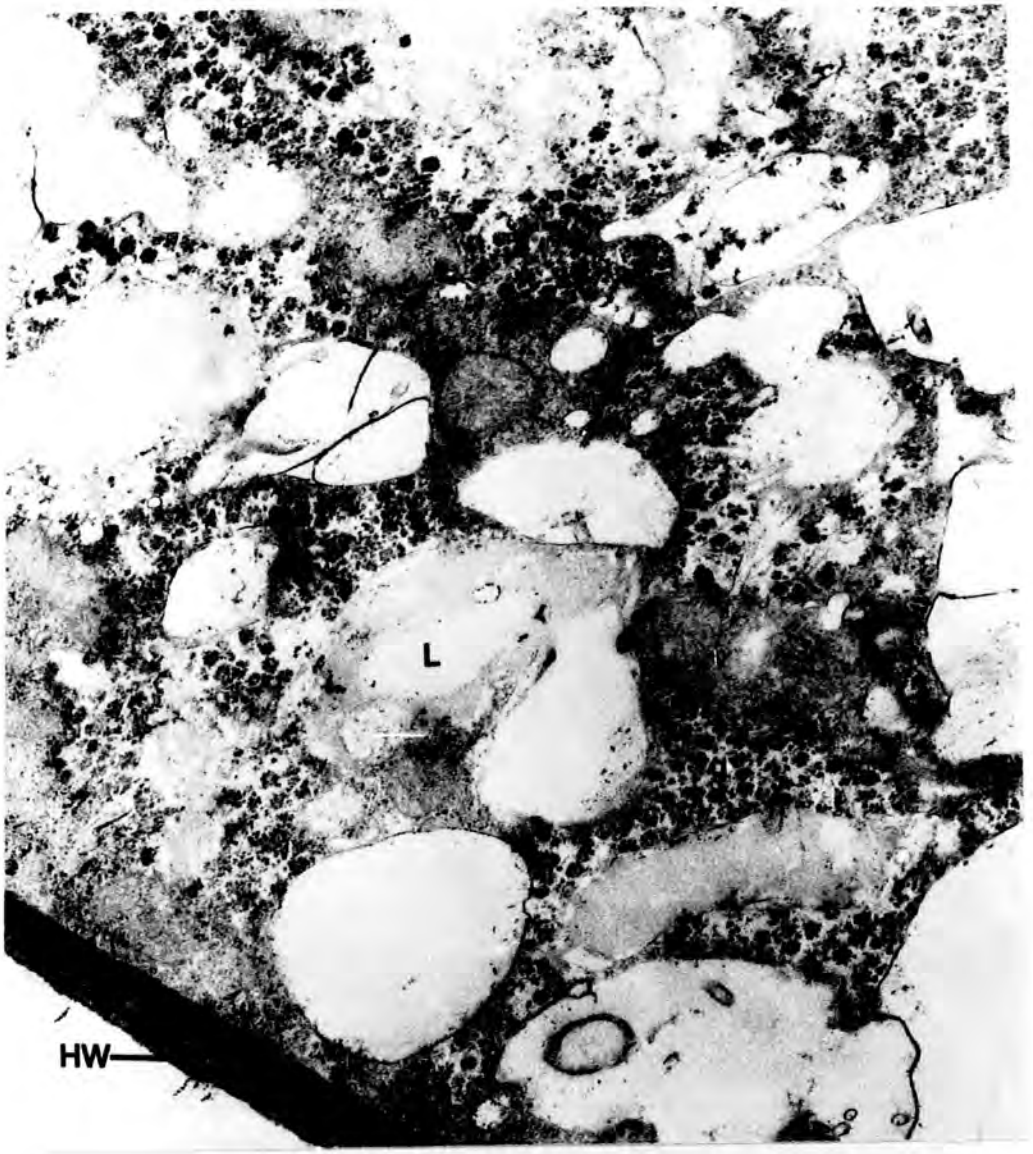


Plate 39. Section through a hypha growing in minimal medium without iron supplement, 43 hours after heat shock.
X 26,750
Glutaraldehyde/formaldehyde-osmium fixation.

