THE FORMATION AND TRANSPORT OF PERIPHERAL VESICLES IN *PHYTOPHTHORA CINNAMOMI*.

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STATEMENT

All research reported in this thesis

is original and my own

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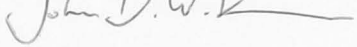

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For Heidi.

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ABSTRACT

This dissertation examines aspects of the process of peripheral vesicle formation in the fungal oomycete *Phytophthora cinnamomi*. Although peripheral vesicles had been extensively studied in sporangia and zoospores, little was known of their behavior in other life cycle stages and the fundamental question whether peripheral vesicles were associated solely with sporulation remained unanswered. This investigation was motivated by two things. A need to further understand the importance of peripheral vesicles to the biology of *P. cinnamomi* 6BR, and secondly, the thought that such a study might elucidate more information on the phenomena of secretory vesicle biogenesis and transport.

In the first chapter I report the examination of various stages in the asexual life cycle of the fungus including chlamydospore development and sporangial development from vegetatively growing populations of germlings and hyphae. The three peripheral vesicles, large peripheral, ventral and dorsal vesicles were not present in vegetatively growing hyphae but appeared during chlamydospore and sporangial development. The vesicles were compared with sporulation specific proteins from other fungi. Peripheral vesicles were present in chlamydospores. These structures perpetuate the fungus under adverse conditions but can germinate rapidly to form hyphae, sporangia and zoospores with a minimum of synthesis required. Large peripheral and ventral vesicles appeared sooner in sporangial development than dorsal vesicles. Also, after the encystment of zoospores, populations of ventral and large peripheral but not dorsal vesicles remained in the cyst cytoplasm for some time. These pieces of evidence suggest that large peripheral and ventral vesicles may act as nutrient reserves in vegetatively growing hyphae. An alternative hypothesis is presented for the small population

of ventral vesicles left in the cyst cytoplasm. The situation may be similar to that in *Saprolegnia ferax* where a small population of adhesive vesicles remained in the cyst cytoplasm in the case of repeated zoospore emergence and encystment. As dorsal vesicles appeared later in sporangial development than large peripheral and ventral vesicles and they were secreted completely from zoospores, they may play solely a role in zoospore encystment. Unlike the situation in many other oomycetes, peripheral vesicles in *P. cinnamomi* are formed in pre-sporangial hyphae perhaps suggesting that the cellular machinery necessary for their synthesis is unavailable during sporangial development. This first chapter showed the advantages of immunofluorescence microscopy over immunodotblotting for more accurately monitoring intracellular antigenic components.

The second chapter reported an investigation into the ultrastructural formation of the peripheral vesicles. Immunological evidence showed clearly that large peripheral and dorsal vesicles were derived from the Golgi apparatus. Although, immunolabelling suggested the ultrastructural origin of the ventral vesicles was the Golgi apparatus, morphological evidence provided the most convincing proof that these vesicles were Golgi-derived. In their mode of formation these peripheral vesicles were similar to secretory and storage proteins of other eucaryotes. Immunolabelling with antibodies to large peripheral and dorsal vesicles suggested the existence of an intermediate E.R.- Golgi apparatus compartment. As this was the first region within the secretory pathway in which the dorsal vesicle antigen appeared, it was probable this intermediate compartment played a role in the maturation of secretory proteins. Double labelling indicated that two vesicle antigens could be present in the same Golgi cisterna, but that a mechanism existed in *trans* Golgi regions which ensured the separation of the antigens into distinct vesicles.

In the third chapter I reported the effects of four drugs on *P. cinnamomi* hyphae. These drugs had previously been shown to perturb the secretory pathway in animal cells. Immunofluorescence microscopy showed that after incubation in the drugs, peripheral vesicle formation was affected. In hyphae treated with tunicamycin the formation of all three vesicles was almost completely inhibited. As this drug inhibits the addition of *N*-linked glycans to glycoproteins it is probable that the vesicle antigens contained such moeities. In electron micrographs no vesicles could be seen budding away from the Golgi apparatus and the E.R./nuclear envelope appeared swollen. Double labelling with the Lpv-1 antibody and the DNA specific nuclear stain DAPI, suggested that the large peripheral vesicle antigen was found in this swollen region. This would suggest that the loss of *N*-linked glycans from the Lpv-1 antigen affects its transport competence and causes the antigen to accumulate in the E.R. Exposing hyphae to the drugs monensin and brefeldin A (BFA) for 1h, proved the ideal period in which to examine the effects of these drugs on the secretory pathway. Both monensin and BFA caused the Golgi apparatus to break down and immunofluorescence microscopy suggested that vesicle antigens were restricted to the E.R. This was an unexpected effect for monensin as there are only a few previous accounts reporting such a cellular response. Cytochalasin D did not impede the formation of peripheral vesicles but appeared to block the transport mechanism which was responsible for the movement of peripheral vesicles away from the Golgi apparatus.

The mechanism of vesicle transport away from the site of vesicle formation and into the developing sporangium was further investigated (Chapter 5). The actin skeleton, comprising actin plaques and microfilaments was prominent in sporangiophores of developing sporangia and suggested that peripheral vesicles

could be transported via this component of the cytoskeleton. Double staining with soy bean agglutinnen (SBA)-rhodamine, which is specific for dorsal vesicles, and antibodies to the tubulin cytoskeleton, showed that peripheral vesicles had no association with microtubules in sporulating hyphae. Microtubules were associated with the nuclei of vegetatively growing hyphae but were not associated with the smaller nuclei which were translocated into the developing sporangium. This might suggest that although microtubules maintain nuclear shape within vegetative hyphae, they break down at sporulation and play no role in organelle transport into the developing sporangium.

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CHAPTER ONE

1.1 A REVIEW OF PERIPHERAL VESICLES IN OOMYCETES.

1.1.1 Introduction

Under appropriate conditions, oomyceteous fungi can produce large numbers of motile, biflagellate zoospores. These propagules swim in water or moist soils and are attracted chemotactically to hosts. Once in the proximity of a potential host, zoospores undergo encystment, become firmly adhered to the host surface and produce a cyst coat and a cell wall. After a short period, encysted zoospores germinate and penetrate the host.

Early ultrastructural studies attempted to ascertain the cellular changes involved in the transition from zoospore to nonmotile cyst (Grove 1970; Hemmes & Hohl 1971; Hoch & Mitchell 1972b). In these first studies, attention was drawn to populations of vesicles located in the peripheral cytoplasm of zoospores. It became clear that these organelles, present in different forms in the various oomycetes, were playing an important role in the infection of hosts.

1.1.2 Peripheral vesicles in the Saprolegniales

The presence of bar-like structures, or bars in *Saprolegnia ferax* sporangia and zoospores was first reported by Gay and Greenwood (1966). These structures, absent from vegetative hyphae, were bound by a single membrane and contained a central region of fine striations or spines. Positioned in zoospores so that they touched the plasma membrane, the authors were led to suggest that they may be secretory in function or may be involved in cell encystment.

Bars disappeared from *Saprolegnia ferax* zoospores at encystment (Heath & Greenwood 1970a) and as this was coincident with the formation of an outer cell wall and that the spines that were present inside bars appeared in the coat, it was suggested that bars were involved in cell wall and coat formation. After the disappearance of bars from encysting primary zoospores, vesicles containing a 'boat hook-like' spherical structure, appeared in the peripheral cytoplasm of primary cysts. These vesicles were termed secondary bars, as opposed to primary bars of primary zoospores and were hypothesised to be secreted from secondary zoospores (Heath & Greenwood 1970a).

Holloway and Heath (1977) confirmed Heath and Greenwood's idea that secondary bars were secreted from secondary zoospores, as 'boat hooks' appeared in the cell coat of secondary cysts. Holloway and Heath (1977) were the first authors to describe oomycete 'fibrous vesicles'. Containing loosely arranged coarse fibrils, these vesicles were shown to appear in primary cysts and to be present in secondary zoospores and cysts, though during secondary cyst germination they enlarged into large vacuoles.

The term, 'encystment vesicles' was preferred by Beakes (1983) for the bars of *Saprolegnia.* Beakes distinguished between primary and secondary encystment vesicles, according to whether the vesicles were secreted from primary or secondary zoospores. Primary encystment vesicles were shown to originate in zoosporangial initials and during zoospore development these organelles became peripherally distributed and aligned perpendicularly to the plasma membrane. Most primary encystment vesicles were shown to be discharged upon encystment of primary zoospores, with the outer primary cyst wall being derived from the peripheral matrix of the vesicle. Differentiation of secondary encystment vesicles was shown to commence during the maturation of the primary cyst. These

vesicles were shown eventually to become situated in clusters in the peripheral cytoplasm of the secondary zoospore until encystment, when they were mostly secreted to form a cyst coat. Beakes (1983) indicated that the fibrous vesicles first appeared during the stage of secondary encystment vesicle formation. Occasionally, thin section profiles of fibrous vesicles could be observed apparently discharging from secondary zoospores and although it was suggested that this may have been part of the encystment process, it was also likely to have been fixation induced. Fibrous vesicles became peripherally situated in secondary zoospores but were not secreted at encystment, being eventually broken down in secondary germlings. In addition to peripheral populations of secondary encystment vesicles and fibrous vesicles in *Saprolegnia*, Beakes (1983) reported an extensive system of flattened cisternae underlying the plasma membrane of secondary zoospores. Hoch & Mitchell (1972a, b) had reported the existence of long and usually narrow vesicles underlying most of the plasma membrane of *Aphanomyces euteiches* zoospores, but had attributed no role to them. Beakes (1983) suggested that during encystment of *Saprolegnia ferax* and *S. parasitica* zoospores, peripheral cisternae fragmented into small vesicles with distinctly fibrillar contents, which, together with Golgi-derived wall vesicles, probably formed the cyst cell wall. It was suggested that peripheral cisternae in secondary zoospores may have an additional role in secondary zoospores in providing some sort of physical support for the plasma membrane.

Hoch & Mitchell (1972b) first observed an unidentified body which contained helical fibres positioned near the kinetesomes at the narrow end of the nucleus in *Aphanomyces euteiches* zoospores. These structures, termed U-bodies, were still observed in encysted zoospores but were only occasionally seen in germinated spores, leading the authors to conclude that they played a role in zoospore

encystment. Because of their constant location next to the kinetesomes of primary and secondary zoospores of *Saprolegnia*, Holloway and Heath (1977) used the name 'K-bodies' for these structures. In their work on an unidentified *Saprolegnia,* two types of K-body were present. K1-bodies were first recognizable in the differentiating sporangium as small, roughly spherical structures containing a granular matrix with a narrow osmiophilic cortex. During primary zoospore development, K1-bodies became larger and had smoother membrane profiles. In mature zoospores the morphology of K1-bodies was quite variable, some being similar to those described for immature zoospores and some being smaller and similar to primary bars. K1-bodies were shown to be present in primary cysts and secondary zoospores, however, before the encystment of secondary zoospores K1-bodies were replaced by K2-bodies. K2-bodies were shown to be in some ways similar in appearance to the larger K1-bodies but were different in having a matrix component of loosely-packed coarse fibres, surrounded by an osmiophilic cortex and a large, central, highly osmiophilic core with closely packed tubules. K2-bodies remained in the encysted secondary zoospore but disappeared before the cyst germinated. The authors suggested that K1-bodies gave rise to primary and secondary bars but conceded that roles for K-bodies were still obscure.