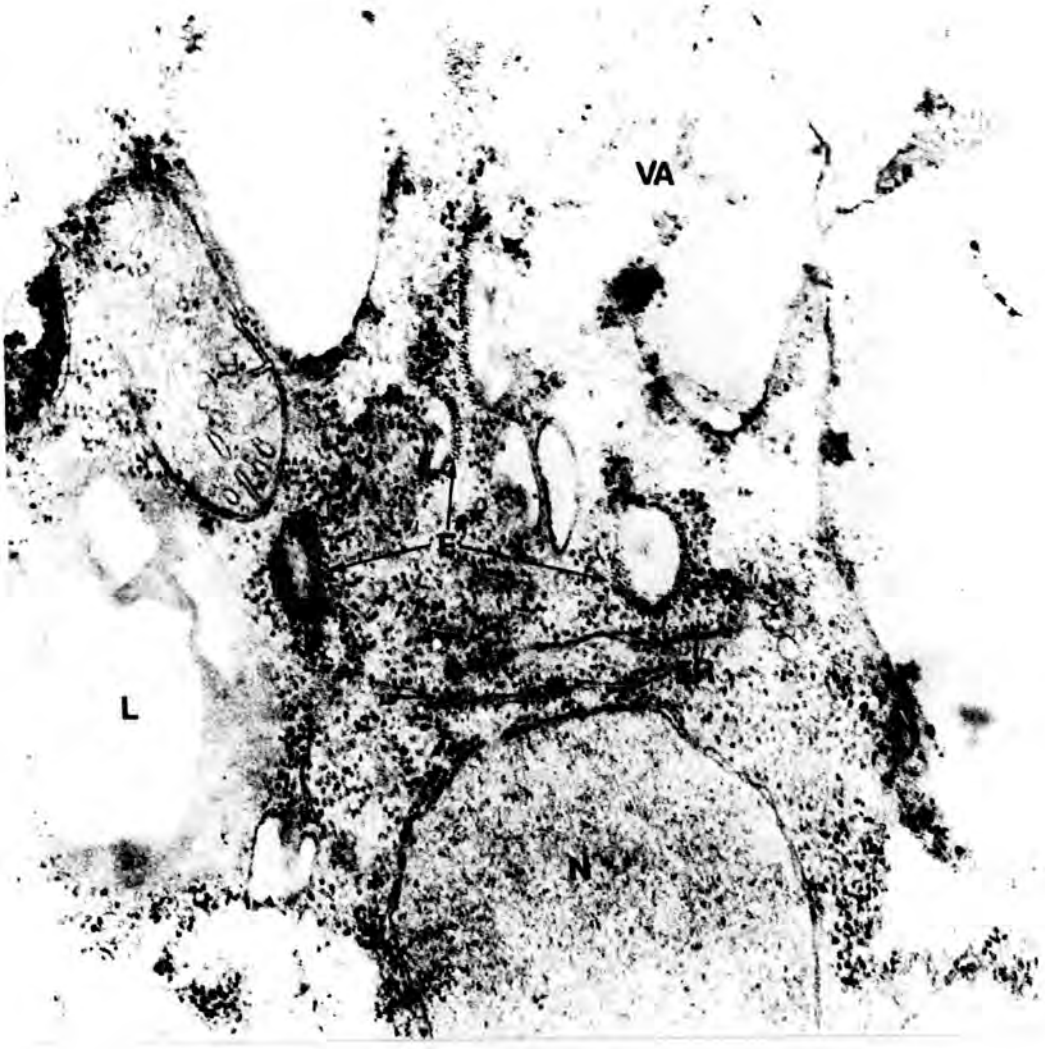


Plate 40. Section through a hypha grown for 43 hours after heat shock in minimal medium without iron supplement, and then for a further 10 hours after addition of iron.
X 15,750
Glutaraldehyde/formaldehyde-osmium fixation.