In a review of the array of microbody-like structures in the oomycetes, Powell *et al.* (1985) suggested possible functions of K-bodies. Because of a proposed phylogenetic link between oomycetes and algae it was considered plausible that K-bodies were some sort of remnant plastid-like structure. Additionally, although K-bodies had been shown to react positively for peroxidase enzymes, an alternative storage role was suggested for these organelles.

Subcellular localisation of carbohydrates in secondary zoospores of *Saprolegnia ferax* was carried out by Lehnen and Powell (1988). The tubule filled cavity and not the granular matrix of the K2-body was shown to contain carbohydrates and sulphydryl groups and as this cavity was consistently located next to the plasmamembrane of zoospores, this was a strong suggestion that K2-bodies had a role in activities at the cell surface. Lehnen and Powell (1989) elucidated an adhesive role of K2-bodies in encysting secondary zoospores of *Saprolegnia ferax.* K2-bodies were shown to be discharged 30-60s after the induction of encystment and the vesicle matrices corresponded to a layer of adhesive material found between the cyst coat and the substrate.

1.1.3 Peripheral vesicles in the Leptomitales

The Leptomitales are considered as intermediates between the Saprolegniales and the Peronosporales (Bessey 1950). Only two ultrastructural descriptions exist for members of this order and one, on *Apodachlya* (Powell *et al.* 1985), reported the presence of a K-body located near the kinetesomes in a mature primary cyst. Gotelli and Hanson (1987) reported K-bodies in *Sapromyces androgynus* zoospores. These authors recorded peripherally located flattened cisternae and a small number of coarse fibrillar vesicles, though there was no discussion on possible functions of these organelles.

1.1.4 Peripheral vesicles in the Lagenidiales

Peripheral vesicles of different forms have been recorded for the family Lagenidiales. In *Lagenidium callinectes,* fibrous vesicles could be seen lining the periphery of the zoospore (Bland & Amerson 1973, Gotelli 1974) and because of

their location, were presumed to have some function in wall synthesis during spore encystment.

In their detailed account of the asexual life cycle of *Lagenisma coscinodisci,* Schnepf *et al.* (1978a) described the complicated formation of encystment vesicles during zoosporogenesis of this species. Mature encystment vesicles eventually lined the periphery of zoospores and were completely secreted at encystment to form the primary cyst wall (Schnepf *et al.* 1978b).

Zoospores of the marine oomycete, *Haliphthoros milfordensis* contain two morphologically distinct peripheral vesicles (Overton *et al.* 1983). One type, the large, fibrous vesicles located posteriorly (but not necessarily peripherally) within zoospores was shown to be secreted from encysting cells. Although there appeared to be a large proportion of this vesicle type left in cysts, the authors suggested that the released contents of these vesicles may be involved in wall formation or spore adhesion. Scattered around the zoospore periphery were numbers of electron dense vesicles, considered to be phospholipid vesicles. In micrographs these vesicles were substantially fewer in number after encystment but were claimed to be internalised and transformed into scallop-fringed vesicles. It was considered that these vesicles might serve as food reserves, or alternatively, it could be suggested, as they appeared late in zoospore development and were absent from vegetative hyphae, they may in fact contribute to cyst wall formation.

Zoospores of *Olpidiopsis saprolegniae* have been shown to contain three types of peripheral vesicles (Bortnick *et al.* 1985). Peripheral cisternae and peripheral vesicles containing hollow, concentrically arranged fibres lined most of the plasma membrane except along the groove. In the anterior end of the zoospore, a single

K-body was positioned near the kinetesome, leading the authors to suggest a role for the K-body in the regulation of flagellar motion.

1.1.5 Peripheral vesicles in the Peronosporales

Ho e*t al.* (1968) published the first ultrastructural account of a Peronsporalean type zoospore, that of *Phytophthora megasperma.* Numerous vesicles were the most conspicuous feature of the zoosporic cytoplasm with some actually appearing to project above the surface of the zoospore, though this was thought to be artifactual. Present along the periphery of the zoospore were some roughly spherical vesicles which were previously undescribed in fungi and were thought to be similar to microbodies of plant and animal cells. These organelles were bound by a well-defined single membrane and contained finely granular, evenly dispersed contents, though sometimes this contained one or more bars of extremely dense material. It was thought that these vesicles may contain sites of enzyme activity and it was considered significant that they were always positioned close to the zoospore surface.

Reichle (1969) examined the ultrastructure of the zoospore of *Phytophthora parasitica* and recorded the presence of two types of peripheral vesicles- granular/fibrous vesicles and vesicles traversed by rods and membranes. Directly underneath the plasmamembrane there were many flat vesicles which lay parallel to the zoospore surface. Hemmes and Hohl (1971) also recognised these flattened vesicles in *Phytophthora parasitica* and suggested that as they disappeared at encystment, they were probably responsible for the deposition of the initial wall layer of the cyst. These latter authors provided more information about the morphology of Peronosporalean microbody-like organelles. Structurally, these

organelles were described to be bullet-shaped and to be enclosed by a unit membrane and consist of a central core of fibres surrounded by one or two cisternae. They were shown to be present throughout encystment and germination and to be found at the cell periphery of secondary zoospores.

Bimpong and Hickman (1975) described the changes in ultrastructure of *Phytophthora palmivora* zoospores at encystment. Microbodies were shown to be no longer restricted to the periphery of cells at encystment but became distributed throughout the cytoplasm of cysts and germlings. When encystment was mechanically induced, there was no appreciable loss of large granular vesicles, though complete loss of flattened vesicles occurred. If zoospores were allowed to encyst in undisturbed conditions, both granular and flattened vesicles disappeared from the zoospore periphery and presumably contributed to the formation of the cyst coat. A number of granular vesicles remained in the cyst under both sets of encystment conditions and these were shown to be broken down during germination. Cytochemical assays were performed on the peripheral vesicles by these authors. The contents of the granular vesicles were shown to be removed by pepsin and to be resistant to the effects of organic solvents. In contrast, the microbodies were shown not to contain reaction product when tested for the presence of catalase with the diaminobenzidine (DAB) technique (c.f. Philippi *et al.* 1975). It was suggested that microbodies may contain other enzymes such as those necessary for the glyoxylate cycle.

Sing and Bartnicki-Garcia (1975b) hypothesised that peripheral (granular) vesicles may have dual roles in cell encystment. A function in cyst adhesion was suggested after electron micrographs revealed newly encysted zoospores adhering to artificial surfaces through the recently discharged contents of peripheral vesicles. An earlier study (Sing & Bartnicki-Garcia 1975a) had shown that the

peripheral vesicles of *P. palmivora* contained glycoprotein with receptor sites for concanavalin A. Sing and Bartnicki-Garcia (1975b) conjectured that the glycoprotein component of peripheral vesicles was involved in the binding of nascent wall microfibrils to the cell surface, as well as each other, during wall formation.

In 1977, Pinto da Silva and Nogueira provided extremely convincing freeze-fracture electron microscopy images of peripheral vesicles fusing with the plasmamembrane of encysting *P. palmivora* zoospores. Their observations put it seemed, beyond a doubt that peripheral vesicles and their equivalents in other oomycetes were involved with adhesion and wall formation during encystment. This idea remained unchallenged for virtually a decade.

In 1987, Hardham presented the first ultrastructural description and serial section reconstruction of the zoospore of *Phytophthora cinnamomi.* Four vesicle types were shown to be present in the peripheral cytoplasm of the zoospore. Large peripheral vesicles, morphologically identical to the fibrous vesicles of the Saprolegniales (Heath & Greenwood 1970a), the fibrillar vesicles of the Leptomitales (Gotelli & Hanson 1987) and the granular or peripheral vesicles already described in most of the Peronosporales (Reichle 1969, Lunney & Bland 1976, Grove & Bracker 1978) were described as elliptical in cross section and appeared finely granular or dispersed and flocculent internally with different fixations (Fig. 1c). Peripheral flattened cisternae were also identified and were discussed as having the morphology of the plasma membrane and not the endoplasmic reticulum (E.R.). Two types of small peripheral vesicle were recognised by the author. One type, largely restricted to the ventral or groove side of the zoospore, was filled with moderately electron dense, granular material and possessed a number of plate-like inclusions (Fig. 1a). These vesicles were

morphologically similar to the microbodies or U-bodies of *P. palmivora* (Philippi *et al.* 1975; Bimpong & Hickman 1975; Powell & Bracker 1986), the spherosomes of *P. megasperma* (Ho *et al.* 1968) and vesicles seen in electron micrographs of *P. capsici* sporangia (Williams & Webster 1970) The second type, confined to the dorsal surface of zoospores, contained electron-lucent regions and lacked plate-like inclusions (Fig. 1b). These bore a close resemblance to the parastrosomes of *P. capsici* (Williams and Webster 1970).

Hardham and coworkers (1986) raised monoclonal antibodies (Mabs) to components of cysts and zoospores of *P. cinnamomi* in an attempt to recognise compounds involved in the infection process. Among this bank of monoclonal antibodies were specific antibodies which reacted with components of large peripheral vesicles (Fig. 1f), peripheral cisternae and the two small peripheral vesicles (Figs 1d,1e) (Gubler & Hardham 1988, 1991). Antibody Lpv-1 recognized three glycoproteins (Mr > 300kDa) that contained mannosyl/glucosyl residues. Immunogold labelling of encysted zoospores with this probe revealed a surprising result. Contrary to the great body of evidence that showed that the contents of (large) peripheral vesicles were secreted to form the cyst wall in oomycetes, Gubler and Hardham (1988, 1990) showed that in *P. cinnamomi* the contents of the large peripheral vesicles were not secreted at any stage of infection. Gubler and Hardham (1988, 1990) showed that after encystment and wall formation, the large peripheral vesicles migrated to the interior of the cyst and were eventually degraded as nutrient stores during cyst germination. The Lpv-1 antibody was shown to react with large peripheral vesicles of other species and this result was confirmed for other *Phytophthora* species including *P. parasitica.*

Antibodies to components of both ventral (Vsv-1) and dorsal peripheral vesicles (Cpa-2) revealed the fate of these different vesicle types at encystment (Gubler & Hardham 1991). Immunolabelling of encysting zoospores showed that both types of small peripheral vesicle were secreted from cells between 1-2min following induction of encystment. Immunogold labelling of encysting cells and immunofluorescence microscopy of zoospores infecting *Eucalyptus seiberi* seedlings revealed that ventral peripheral vesicles secreted their glycoprotein contents onto the ventral surface of the cyst that faces the root indicating that these vesicles were probably involved in the adhesion of cysts to hosts. In contrast, dorsal peripheral vesicles secreted their contents to form a thin glycoprotein coat on the dorsal surface of the encysted cell, which faces away from the root surface. The exact role of this dorsal cyst coating was not determined. It is possible that this coating may play a protective role or may prevent osmotic swelling in the crucial transition stage during encystment when the cell switches from osmoregulation via the operation of a water expulsion vacuole (zoospore) to the maintenance of cell turgour with the formation of the cyst cell wall. The dorsal cell coat may also provide a suitable matrix for the assembly of the microfibrillar cell wall, a function previously attributed to the large peripheral vesicles (Sing & Bartnicki-Garcia 1975b, Bartnicki-Garcia & Hemmes 1976).