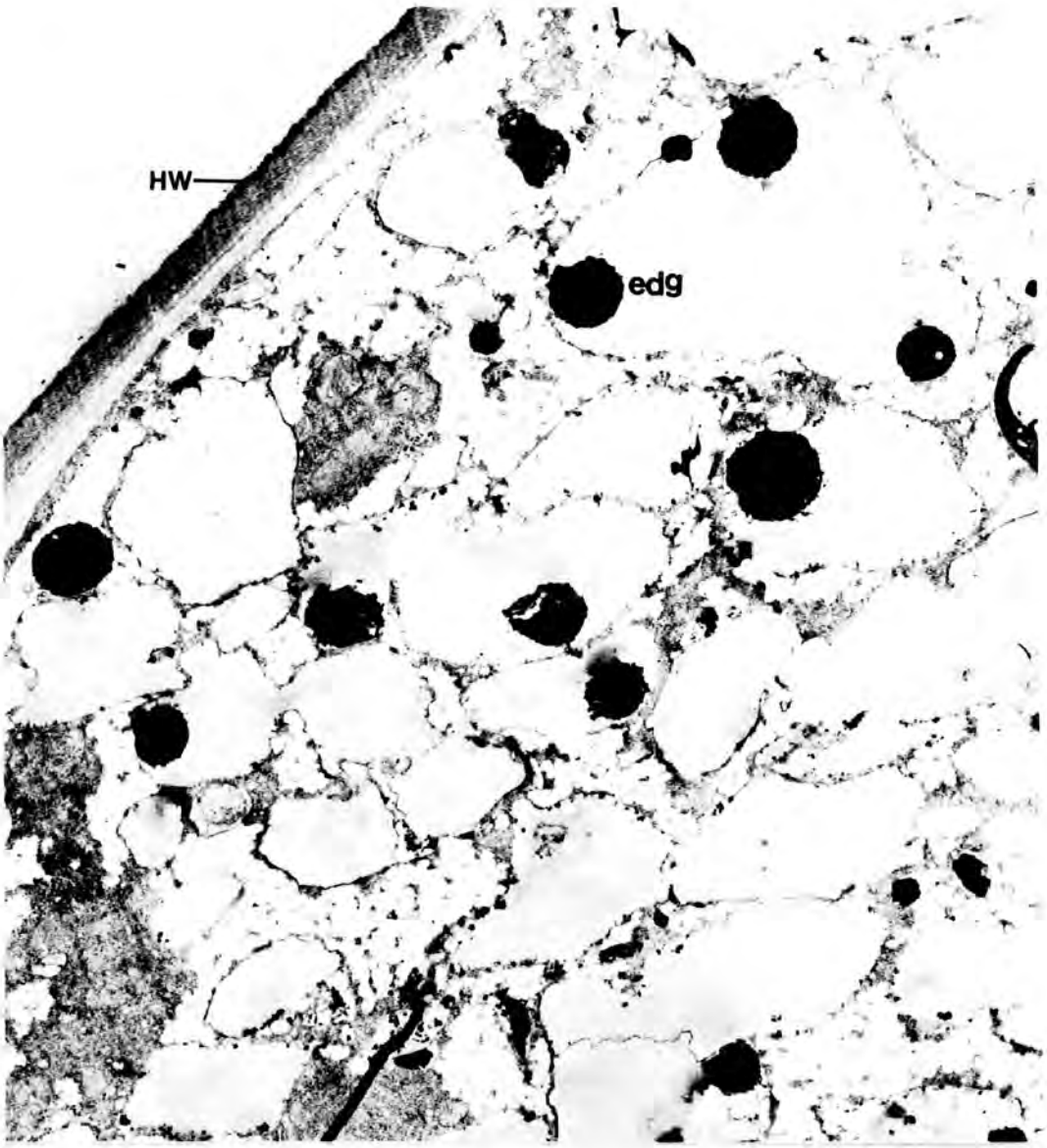


Plate 41. Section through a hypha grown for 43 hours after heat shock in minimal medium without iron supplement, and then for a further 20 hours in presence of iron and cycloheximide. X 16,620.
Glutaraldehyde/formaldehyde-osmium fixation.

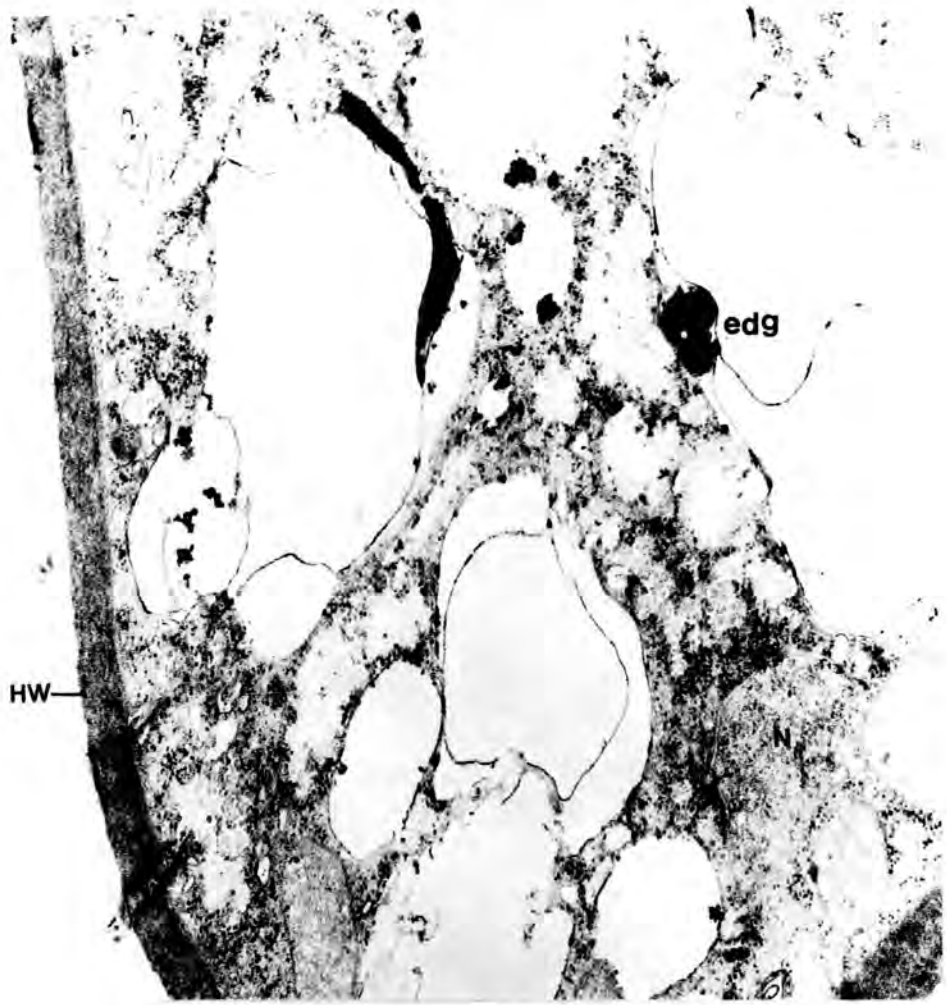


Plate 42. Section through a hypha grown for 43 hours after heat shock in minimal medium without iron supplement, and then for a further 20 hours in presence of iron and actinomycin D. X 30,240. Glutaraldehyde/formaldehyde-osmium fixation.