Immunogold labelling with an antibody specific to the peripheral cisternae of *P.cinnamomi* (Cpw-1) revealed the fate of this organelle at encystment (Gubler & Hardham 1991). One to 2min after the induction of encystment, the peripheral cisternae move away from the zoospore plasmamembrane and break into small vesicles. Cpw-1, which weakly labels the peripheral cisternae, also labels these vesicles. After 2-5min these vesicles undergo exocytosis and by 10min after encystment Cpw-1 positive material can be seen incorporated in the new cell

wall. Thus, immunogold labelling with an antibody specific to the peripheral cisternae of zoospores confirmed earlier suggestions (see Hemmes & Hohl 1971, Bimpong & Hickman 1975) that this organelle was involved in cell wall formation. The work of Gubler and Hardham has, to date, provided the best evidence for the roles of the different peripheral vesicle types in *Phytophthora* and, by analogy for many oomycetous species.

The first account of the ultrastructural changes associated with encystment of *Pythium* zoospores showed that the cyst wall of *Pythium aphanidermatum* was formed from the discharged contents of preformed vesicles at the cell periphery (Grove 1970). In *Pythium proliferum* zoospores, Lunney and Bland (1976) recognised peripheral vesicles with fibrillar and granular contents, flattened peripheral vesicles and microbodies. Peripheral vesicles were shown frequently to discharge their contents extracellularly and together with the peripheral cisternae were suggested to be involved with initial wall deposition. The microbodies which were still present in encysted zoospores, were considered to contain specific enzymes. Grove and Bracker (1978) showed that at encystment, peripheral vesicles fused with the plasma membrane to form a loosely organised surface coat of flocculent appearance. Although, 1min after encystment most peripheral vesicles had disappeared, some remained in the cytoplasm of cysts until germination when they became autophagous with multivesicular bodies. These authors also showed the disappearance of peripheral cisternae coincided with the formation of the new cell wall about 2min after encystment. Microbodies similar to the parastrasomes of Williams and Webster (1970) and the U-bodies of Philippi *et al.* (1975) were shown to become scarce in cysts, though other forms of microbodies, often with distinct crystalline inclusions, remained in the cyst and could still be seen at germination.

Estrada-Garcia *et al.* (1990) used antibodies to recognize glycoprotein components of large peripheral vesicles in encysting cells of *P. aphanidermatum.* In contrast to the work of Gubler and Hardham (1988), these authors showed large peripheral vesicle specific antibodies labelling cyst coat components. Although these authors claimed that large peripheral vesicles secreted their contents onto the surface of encysting cells, large peripheral vesicles were still very common in micrographs of the cysts.

Recent work by Cope and Hardham (1992) has clarified the fate of the large peripheral vesicles in *P. aphanidermatum.* Cpa-2, the Mab specific for dorsal peripheral vesicles in *P. cinnamomi* zoospores is shown to react with the large peripheral vesicles of *P. aphanidermatum* and is not found on the cyst surface at encystment but is present in unsecreted large peripheral vesicles within the cyst. Antibodies to ventral vesicles of *Phytophthora cinnamomi* (Vsv-1) also react with a population of ventrally located vesicles in *P. aphanidermatum* and these do undergo exocytosis and may be adhesive in function. This last series of results tends to suggest that the biology of encystment within the Peronosporales may be very similar. In the future, following immunocytochemical studies of other oomyceteous zoospores, it is conceivable that peripheral vesicles may be found to be a lot less diverse in kind and function.

1.2 OBJECTIVES OF THE PROJECT AND APPROACHES

In this project, attempts have been made to gather more information about the large peripheral, ventral and dorsal vesicles of *P. cinnamomi* and through this gain further insight into the processes of secretory vesicle formation and transport. As

these organelles have been documented in a variety of life cycle stages such as chlamydospores (Hemmes & Wong 1975) and oospores (Hemmes 1983), the first approach was to elucidate whether these three organelles were associated exclusively with sporulation or were present in much of the life cycle of the species. With this first objective completed, it was likely that I would have determined a stage in the life cycle in which large numbers of peripheral vesicles were synthesised. This would enable me to undertake an immuno-ultrastructural study of the process of vesicle formation - the first account of its kind in a fungal system. A fourth chapter would report on the the effects of a number of drugs on peripheral vesicle formation. The results from this work may reveal further information on the nature of the glycoproteins within the vesicles and the properties of the secretory pathway. It may also allow the assessment of the effectiveness of these drugs, in some cases for the first time on a fungal system, in perturbing the secretory pathway. The process of secretory vesicle transport has been documented to involve microtubules (Allen *et al.* 1985, Koonce & Schliwa 1986) and actin microfilaments (Kohno & Shimmen 1988). Previous studies on a related oomycete, *Saprolegnia ferax* (Heath & Kaminskyj 1989, Heath & Harold 1992) have suggested that the transport of secretory vesicles in hyphae is mediated by the actin cytoskeleton. In chapter five, studies on the actin and tubulin cytoskeletons from vegetative and sporulating hyphae are reported. The motivation behind such studies was to assess a possible involvement of the elements of the cytoskeleton in vesicle transport into the developing sporangium.

In these investigations I have used the three monoclonal antibodies Vsv-1, Cpa-2 and Lpv-1 which react with the contents of zoospore ventral, dorsal and large peripheral vesicles respectively. The major techniques used were immunofluorescence microscopy of cryosectioned material (Chapters 2,3,4,5),

immunodotblotting (Chapter 2), standard transmission electron microscopy (Chapters 3,4), immunoelectron microscopy (Chapter 2,3) and cytochemical staining of intact hyphae (Chapter 5).

Figs 1.1a- 1.1f. Peripheral vesicles in *P. cinnamomi*

Fig. 1.1a. Ventral vesicle (material embedded in Spurr's resin). These vesicles contained moderately electron dense granular material and a number of plate-like inclusions. X 64,800.

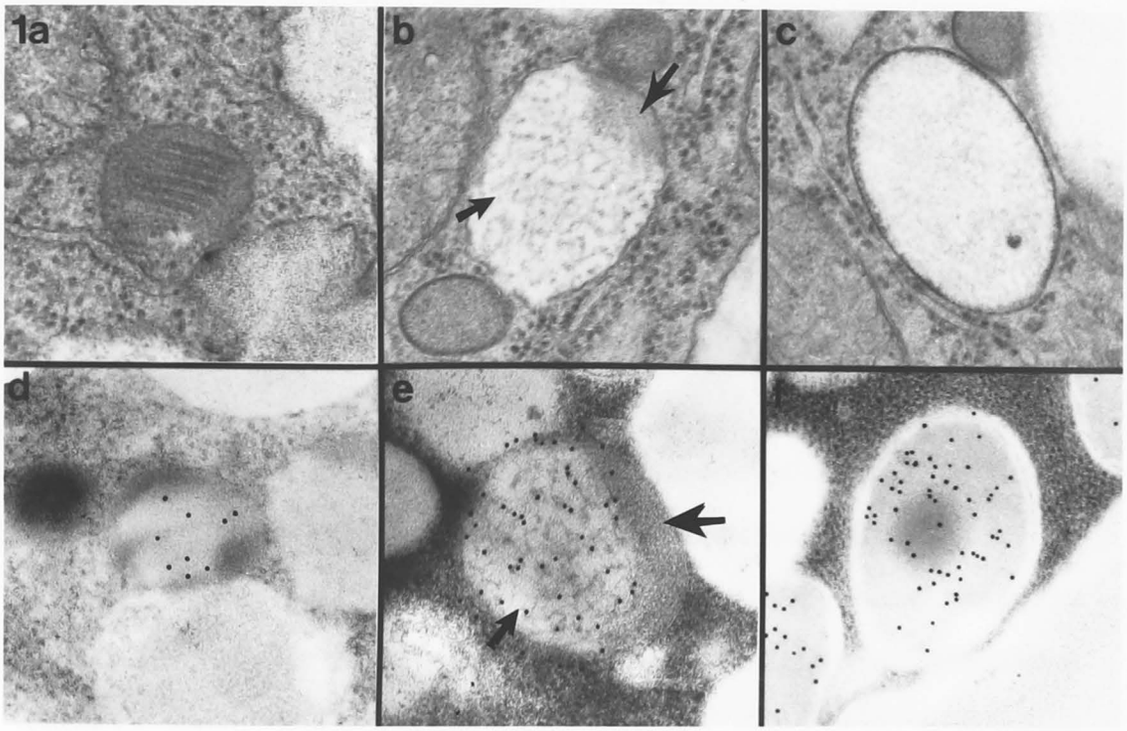
Fig. 1.1b. Dorsal vesicle (material embedded in Spurr's resin). Dorsal vesicles were heterogeneous in content and contained a region of moderately electron dense granular material (large arrow) and a more electron-lucent region of flocculent appearance (small arrow). X 57,700.

Fig. 1.1c. Large peripheral vesicle (material embedded in Spurr's resin). These vesicles were eliptical in cross section and appeared finely granular or dispersed and flocculent internally with different fixations. X 51,000.

Fig. 1.1d. Ventral vesicle labelled with monoclonal antibody Vsv-1. The antibody appeared to label the plate-like inclusions within the vesicle. X 55,600.

Fig. 1.1e. Dorsal vesicle labelled with monoclonal antibody Cpa-2. The antibody appeared to react with the flocculent material within the electron lucent region (small arrow) and not the electron dense region of the vesicle (large arrow). X 45,000.

Fig. 1.1f. Large peripheral vesicle labelled with monoclonal antibody Lpv-1. X 45,000.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CHAPTER TWO: PERIPHERAL VESICLES IN *PHYTOPHTHORA CINNAMOMI* ARE SPORULATION SPECIFIC.

2.1 INTRODUCTION

Phytophthora cinnamomi Rands, the dieback fungus, is an oomycete pathogen with a broad host range (Zentmyer 1980). Motile biflagellate zoospores are the major infective agent in *P. cinnamomi* and are produced by the cleavage of multinucleate sporangia (Hardham 1989). Invasion of hosts is mediated by the chemotaxis of the zoospores and the subsequent encystment of these cells on plant roots. Under conditions not suitable for growth and dissemination through the formation of sporangia, *P. cinnamomi* will form chlamydospores which can perpetuate the fungus for long periods of time in the absence of hosts (Mircetich & Zentmyer 1966). When favorable conditions return, chlamydospores germinate and grow to form mycelia, sporangia or new chlamydospores (Weste 1983).

Immunologically distinct vesicles localised to the peripheral cytoplasm in *P. cinnamomi* zoospores play important roles in zoospore encystment and germ tube growth (Gubler & Hardham 1991; Hardham & Gubler 1990; Hardham *et al.* 1991a). Ventral vesicles have been shown to contain glycoproteins which effect the adhesion of spores to hosts (Hardham & Gubler 1990), and large peripheral vesicles are stores of protein used in the growth of the germ tube (Gubler & Hardham 1990). The exact role of the cyst coat secreted by the dorsal vesicles remains to be determined. It has been suggested that the coat may be involved in cell protection or it may provide a suitable matrix for the assembly of the microfibrillar cyst wall (Gubler and Hardham 1991). Alternatively, it may

prevent the cyst from bursting in the critical transition stage during encystment when the cell switches from osmoregulation through the operation of a water expulsion vacuole to maintenance of cell turguor with the formation of a cell wall (Hardham *et al.* 1991a).