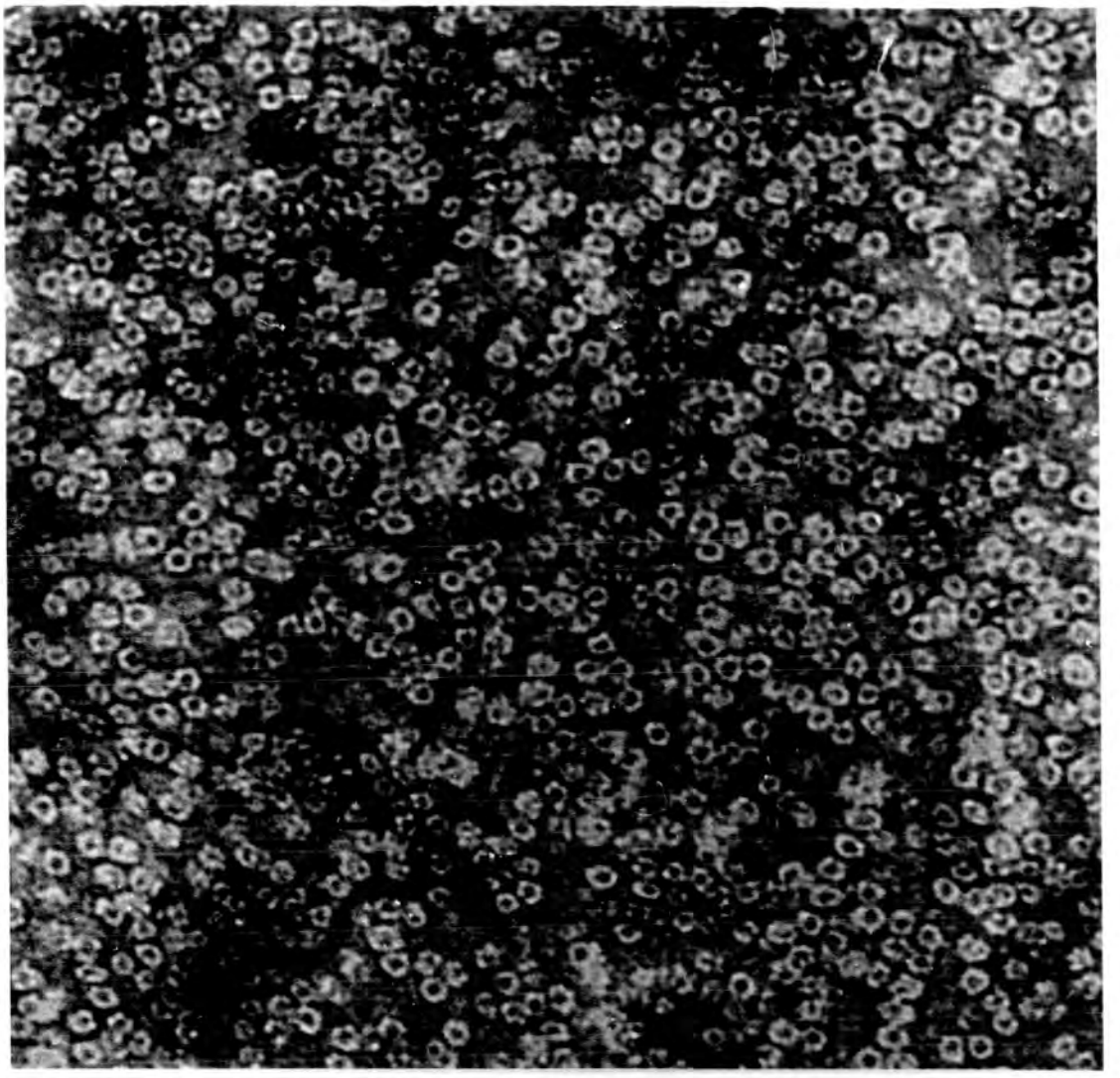


Plate 43 Negatively stained phycoferritin molecules. A drop of the protein solution was applied onto a carbon-coated grid for one minute followed by brief washing with water then a drop of saturated aqueous uranyl acetate was applied for 30 seconds, drained and dried.

X 300,000 approximately

Discussion

Phycoferritin isolated from mycelia, grown in high iron supplemented medium gave a maximal yield of up to 25 mg per 100 g mycelium. This was considered to be a high yield compared to phytoferritins obtained from seeds of pea (8 mg/kg) and lentil (20 mg/kg) as reported by Crichton et al., (1978).

Using only three markers, David & Easterbrook (1971) first reported a subunit molecular weight of 25,000 daltons for phycoferritin by using the method of Weber & Osborn (1969). This figure was slightly higher than for horse ferritin subunit (known to be 24,000 daltons at that time). Later, according to personal communication with Crichton, David (1974) reported a subunit molecular weight of 18,500 daltons for phycoferritin. Crichton et al., (1975), in a table, reported a subunit molecular weight of 18,000 daltons rather than 18,500 quoted by David (1974). Such a situation necessitated the use of several protein markers of a narrow range of subunit molecular weight to resolve some doubts about a realistic subunit molecular weight for phycoferritin. In this study it was shown first, based on gel co-electrophoresis, that phycoferritin and horse ferritin subunits had discrete bands, thus eliminating the possibility of a nearly equal molecular weight as was reported by David & Easterbrook (1971). The use of several protein markers and the reasonable reproducability gave a clearer picture. The estimated subunit

molecular weight reported here (20,200 daltons) resembled that of the mycoferritin of Mortierella alpina which was reported to be 19,300 by Bozarth & Goenaga (1972) and that of phytoferritins of pea (20,300) and lentil (21,400) reported by Crichton et al., (1978).

The presence or absence of iron in the medium seems to exert its influence on the mycelial growth, colour and the amount of phycoferritin obtainable. The nearly equal amounts of phycoferritin from either two or three day-old mycelia (grown in presence of iron) suggests that the protein was produced as one batch at a certain stage of growth which coincided with a noticeable carotenogenesis activity. This was supported by the finding that the addition of iron to growing mycelia (48 hours after inoculation) resulted, one day later, in a nearly equal yield of phycoferritin compared with cultures which received iron prior to inoculation. The only difference observed, in the latter case, was in the level of phycoferritin iron being slightly higher in phycoferritin obtained upon the addition of iron to growing mycelia. This could, perhaps, be due to a more efficient uptake and/or incorporation of iron as phycoferritin at this stage.

The presence of a high concentration of zinc salt in the medium was shown to affect the organism as a whole and ferritin-iron relationship in particular. The reduced

yellowish colouration observed could be attributed to an overall attenuation of the carotenogenesis capacity during the period studied. Equally zinc deficiency in the medium of growing mycelia of Phycomyces was reported to affect the activity of tryptophansynthetase (Hilgenberg & Hofmann, 1977).