Peripheral vesicles have been shown to be present in stages of the life cycle other than zoospores. Hemmes and Wong (1975) have shown large peripheral vesicles to be present in developing, mature and germinating chlamydospores. Hyde *et al.* (1991a) have shown the three vesicle types to be present in sporangia that have yet to undergo cleavage to produce zoospores. Hemmes (1983) records peripheral vesicles in *Phytophthora* hyphae and suggests that they flow into the sporangium during the sporangial expansion phase

In the work reported in this chapter, a fundamental question about these cell components is addressed. Are these vesicles present in much of the life cycle of *P. cinnamomi* or are they associated exclusively with spore formation? To answer this I document the occurrence of the three peripheral vesicles in various stages of the asexual life cycle of *P. cinnamomi.* I examine the disappearance and subsequent reappearance of peripheral vesicles in newly formed cysts that are induced to germinate, and investigate the behavior of peripheral vesicles in the development of chlamydospores and sporangia.

This work is important as studies of when peripheral vesicles are present in stages of the life cycle other than zoospores may further clarify the importance of these vesicles to the biology of the organism.

2.2 MATERIALS AND METHODS

2.2.1 Culture

Culturing and the production of zoospores from *P. cinnamomi* (6BR; DAR 52646) followed the methods described in Hardham *et al.* (1991b). Seven small squares (5mm by 5mm) of mycelia were cut from the edge of a colony of *P. cinnamomi* growing on 0.5cm deep layer of V8 nutrient agar containing 10% V8 juice (Campbell's Soups Pty Ltd, Lemnos, Australia), 0.002% β-sitosterol (Sigma Chemical Co., St. Louis, MO), 0.01% $CaCO_3$ and 1.7% Bacto agar (Difco, Detroit, MI) in a 9cm diameter petri dish. The squares were inoculated onto a sterile miracloth disc overlying V8 nutrient agar and incubated for 5 days at 25°C in the dark. Each disc was then transferred to 100ml of 5% V8 broth (5% V8 juice, 0.01% $CaCO_2$, 0.002% β-sitosterol) and shaken overnight at 150 rpm at 22°C. The cultures were then washed three times in mineral salts solution (10mM Ca $(NO_3)_2$, 5mM KNO_3, 4mM $MgSO_4$ and 2ml l^{-1} of a solution containing 10mM $FeSO_4$ and 10mM Na_2 EDTA) and incubated for 24h at 22°C in 100ml of this solution, during which time sporangia developed. Cleavage of sporangia was induced by rinsing miracloth cultures three times in cold double distilled water, and incubating them at 4°C for 13min in 10ml of distilled water. The discs were then transferred to 18°C on a light box, where cleavage occurred. Zoospores were released during the following 75min. Zoospores were induced to encyst by 20s agitation in a sterile 100ml Schott bottle (Duran, Mainz, West Germany). To produce germlings approximately 2-5 x 10^6 germinated cysts were inoculated into 10ml of 10% V8 broth and incubated at 22°C in 15ml culture tubes (Disposable Products, Adelaide, Australia).

Chlamydospores were produced via the method of Englander and Turbitt (1979). A small piece of V8 agar (5mm by 5mm) with mycelium taken from the margin of a 5 day old colony, was placed onto the centre of a shallow (0.5cm deep) V8 nutrient agar plate and incubated in the light at 22°C. Samples were taken daily to determine the timecourse of chlamydospore production.

For sampling of mycelia in mineral salts solution, small (5mm x 5mm) pieces of agar with mycelium were taken from the growing edge of a colony on V8 agar. Mycelial plugs were incubated in 9cm petri dishes in the dark in 15ml of 5% V8 broth at 25°C for 24h. The plugs were then washed three times in mineral salts solution and incubated in a final volume of 15ml of mineral salts in the light at 22° C for 8h.

2.2.2 Immunofluorescent staining

Cysts, germlings and hyphae were fixed in 4% paraformaldehyde in 50mM Pipes (piperazine-N', N'-bis [2-ethane-sulphonic acid]) buffer (pH 7.0) for 1h at room temperature. After fixation, a number of enzyme digestion protocols were utilised in an attempt to degrade the hyphal wall. These included protocols that had previously been successful in removing the cell wall of *P. megasperma* hyphae, i.e. 30min incubation in 5mg/ml Novozym (Novo laboratories) in 50mM Pipes buffer (pH 6.8) at 28°C (Howlett 1989), pollen tubes, i.e. 40min incubation in 2% *Trichoderma viride* cellulase (Miles Mck. Kali Chemie) in phosphate buffer (pH 6.5) at 37°C (Runeberg *et al.* 1986), or 10min incubation in 10% cellulysin (Calbiochem, La Jolla, CA) in phosphate buffer (pH 6.5) at 37°C (Derksen *et al.* 1985), root hairs, i.e. 5% Onozuka R-10 cellulase (Yakult, Honsha, Tokyo) in 50mM Pipes buffer (pH 6.8) at 37°C (Traas *et al.* 1985), and seed hairs, i.e. 20min incubation in a mixture containing 1% pectinase (Serva, Heidelberg, Germany),

1% driselase (Fluka, Buchs, Switzerland) and 2% cellulase (Boehringer Mannheim, Castle Hill, Australia) in microtubule stabilising buffer (MSB, which contained 100mm Pipes, 1mM MgCl2 and 5mM EGTA, pH 6.8) at room temperature (Quader *et al.* 1987) and moss protonemata, i.e 20min incubation in a mixture of 2% driselase (Fluka), 50µg/ml leupeptin (Sigma, Castle Hill, Australia) and 5% mannitol (Sigma) in MSB (pH 6.8) at room temperature (Doonan *et al.* 1985). After fixation the hyphal material was also processed for cryomicrotomy. The material was rinsed twice in Pipes buffer and frozen in Tissue Tek embedding compound (Miles Inc., Elkhart, IN) in plastic moulds by plunging in liquid nitrogen. Ten micrometer thick cryosections of germlings and twelve micrometer thick sections of hyphae were cut on a Reichert-Jung 2800 Frigocut E cryotome and dried at room temperature onto poly-L-lysine coated slides. Immunostaining of enzyme-digested material and cryosections was carried out in 10cm square petri dishes lined with distilled water moistened filter paper. The samples were incubated for 45min at 37°C in 1µg ml^{-1} purified Vsv-1 antibody, 0.5 µg ml^{-1} purified Cpa-2 antibody or 1:10 Lpv-1 hybridoma supernatant in phosphate buffered saline (PBS; 20mM sodium phosphate, 150 mM NaCl) containing 1% bovine serum albumin (BSA). After three washes in PBS, the enzyme-digested material and cryosections were incubated in sheep anti-mouse Ig immunoglobulin-fluorescein isothiocyanate (SAM-FITC [Silenus, Dandenong, Australia]) diluted 1:60 in 1% BSA-PBS for 45min at 37°C. Samples were subsequently washed three times in PBS and once in distilled water. They were then dried and mounted in mowiol containing 0.1% paraphenylenediamine (PPD). The samples were examined and photographed with a Zeiss Axioplan microscope equipped with epifluorescence optics with an F1 filter cube (excitation 450-490nm; dichroic mirror 510nm; barrier 515-565nm).

2.2.3 Immunodot blot analysis

Germlings were frozen in liquid nitrogen, lyophilized, then ground in eppendorf tubes using a glass rod. The ground material was extracted for 20min with 6M guanidine hydrochloride, then diluted 50 times with Tris buffered saline (TBS, 10 mM Tris-HCl, 150mM NaCl, pH 7.4). The samples were incubated on ice for 10min before centrifuging at 13,000 rpm for 10min. Solubilised material was transferred to nitrocellulose in an immunodot blot apparatus (Biorad, Nth Ryde, N.S.W., Australia.). The efficacy of the transfer was monitored by staining with 0.2% Ponceau S in 3% trichloroacetic acid for 5min. After blocking nonspecific binding sites for 1h in 5% milk powder in TBS and washing three times in TBS-0.1% Tween-20, the nitrocellulose was incubated for 45min in undiluted supernatants of Vsv-1 or Lpv-1, or 3 $\mu g\ ml^{-1}$ purified Cpa-2 in 1% BSA-PBS. Bound antibody was visualised with peroxidase-conjugated SAM (Silenus) using 4-chloro-1-napthol as substrate.

2.2.4 Morphometric analysis and immunolabelling of flat embedded sporangia.

Ultrathin sections of zoospores and cysts, fixed and embedded in Lowicryl K4M (Gubler & Hardham 1988) were immunolabelled with Vsv-1-Au10 direct gold probe prepared by the methods of Gubler & Hardham (1988). Twenty micrographs, with a final print magnification of 16,000 were taken of both stages. Vesicles labelled with Vsv-1-Au10 were traced on a digitiser pad (Houston Instruments, Austin, Texas) and were analysed with a Sigma Scan 3.1 program (Jandel Scientific, Sausalito, CA) to determine the percentage volume taken up by the vesicles at both stages.

Sporulating hyphae were fixed and processed for immunocytochemical labelling as above. One main difference was that samples were embedded between two

microscope slides, which had previously been coated with mould parting compound (Electron Microscopy Sciences, Fort Washington, PA, USA). Samples were selected for ultrathin sectioning with the aid of a diamond tipped objective (Zeiss, West Germany) and were stuck to the surface of a blank resin block with Supa Glue (Selleys Pty Ltd. Padstow, NSW, Australia). Ultrathin sections of developing sporangia were immunolabelled initially with hybridoma supernatants of Vsv-1, Cpa-2 and Lpv-1, followed by one hour incubation in Sheep anti-mouse IgAu10 (Gubler & Hardham 1988).

2.3 RESULTS

2.3.1 Background

None of the enzyme digestion techniques were successful in consistently exposing antigenic material. The most effective method of gaining access to cytoplasm contents proved to be thick sectioning cryomicrotomy. This technique consistently allowed access to both surface and internal components and was the major technique reported in the chapter.

2.3.2 Peripheral vesicles in germlings growing in 5% V8 broth

Labelling of 5min old cysts with Vsv-1 showed that much of the ventral vesicle antigen was secreted and coated part of the surface of the cysts. However, some ventral vesicles remained in the cytoplasm of the cyst (Fig. 2.1a). Morphometric analysis of immunogold labelled sections of indicated that 33% ±8% (mean of 20) of zoospore volume was taken up by labelled ventral vesicles whereas in 5min old

cysts this value was 3% ±1%. This meant that 9% of ventral vesicles were still present in the cytoplasm after encystment (Fig. 2.2a-2.2d). At 2h and 4h after encystment, ventral vesicles were still observed in germ tubes (Figs 2.1b, 2.1c) but by 10h after encystment (Fig. 2.1d), all ventral vesicles had disappeared from the germlings. The secreted Vsv-1 antigen remained associated with the cyst surface during this period (Figs 2.1a-2.1d).

Labelling of 5min old cysts with Cpa-2 showed that the contents of the dorsal vesicles were completely secreted during encystment to form a coat on the outside of the cyst (Fig. 2.1e). This coat remained on the surface of the cyst during the next 10h of germling growth (Figs 2.1f-2.1h). No dorsal vesicles were seen in germlings throughout the 10h period.

Labelling of 5min old cysts with Lpv-1 showed that the large peripheral vesicles were not secreted during encystment (Fig. 2.1i). These remained in the cyst and were present in germ tubes at 2h (Fig. 2.1j), but by 4h these vesicles had completely disappeared (Fig. 2.1k). They were still absent at 10h (Fig. 2.11). At no stage was the Lpv-1 antigen observed on the surface of the germlings.