The effect of high zinc did not impair the production of ferritin, whether iron was present initially or was added during the growth of mycelia. The only observed effect was in the amount of iron present in the two samples. This was approx. twice as much as in ferritin obtained from mycelia grown initially in the co-presence of high iron and zinc. The addition of iron to two day high zinc-fed culture, unexpectedly, did not affect the ferritin iron. This is somewhat different from observations of the effect of a high zinc diet on animal ferritin. Coleman & Matrone (1969) showed that rats fed on a high zinc diet (0.75%) had a low iron content of their liver ferritin. Nevertheless, injection of iron salts still provoked an increase in liver ferritin synthesis and accumulation, even though the ferritin continued to have a low iron content. Zinc ions were found to inhibit the oxidation of ferrous to ferric iron by apoferritin and thus interfered with the capacity of ferritin to store iron as ferric oxyhydroxide (Munro & Linder, 1978). Finally, Treffry et al., 1977 reported a competition between zinc and

terbium (Tb^{3+}) ions binding to apoferritin and a competition between these ions and the uptake of iron. Whether zinc ions interfere in a similar fashion with Phycomyces ferritin requires further investigations since this organism is well suited for such studies due to easier manipulations than the more complex animal material.

David (1974) used (^{59}Fe) in his studies on Phycomyces ferritin in which he observed a fiftyfold increase in ferritin iron as a result of growing the fungus in a high iron supplemented medium. He concluded that such an increase was "probably too great to be accounted for simply by the addition of iron to unfilled ferritin molecules". He thus suggested that a de novo synthesis of apoferritin had occurred in a similar fashion to the induction of apoferritin synthesis upon iron administration in animal tissues. Prior to these suggestions, he (cited in Bergman et al., 1969) demonstrated that in Phycomyces the uptake of ^{3}H-leucine into three day-old mycelia was very low compared to Neurospora crassa under similar conditions.

Bearing the above mentioned findings and suggestions in mind, the question raised by Bergman et al., (1969) whether apoferritin synthesis was regulated by the iron level in the medium was approached by the use of radioactive amino acids.

The results of the present study suggest first that for the sake of obtaining positive and sufficient incorporation, to demonstrate iron stimulation of phycoferritin synthesis, very high concentration of the label should be used together with conditions that would provide maximal mycelial yield. Thus the use of ^{3}H-leucine at a final concentration of 120μCi/ml MM, reasonably gave authentic counts of sliced gel of the electrophoresed native and denatured phycoferritin. The fact that high levels of ^{3}H-leucine were required to give adequate incorporation into ferritin suggests that the synthesis of the protein is not limited by amino acid pool size. The specific activities of the labelled phycoferritin showed a sixfold increase in phycoferritin synthesis induced by the presence of iron. This is compatible with results reported for animal ferritin (Drysdale & Munro, 1966). These results also indicate that, in the presence of iron, phycoferritin was synthesized at a certain stage of the differentiating and growing mycelia. Thus the electron microscopical observation reported in the first chapter about the reappearance of ferritin upon the availability of lipid droplets are greatly supported by these findings. The use of Actinomycin D and cycloheximide in the labelling experiment confirmed preliminary findings that iron presence or addition induced phycoferritin synthesis. That such an induction was not inhibited by Actinomycin D seemed

compatible with results obtained for animal ferritin, when the antibiotic was used at a reasonable dose (80-150µg/100g of rat), as shown by Drysdale & Munro (1966) and Miller et al., (1970). Higher dosage of the drug (1500µg/100g body weight) was reported to have an inhibitory effect (Yoshino et al., 1968) but such a dose was known to impair not only mRNA synthesis but also the whole synthetic machinery of the cell (Fagard & Saadi, 1977). The finding for Phycomyces indicated that the mechanism of iron induction probably did not occur at a transcriptional level.

The protein inhibitor, cycloheximide has been reported to exert deleterious effects beside its inhibition capacity (McMahon, 1975). Admittedly such effects were observed in mycelia of Phycomyces growing in the presence of the drug when its concentration exceeded 20µg/ml MM. This was manifested in what looked like a lysis or disintegration of mycelial walls and a consequent outflow of cell contents as judged by the yellowish colouration of mycelial filtrates. Nevertheless, the concentration employed in the labelling experiment did not clearly show such an effect, neither did it cause a complete inhibition of radioactivity incorporation into the protein sample obtained. However, the result clearly indicated an inhibitory effect of the drug on iron induction of ferritin synthesis. This, again,

is compatible with findings reported for animal ferritin (Drysdale & Munro, 1966; Millar et al., 1970; Saadi et al., 1974; Zahringer et al., 1975) and indicated that the stimulant effect of iron might have occurred at the translational level. An up to date full discussion on the mechanism by which iron induces ferritin synthesis in animal tissues has been recently reviewed (Munro & Linder, 1978).