Changes in the relative abundance of the three antigens in germlings growing in 10% V8 broth were analysed using immunodot blotting of cell extracts (Fig. 2.3). The level of ventral vesicle antigen did not change during the first 2h after encystment, but decreased during subsequent growth. No changes in the level of dorsal vesicle antigen were detected over the 10h period. A decline in large peripheral vesicle antigen was observed between 2 and 4h.

2.3.3 Peripheral vesicles in germlings transferred to mineral salts solution

The effect of removing the exogenous nutrient source was investigated by replacing the V8 broth with mineral salts solution (see 2.2.1) after the germlings had been growing in V8 broth for 6h. Zoospore peripheral vesicle antigens were then monitored by immunofluorescent labelling (Fig. 2.4) or immunodot blot analysis (Fig. 2.5).

At the time of transfer to mineral salts solution (6h after encystment) the germlings did not contain any ventral, dorsal or large peripheral vesicles (Figs 2.4a, 2.4e, 2.4i) although secreted ventral and dorsal vesicle material coated the cyst surface (Figs. 2.4a, 2.4e). Both ventral (Fig. 2.4b) and large peripheral (Fig. 2.4j) vesicles were present 6h after transfer to mineral salts solution. The number of ventral (Figs 2.4c, 2.4d) and large peripheral (Figs 2.4k, 2.4l) vesicles in the germ tubes increased over the following 24h. Dorsal vesicles were not present until 30h after transfer to mineral salts solution (Figs 2.4f-2.4h). The timing of the appearance of the dorsal vesicles at 30h coincided with the first appearance of sporangia in the germling culture (inset, Fig. 2.4h).

Immunodot blotting of extracts from germlings incubated in mineral salts solution detected the appearance of Vsv-1 antigen after 6h and its increase over the next 12h (Fig. 2.5). No changes in the level of Cpa-2 antigen were evident. Lpv-1 antigen was first detected 18h after transfer to mineral salts solution.

2.3.4 Peripheral vesicles in mycelia, sporangia and chlamydospores.

When mycelia that had been growing on agar plates for 5 days were transferred to 5% V8 broth, all three vesicles were present at the time of transfer (Table 2.1, Fig. 2.6) but all peripheral vesicles disappeared by 24h (Table 2.1, Fig. 2.7).

Subsequent incubation of the plugs in mineral salts solution led to the reappearance of ventral and large peripheral vesicles between 4 and 6h (Fig. 2.7, Table 2.1). Dorsal vesicles reappeared between 6 and 8h, which coincided with the first appearance of sporangia (Fig. 2.7). Therefore all three peripheral vesicles were present in the developing sporangia (Fig. 2.8).

After growing for 5 days on V8 agar in the dark, hyphae at the edge of the colony contained all three of the vesicle types (Table 2.2, Fig. 2.6). If a small square of this mycelium was inoculated onto a new shallow V8 agar plate, cryosectioning after one day revealed that the hyphae did not contain any of these three vesicle types (Table 2.2). By 2 days, however, all three vesicles were again present. This was coincident with the first appearance of chlamydospores (Fig. 2.9).

2.4 DISCUSSION

This study has revealed important new details about the occurrence of peripheral vesicles in *P. cinnamomi* 6BR. The vesicles were absent from vegetative stages of the asexual life cycle and appeared concommitantly with chlamydospore formation and during sporangial development.

Sporulation specific proteins have been recorded for a number of fungi. In *Neurospora crassa* and in other related members of the ascomycetes, a major protein has been shown to have a specific association with perithecial development (Nasrallah & Srb 1977). This protein is not present in ascospores but its presence in perithecia has been suggested to be related to the nutrition of the developing spores (Nasrallah & Srb 1978). In *Aspergillus nidulans* a prominent protein has been shown to appear with conidiation (Champe *et al.* 1981). This protein

however appears to have no causative role in the conidiation process as mutant forms of the fungus develop conidia normally in its absence. Glycoproteins in large peripheral vesicles have been likened to the pycnidiospore specific protein species in *Botryodiplodia theobromae* (Gubler & Hardham 1990). This protein, like that in large peripheral vesicles, also may have some storage role as it degraded during spore germination (van Etten *et al.* 1979). Another sporulation specific protein, the conidiation specific Con10 protein of *Neurospora crassa* (Springer *et al. 1992*), behaves similarly to *P. cinnamomi* peripheral vesicles. It appears about 8h into conidiation, is shown to be present evenly distributed in the cytoplasm of macroconidia and is degraded between 2 and 4h after germination. Some of the work on sporulation specific proteins in the zoosporic chytrid, *Blastocladiella,* shows similarities with the work of this study. Lodi and Sonneborn (1974) have shown that a caseinolytic protease first appears and increases in concentration slowly during sporulation in *B. emersonii.* The enzyme is retained in zoospores but is absent from vegetatively growing cells. The gamma particles formed during sporulation in *Blastocladiella* (Barstow & Lovett 1975) resemble in behavior and function the peripheral vesicles of *P. cinnamomi.* These structures are probably involved in wall formation during zoospore encystment and dissappear during germination (Truesdell & Cantino 1970).

The formation of chlamydospores is coincident with the presence of all three vesicle types. Large peripheral vesicles have been recorded in chlamydospores of *P. cinnamomi* by Hemmes and Wong (1975). Gubler and Hardham (1990) suggested that they may be broken down during germination of these spore types. Chlamydospores germinate under appropriate conditions to form sporangia and zoospores (Weste 1983). I suggest that the presence of peripheral vesicles during the initiation of chlamydospores may provide for the rapid production of

sporangia after chlamydospore germination with a minimum of synthesis required. The presence of chlamydospores in 5 day old mycelial colonies (data not shown) may also explain the presence of the three peripheral vesicles in cryosectioned and immunostained samples of such colonies.

During sporangium formation in *P. cinnamomi* there is differential synthesis of peripheral vesicles. In other fungal species, the formation of different proteins at different stages during sporulation has been widely documented. Huang and Staples (1982) have reported the synthesis of different proteins at early and late stages in appressorium formation in *Uromyces.* During perithecial development in *Sordaria brevicollis,* the appearance of specific polypeptides is shown to corrrelate with different morphogenetic events (Broxholme *et al.* 1991). Gwynne and Brandhorst (1982) have indicated that during sporulation of *Achyla ambisexualis* some groups of proteins are initially synthesised in response to starvation while the timing of synthesis of other proteins is linked with sporangium formation. The synthesis of large peripheral and ventral vesicles but not dorsal vesicles by 6h after being incubated in a mineral salts solution of *P. cinnamomi* germlings and hyphae is suggestive that these former vesicles may be part of an initial starvation response. This idea is strengthened by the fact that large peripheral vesicles and a small population of ventral vesicles are not released at encystment. As well as the function of ventral vesicles in cyst adhesion, these two vesicle types may act as endogenous nutrient stores during the growth of the germ tube and during sporangium development.

There is an alternative explanation for the presence of the small population of ventral vesicles that remains in the cyst cytoplasm and which is still present in germ tubes up to 4h after encystment. A small reserve of ventral adhesive vesicles remaining after encystment may be important under conditions where zoospores

undergo repeated emergence. This would provide for the attachment of a subsequently released zoospore to its host. A small reserve of K2-bodies which is similarly located in *Saprolegnia* zoospores and is also involved in cell adhesion (Lehnen & Powell 1989) remains in the zoospores after encystment (Lehnen pers. comm.). Although *P. cinnamomi* has not been documented to undergo repeated emergence, these findings imply possible phylogenetic links between the Saprolegniales and the Peronosporales and suggest that ventral vesicles are relics of repeated emergence.

There is strong evidence that dorsal vesicles play solely a role in zoospore encystment. These vesicles develop in synchrony with sporangia and they are completely secreted from the zoospores at encystment. Dorsal peripheral vesicles and sporangia form sooner in mycelia than they do in germlings, perhaps because of the degree of competence acquired by the mycelial colony. A certain period of vegetative growth may be necessary before *P. cinnamomi* germlings can form sporangia. Champe *et al.* (1981) have shown that a certain period of vegetative growth is necessary to achieve conidiation competence in *Aspergillus nidulans.* The inability of the immunodot blots to detect any changes in Cpa-2 antigen levels in the experiments which were involved with growing germlings in a nutrient deficient medium is an indication of the masking effects of the secreted cell coat which is still highly immunoreactive even 36h after encystment (not shown). This technique also failed to detect initial large peripheral vesicle synthesis in germlings growing in mineral salts, but it is suggested that the extraction procedure may not have been as efficient as with older germlings.

Before this study, little was known about the exact stage of origin of the peripheral vesicles in *Phytophthora.* Hemmes (1983) suggested that they may form at an unknown site in the mycelium, or in the cortical cytoplasm of germinating

chlamydospores and oospores. In a study on the formation of zoospores within sporangia of *P. cinnamomi*, Hyde *et al.* (1991a) concluded that since large peripheral vesicles, dorsal vesicles and ventral vesicles were present in the sporangium before the induction of zoospore cleavage, they must move in from the hyphae or be produced prior to, or during, sporangial maturation. I have shown that formation of peripheral vesicles in *P. cinnamomi* occurs in pre-sporangial hyphae and their presence in sporangia and chlamydospores is presumably mediated by cytoplasmic inflow. This observation contrasts with what is known about peripheral vesicle formation in other oomycetes. Grove and Bracker (1978) suggested that peripheral vesicles in *Pythium aphanidermatum* formed in immature sporangia while Lunney and Bland (1976) first observed peripheral vesicles during the early stages of cleavage of *Pythium proliferum* sporangia. Holloway and Heath (1977) and Beakes (1983) have suggested that fibrillar vesicles, which are similar in morphology to large peripheral vesicles are first synthesised in primary cysts of *Saprolegnia.* Schnepf *et al* (1978a) reported that encystment vesicles in *Lagenisma coscinodisci* first developed in sporangia, whereas microbody-like structures developed in the maturing cyst (Schnepf *et al.* 1978b). Pueschel and van der Meer (1985) reported that in the marine parasitic oomycete *Petersenia palmariae*, small electron dense vesicles that disappeared at encystment appeared during sporangial cleavage. In *Saprolegnia ferax*, Lehnen and Powell (1989) have shown that the adhesive K2-bodies are probably derived from smooth surfaced fenestrated cisternae within primary cysts. K1-bodies have been recognised in differentiating sporangia (Holloway & Heath 1977).

In the life cycle stage in which they originate, peripheral vesicles in *P. cinnamomi* are analogous to the primary bars in *Brevilegnia minutandra,* which are shown to appear in pre-sporangium hyphae (Armbruster 1982), and the primary encystment

vesicles of *Saprolegnia ferax* which are fully differentiated in pre-septum zoosporangium initials (Beakes 1983). Why should peripheral vesicles form in pre-sporangial hyphae of *P. cinnamomi*? One approach could be that if conditions that favor vegetative growth return before sporangia have appeared, hyphae can recycle the contents of the large and ventral peripheral vesicles during such vegetative growth. A further explanation is that the cellular machinery necessary for vesicle synthesis may be taken up with other major functions during sporangial development. For example the Golgi apparatus, from which the peripheral vesicles may be derived (see chapter 3), appears to play a major role in wall synthesis during sporangial development (Christen & Hohl 1972) and is the source of materials for membrane formation during sporangial cleavage (Hyde *et al.* 1991b).

This work has elucidated previously unrecognised stages in the vegetative life cycle of *P. cinnamomi* at which peripheral vesicles occur. The vesicles are present under conditions of spore formation and are similar in behavior to a number of other sporulation specific proteins found in fungi. Large peripheral and ventral vesicles are produced earlier in sporulation than dorsal vesicles. In addition, large peripheral vesicles and a small population of ventral vesicles remain after encystment whereas dorsal vesicles are completely secreted from zoospores. These observations suggest that, in addition to the adhesive function of ventral vesicles, they and large peripheral vesicles may act as endogenous nutrient reserves during the asexual life cycle of *P. cinnamomi,* wheras dorsal vesicles serve a function, as yet still obscure, in zoospores only.

Figs 2.1a-2.1l. Peripheral vesicles in cysts and germlings growing in 5% V8 broth. bar =17μm

Fig. 2.1a. 5min old cyst labelled with Vsv-1, showing that although much of the ventral vesicle antigen was secreted and coated part of the surface of the cyst, some ventral vesicles remained in the cyst cytoplasm.

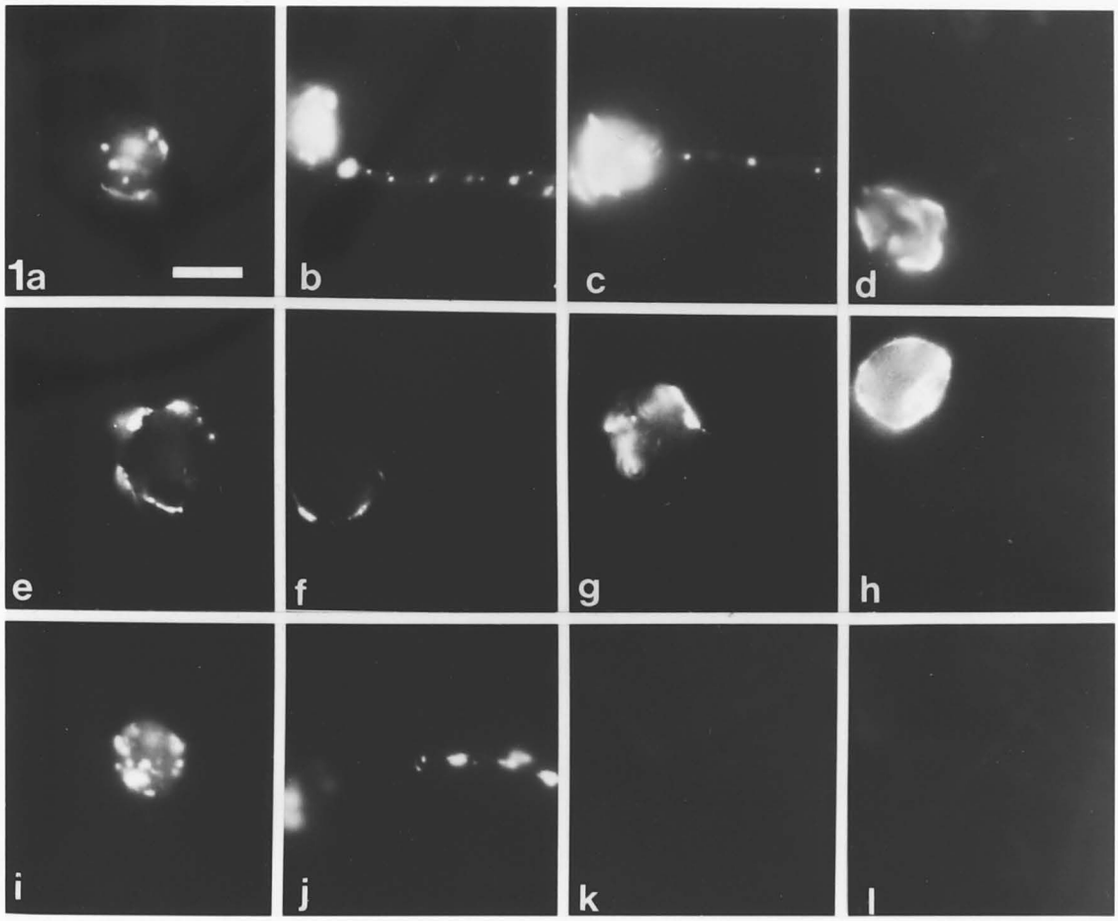
Figs 2.1b-2.1c. At 2h (2.1b) and 4h (2.1c) after encystment, ventral vesicles could be observed in germ tubes.

Fig 2.1d. By 10h after encystment, ventral vesicles had disappeared from germlings. Vsv-1 antigen still remained associated with the cyst surface.

Figs 2.1e-2.1h. Labelling of 5min old cyst with Cpa-2 showed that the contents of the dorsal vesicles were completely secreted during encystment to form a coat on the outside of the cyst (2.1e). This coat remained on the surface of the cyst during the next 10h of germling growth.

Figs 2.1i-2.1j. Labelling of 5min old cysts with Lpv-1 showed that the large peripheral vesicles were not secreted during encystment (2.1i). These vesicles remained in the cyst and were present in germ tubes at 2h (2.1j).

Figs 2.1k-2.1l. By 4h large peripheral vesicles had disappeared from germlings (2.1k) and they were still absent at 10h (2.1l). At no stage in the 10h period was the Lpv-1 antigen observed on the surface of the germlings.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j
k
l

Figs 2.2a-2.2d. A zoospore and cyst of *P. cinnamomi* indicating that not all ventral vesicles are secreted at encystment.

Fig 2.2a. Low magnification T.E.M. of *P. cinnamomi* zoospore. X 6250.

Fig 2.2b. Low magnification T.E.M. of *P. cinnamomi* 5min old cyst. X 6150.

Fig 2.2c. Higher magnification T.E.M. of same zoospore in 2.2a. Arrows indicate Vsv-1-Au10 labelled ventral vesicles. X 33,850.

Fig 2.2d. Higher magnification T.E.M. of same cyst in 2.2b. Arrow indicates Vsv-1-Au10 labelled ventral vesicle. X 32,780.

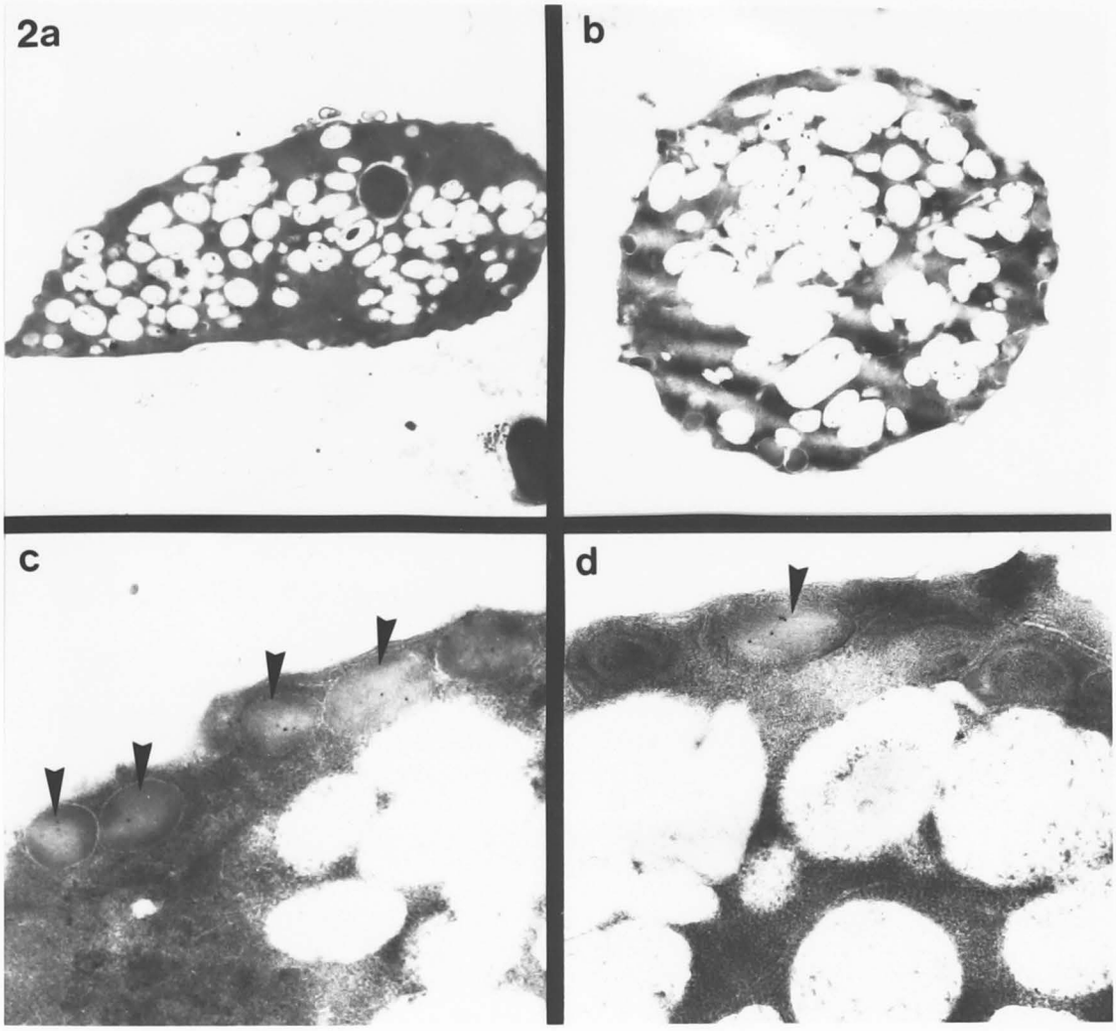

2a
b
c
d

Fig 2.3. Immunodot blot of cell extracts used to detect changes in the relative abundance of the three antigens in germlings growing in 5% V8 broth for 10h. The level of ventral vesicle antigen did not change during the first 2h after encystment, but decreased between 2 and 10h. No changes in the level of dorsal vesicle antigen were detected over the 10h period. A decline in large peripheral vesicle antigen was observed between 2 and 4h.

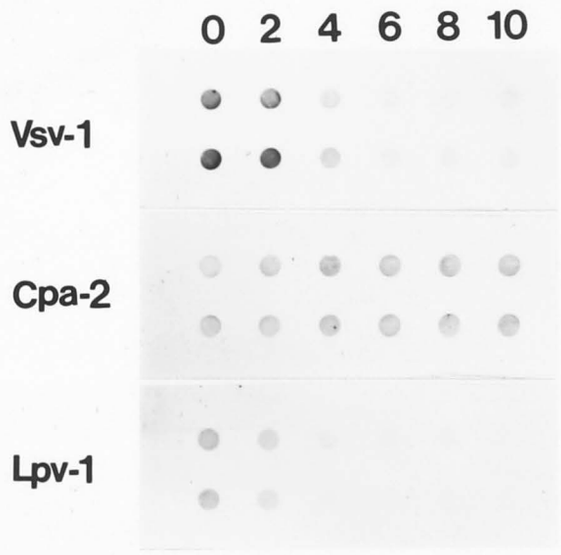


Fig 3

Figs 2.4a-2.4l. Peripheral vesicles in germlings transferred to mineral salts solution. bar=22.5μm

Figs 2.4a-2.4d. Ventral vesicles were absent at the time of transfer to mineral salts (6h old germlings, 2.4a) but after 6h in the mineral salts solution, they had reappeared (2.4b) and they increased in number over the next 12 (2.4c) and 24h (2.4d).