General Discussion and Conclusion

According to Munro & Linder (1978) the estimated total iron content of the body of the adult human is about 3g, being somewhat greater in males. Of this iron about 65% is accounted for by circulating haemoglobin, and some 10% is present in specific functional tissue compounds, primarily myoglobin, with small amount of iron in the cytochromes of mitochondria and in iron containing enzymes (e.g. catalase, peroxidase, succinic dehydrogenase). Iron retained in the storage forms of ferritin and hemosiderin accounts for the remaining 20%. This quotation justifies the need for the existence of ferritin in animal tissues as an iron store destined to meet the supply and demand of iron so that the body would maintain its proper physiological machinery.

Although the role of animal ferritin is reasonably well understood and established in the literature, very little is known about the function of phytoferritin. The appearance of phytoferritin in the leaves of 'iron-treated' plants could serve as a buffering intermediate in the iron metabolism by protecting the cell against the toxicity of the over-absorbed iron (Seckbach, 1972). The naturally occurring phytoferritin in pea embryos and cotyledons and in young bean leaves suggests that phytoferritin represents the iron store-house from which developing plastids draw iron during development of the photosynthetic apparatus (Hyde et al., 1963).

The role of phycoferritin in Phycomyces has not yet been fully elucidated. However, the facts are that phycoferritin iron is selectively accumulated in vegetative spores (David & Easterbrook, 1971) and that its release, into soluble pools, is controlled upon the germination of spores of this organism (David, 1974). This suggests its involvement in biosynthetic processes that require iron rather than having a sole function as an iron detoxification agent. This is supported by the findings in this study that phycoferritin could not be detected or isolated after the establishment of mycelia (24 hours after inoculation)

the latter were grown in the presence or absence of iron. Moreover, the EM detection and the isolation of phycoferritin at a certain stage during mycelial differentiation could have more than one implication. Firstly, it was shown that phycoferritin could only be detected in sections of mycelia grown in the presence of iron. This detection coincided always with the availability of lipid droplets. Since β-carotene has been shown to be located in such lipid droplets (Bergman et al., 1969), it could be suggested that ferritin appearance or synthesis is somewhat coordinated with carotenogenesis. That such an appearance was a result of a de novo synthesis of the protein moiety, whether iron was initially present or whether it was added at the initiation of carotenogenesis, strongly indicates that ferritin synthesis

during differentiation is critically timed. Based upon these findings one could speculate that, since the initiation of sporangiophores, 2-3 days after inoculation on a solid medium, is preceded by a clearly observed yellowish colouration of the supporting mycelia, it could be at this stage that ferritin is synthesized and located on the surface of lipid droplets ready for its final destination in the vegetative spores. This migration was found to be accompanied by maturation of iron content of individual protein molecules as was shown by the broad spectrum of Svedberg values of ferritin isolated from growing sporangiophores (David, 1974). This again strengthens the possibility of a functional role for phycoferritin during germination. Whether such a function would have a link with the mitochondrial changes observed prior to the carotenogenic appearance of mycelia, grown in different media, needs critical investigation. It should also be established whether the mitochondrial changes are innate or adaptive characteristics. In this regard, Keyhani et al., (1972) studied the cytochrome system of two strains of Phycomyces before and during germination by using a reduced-minus-oxidized difference spectrophotometer. They found that the ungerminated spores contained the complete cytochrome system and that the cytochrome content was the same before and just after the heat shock treatment. During

germination the amount of cytochromes increased markedly. This increase would probably involve synthesis of more cytochromes and thus a considerable demand for iron.

It is believed that the sixfold increase in the synthesis of the protein moiety of phycoferritin observed in this study, as a result of a high iron concentration in the medium, is a stronger evidence for iron induction of ferritin synthesis than the fiftyfold increase in ferritin iron reported by David (1974). The combined use of labelled amino acids and antibiotics revealed that such a de novo synthesis of apoferritin might have occurred in the presence of a preformed mRNA molecule and that the iron induction might have taken place at a translational level.