Figs 2.4e-2.4h. Dorsal vesicles were not present at the time of transfer (2.4e) or after 6h (2.4f) or 18h (2.4g) in the mineral salts solution. After 30h in the mineral salts solution dorsal vesicles were present (2.4h) and at this time sporangia had begun to appear (inset 2.4h.).

Figs 2.4i-2.4l. Large peripheral vesicles, like ventral vesicles, were absent at the time of transfer to mineral salts (6h old germlings, 2.4i) but after 6h in the mineral salts solution, they had reappeared (2.4j) and they increased in number over the next 12 (2.4k) and 24h (2.4l).

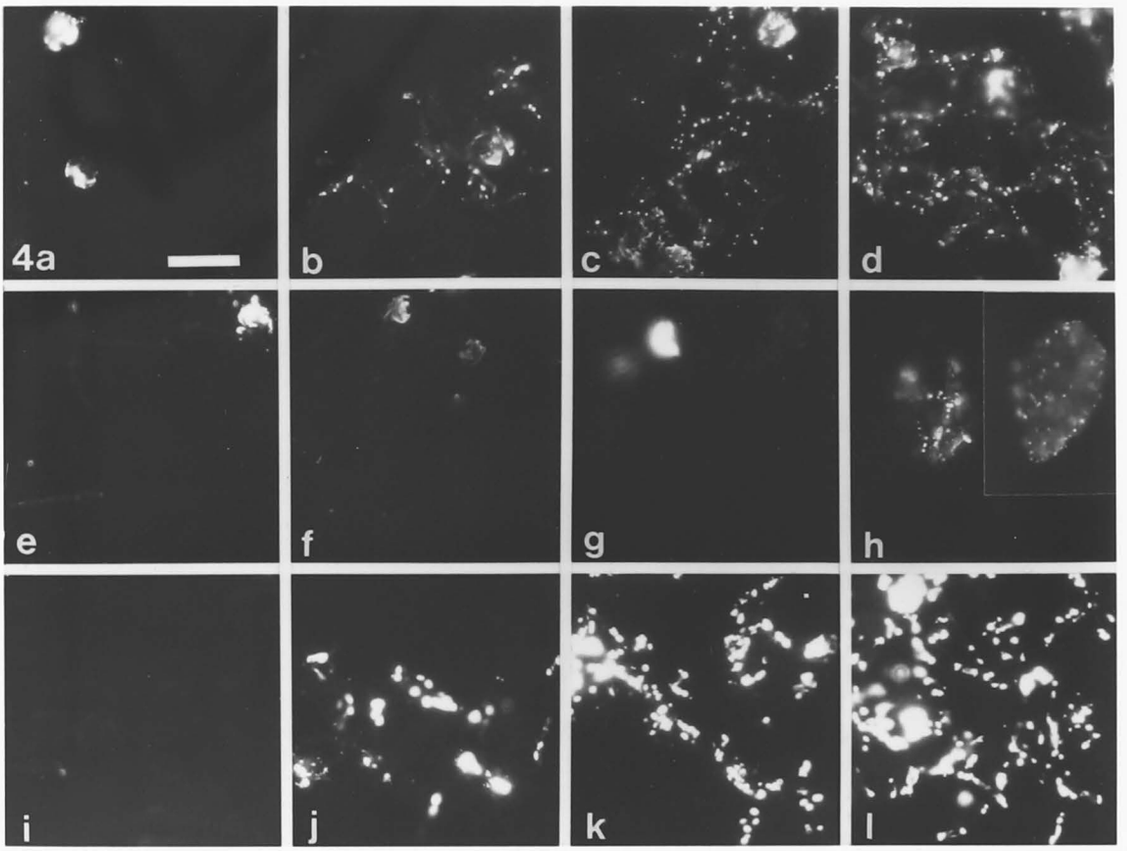

4a
b
c
d
e
f
g
h
i
j
k
l

Fig. 2.5. Immunodot blotting of cell extracts used to determine changes in the relative abundance of the three antigens transferred to mineral salts, after 6h in V8 broth. After 6h incubation in the mineral salts solution the Vsv-1 antigen was detected and then levels of the antigen increased over the next 24h. No changes were evident in the level of Cpa-2 antigen over the 30h period. Lpv-1 antigen was first detected 18h after transfer to mineral salts solution.

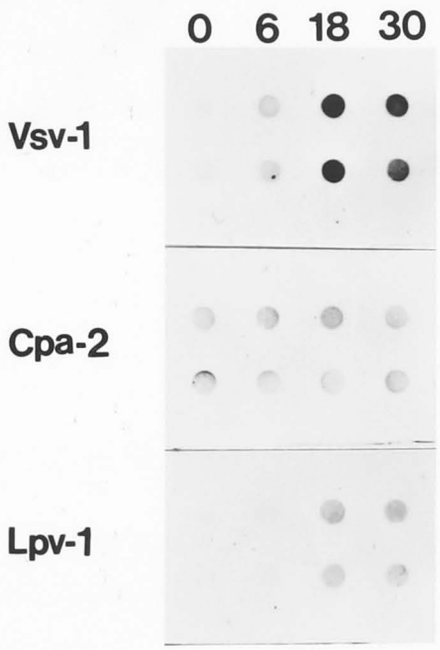


Fig5

Table 2.1. The disappearance and reappearance of peripheral vesicles in mycelial colonies grown in 5% V8 broth for 24h and then incubated in mineral salts solution for 8h. After 5 days growth on V8 agar, all three peripheral vesicles were present in cryosectioned hyphae. Following 24h incubation in 5% V8 broth all three peripheral vesicles had disappeared from the hyphae. Subsequent incubation of the plugs in mineral salts solution led to the reappearance of ventral and large peripheral vesicles between 4 and 6h. Dorsal vesicles reappeared between 6 and 8h which coincided with the first appearance of sporangia.

+ vesicles present.

- vesicles absent.

(s) sporangia present.

vesicle	V8 agar	0h	2h	4h	6h	8h (s)
ventral	+	-	-	-	+	+
dorsal	+	-	-	-	-	+
large	+	-	-	-	+	+

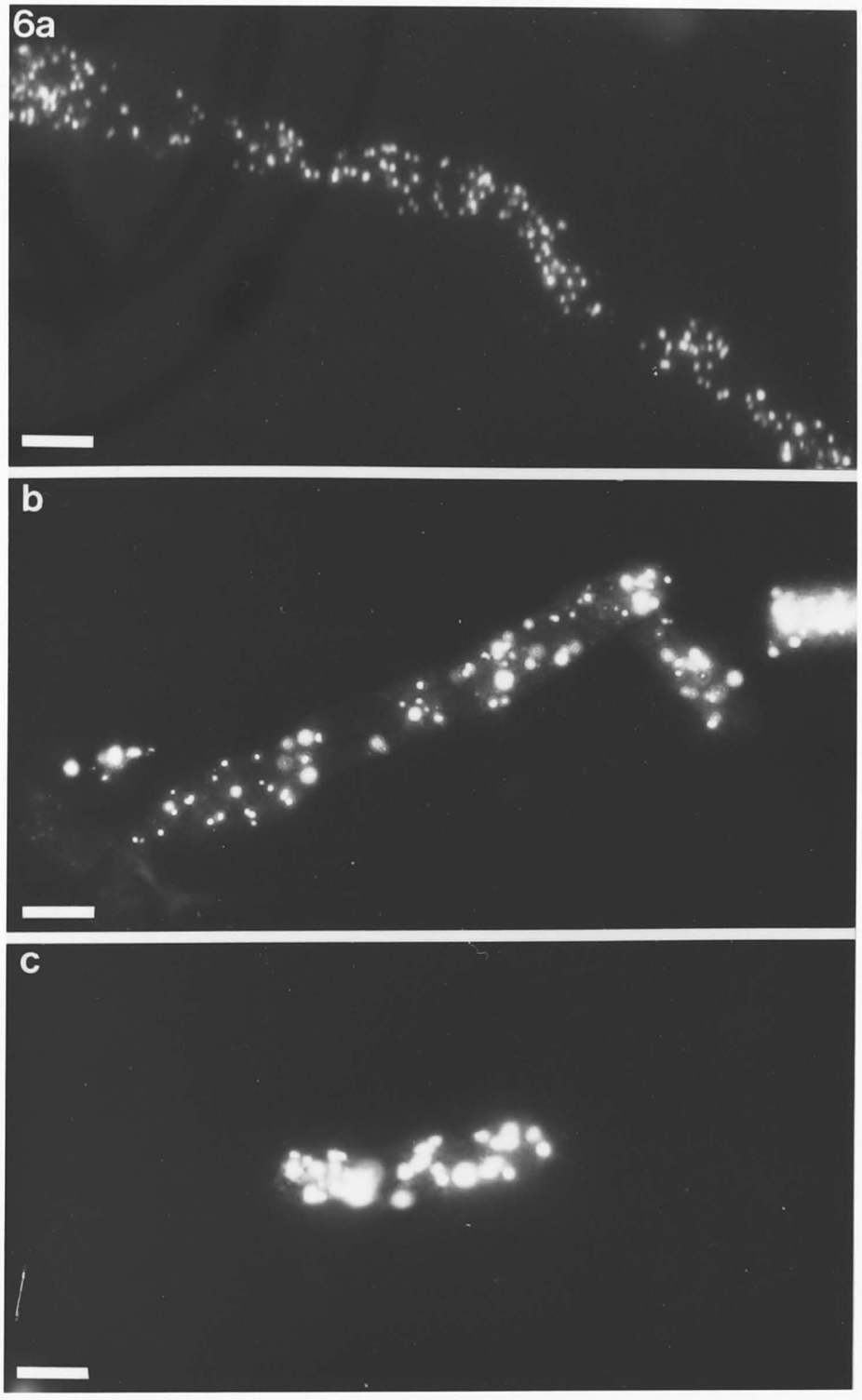
Table 2.1.

Figs 2.6a- 2.6c. Peripheral vesicles in cryosectioned hyphae taken from the edge of a 5 day colony growing on V8 agar.

Fig. 2.6a. Ventral vesicles. bar=9.7µm.

Fig. 2.6b. Dorsal vesicles. bar=10.8µm

Fig. 2.6c. Large peripheral vesicles. bar=8.8µm


6a
b
c

Figs 2.7a-2.7h. Peripheral vesicles were absent from hyphae growing vegetatively in 5% V8 broth. The three peripheral vesicles were present in large numbers after subsequent incubation of mycelial colonies in mineral salts solution.