Further studies on phycoferritin are needed to elucidate whether such a synthesis occurred predominantly on free polysomes or on bound polysomes as was reported for animal ferritin (Zahringer et al 1977). It is anticipated that phycoferritin synthesis is likely to occur on free polysomes since, from my experience, typical membrane-bound ribosomes have not been observed in the numerous micrographs of sections through Phycomyces mycelia. This is supported by the findings of Pearson & Thomas (personal communication) who found that polysomes isolated from mycelia of this fungus, at various times, consisted almost entirely of free polysomes. This

pattern was not affected by the addition of iron to growing mycelia neither did the profiles of ribosomal RNA's change with this addition.

In conclusion it should be mentioned that Phycomyces germinating spores and mycelia represent a very amenable model for studying many aspects of iron metabolism. This is facilitated by the advantage of having a known time at which this protein could be synthesized in the mycelia of this fungus. In addition it was found that accumulation of β-carotene by wild-type Phycomyces can be strongly stimulated by external agents such as light and vitamin A (see reviews by Cerda-Olmedo, 1974, 1977) and Sandman & Hilgenberg (1978) thus providing more control on the regulation of carotene synthesis and hence its correlation with phycoferritin appearance. Finally, the conspicuous location of phycoferritin on the surface of β-carotene-containing lipid droplets is very attractive and offers a much simpler system than that of animals for the study of this specialized protein.

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Appendix 1

List of some ultrastructural studies performed on germinating spores or conidia of fungi from major classes of the Eumycotina

1. Chitridiomycetes	Blastocladiella emersonni	(Cantino et al., 1963)
2. Oomycetes	Phytophthora parasitica	(Hemmes et al., 1971)
3. Zygomycetes	Rhizopus species	(Hawker & Abbot, 1963)
	Rhizopus arrhizus	(Ekundayo, 1966)
	Rhizopus arrhizus	(Hess & Weber, 1973)
	Rhizopus arrhizus stolonifer	(Buckley et al., 1968)
	Rhizopus sexualis	(Hawker & Beckett, 1971)
	Mucor rouxii	(Bartnicki-Garcia, 1968b)
	Cunninghamella elegans	(Hawker et al., 1970)
	Cunninghamella echinulata	(Khan, 1975)
	Piptocephalis unispora	(Jeffries & Young, 1976)
	Phascolomyces articulosus	(Jeffries & Young, 1976)
4. Ascomycetes	Saccharomyces cerevisiae	(Hashimoto et al., 1958)
	Saccharomyces cerevisiae	(Steele & Miller, 1974)

	Saccharomyces cerevisiae	(Kreger-Van Rij, 1978)
	Aspergillus oryzae	(Tanaka, 1966)
	Aspergillus niger	(Tsukahara, 1968)
	Aspergillus nidulans	(Border & Trinci, 1970)
	Aspergillus nidulans	(Florance et al., 1972)
	Penicillium megasporium	(Remsen et al., 1967)
	Penicillium griseofulvum	(Fletcher, 1971)
	Penicillium chrysogenum	(McCoy et al., 1971)
	Penicillium notatum	(Martin et al., 1973)
	Neurospora tetrasperma	(Lowry & Sussman, 1968)
	Sphaerotheca macularis	(Mitchell & McKeen, 1970)
5. Basidiomycetes	Psilocybe species	(Stocks & Hess, 1970)
	Coprinus lagopus	(Heintz & Niederpruem, 1971)
	Coprinus cinereus	(McLaughlin, 1977)
6. Deuteromycetes	Alternaria brassicola	(Campbell, 1970)
	Botrytis fabae	(Richmond & Pring, 1971)
	Botrytis cinerea	(Gull & Trinci, 1971)

Fusarium culmorum	(Marchant, 1966)
Fusarium oxysporum	(Griffiths, 1973)
Fusarium sambucinum	(Stalhammer-Carlemalm, 1976)
Trichoderma viride	(Rosen et al., 1974)
Geotrichum candidum	(Steele & Fraser, 1973)

Appendix 2

Amino acid composition of phycoferritin. The single sample was processed as mentioned in Materials and Methods (Chapter two).

Amino Acid	No. Residues/mol
Asp	5
Thr	3
Ser	3
Glu	10
Pro	12
Gly	4
Ala	6
Val	3
Met	1
Ileu	2
Leu	6
Tyr	2
Phe	1
His	1
Lys	3
Arg	2
Cys	1