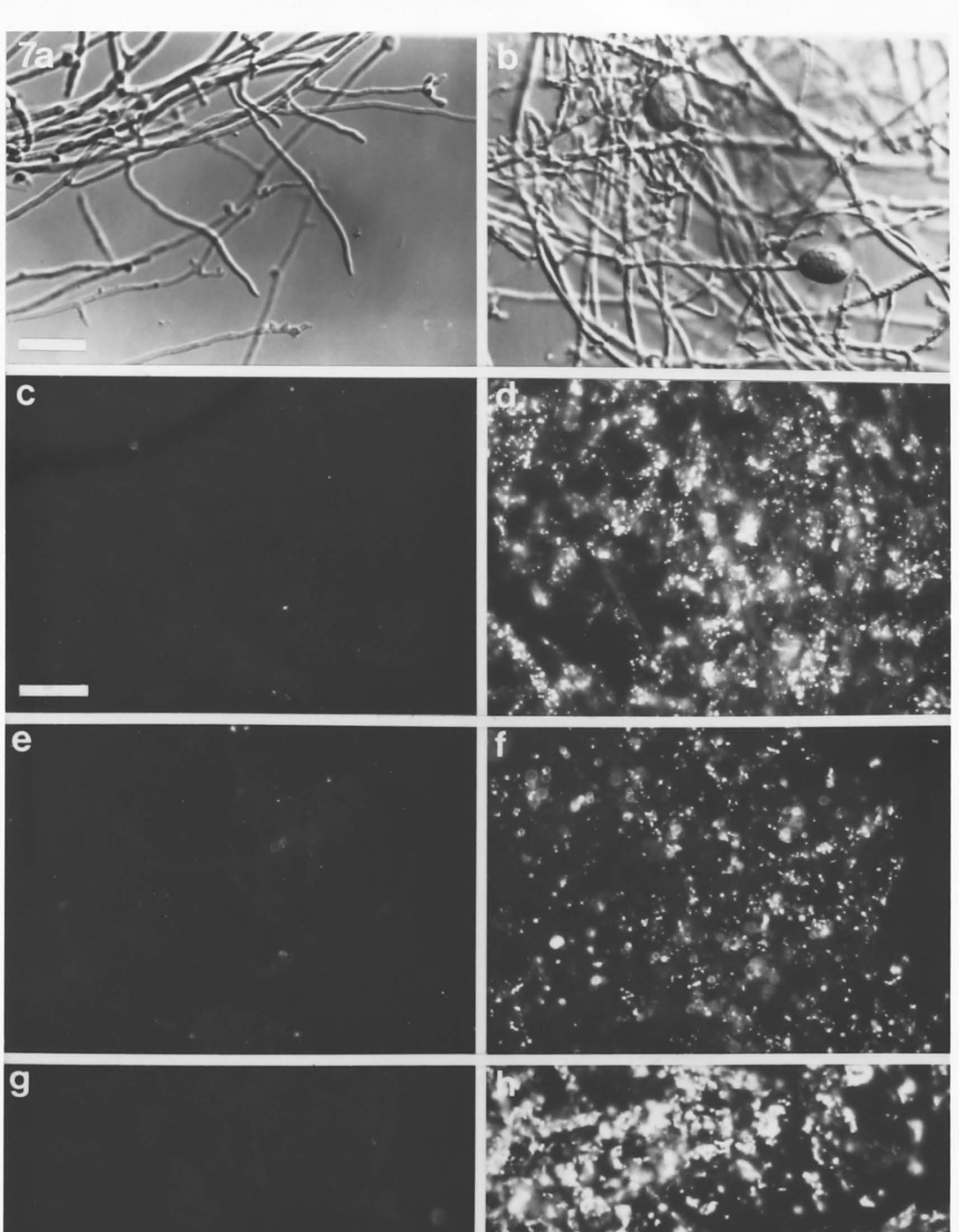
Fig. 2.7a. Light micrograph of hyphal culture after 24h incubation in V8 broth, showing many growing hyphal tips. bar=49.6µm.

Fig. 2.7b. Light micrograph of hyphal culture after 8h incubation in mineral salts solution. Sporangia began to appear at this time.

Fig. 2.7c-2.7d. Ventral vesicles were absent from vegetatively growing colonies (2.7c), but were present in large numbers after 8h incubation in mineral salts solution (2.7d). bar=29.7µm

Fig. 2.7e-2.7f. Dorsal vesicles were absent from vegetatively growing colonies (2.7e), but were present in large numbers after 8h incubation in mineral salts solution (2.7f).

Fig. 2.7g-2.7h. Large peripheral vesicles were absent from vegetatively growing colonies (2.7g), but were present in large numbers after 8h incubation in mineral salts solution (2.7h).


7a
b
c
d
e
f
g
h

Figs 2.8a-2.8d. The presence of all three peripheral vesicles in developing sporangia

Fig. 2.8a. Light micrograph of a young, developing sporangium, flat embedded in Lowicryl K4M. bar=20 μm.

Fig. 2.8b. Ventral vesicles (arrows) in same sporangium as in 2.8a. X 27,500.

Fig. 2.8c. Dorsal vesicles (arrows) in same sporangium as in 2.8a. X 36,250.

Fig. 2.8d. Large peripheral vesicles (arrows) in same sporangium as in 2.8a. X 42,600.

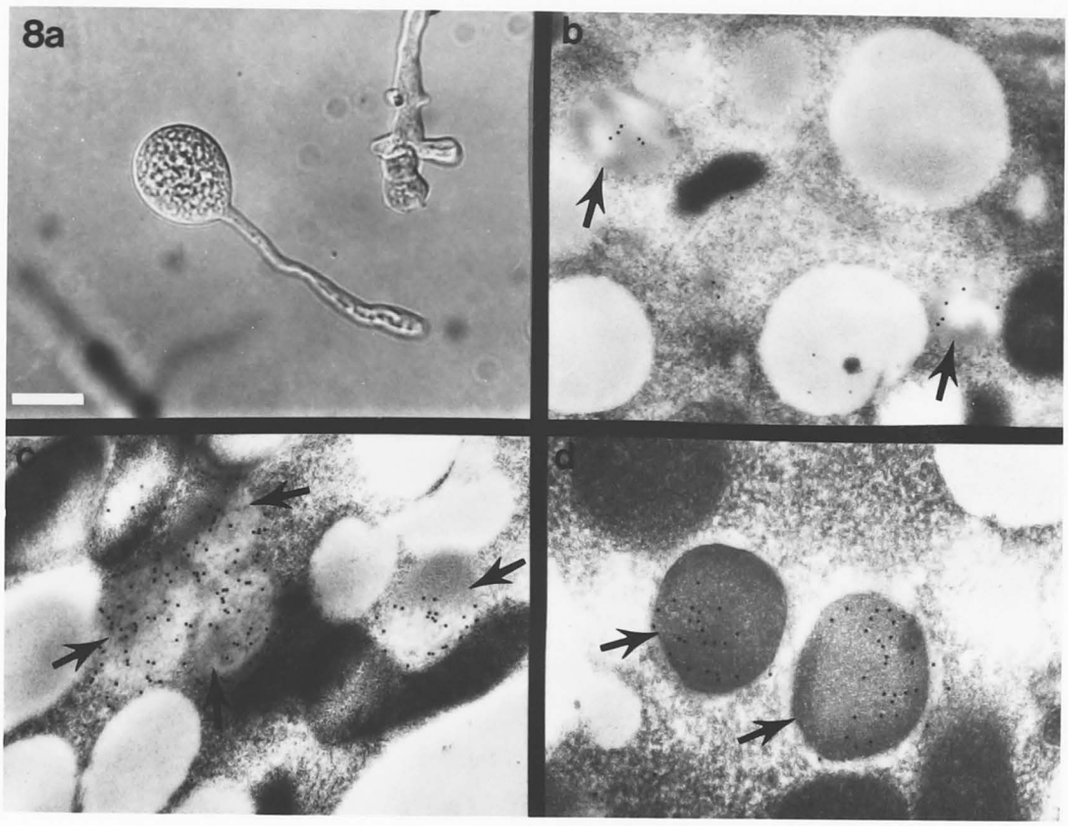

8a
b
c
d

Table 2.2. The presence and absence of peripheral vesicles during chlamydospore development. After growing for 5 days on V8 agar in the dark, hyphae at the edge of the colony contained all three of the vesicle types. If a small square of this mycelium wass inoculated onto a new V8 agar plate, cryosectioning after one day revealed that the hyphae did not contain any of these three vesicle types. By two days all three vesicles were again present and this was coincident with the first appearance of chlamydospores.

+ vesicles present.

- vesicles absent.

(c) chlamydospores present.

vesicle	0 day	1 day	2 day (c)
ventral	+	-	+
dorsal	+	-	+
large	+	-	+

Table 2.2.

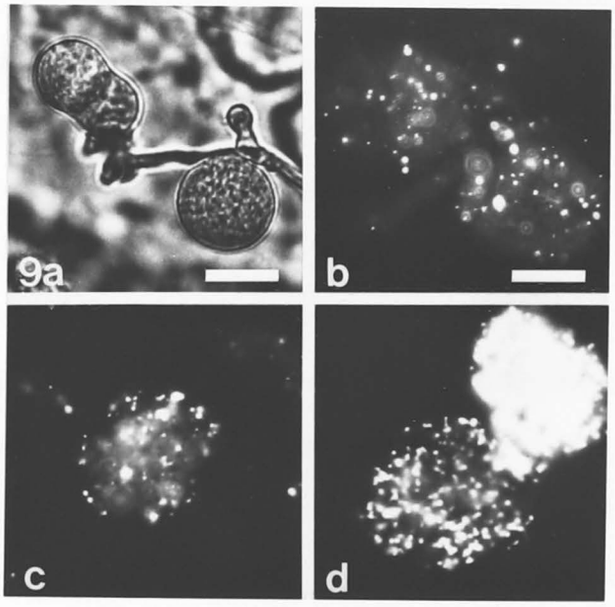
Figs 2.9a-2.9d. The presence of all three peripheral vesicles in chlamydospores.

Fig. 2.9a. Light micrograph of chlamydospores from 2 day old mycelial colony. bar= 14μm

Fig. 2.9b. Cryosectioned chlamydospores incubated with Vsv-1, indicating large numbers of ventral vesicles. bar= 18μm.

Fig. 2.9c. Cryosectioned chlamydospore incubated with Cpa-2, indicating large numbers of dorsal vesicles.

Fig. 2.9d. Cryosectioned chlamydospores incubated with Lpv-1, indicating large numbers of large peripheral vesicles.


9a
b
c
d

CHAPTER THREE: THE ULTRASTRUCTURE OF PERIPHERAL VESICLE FORMATION.

3.1 INTRODUCTION

This chapter reports on the ultrastructural formation of large peripheral, ventral and dorsal vesicles. There are few descriptions of the formation of peripheral vesicles in oomycetes and those that do exist are based on morphological observations provided by transmission electron microscopy. Oomycete peripheral vesicles are thought to be derived from either the Golgi apparatus or the endoplasmic reticulum (E.R.) or indeed a combination of both occurs in the formation of the attack apparatus in *Haptoglossa* (Robb & Lee 1986). Schnepf *et al.* (1978a) discussed the complicated ontogeny of encystment vesicles from Golgi-derived precursor vesicles in *Lagenisma,* but presented very little evidence to substantiate their description. Beakes (1983) recorded that fibrous vesicles first appeared around the apex of the nucleus in primary and secondary cysts of *Saprolegnia,* and suggested that they were probably derived from active dictyosomes which lay in close proximity. Lehnen and Powell (1991) showed that the precursors for K2-bodies in the primary cysts of *Saprolegnia ferax* formed from smooth-surfaced fenestrated cisternae, probably a simplified form of Golgi apparatus.

A number of authors propose an E.R. derivation for Oomycete peripheral vesicles. In *Lagenisma,* Schnepf *et al.* (1978b) recorded that microbody-type organelles form during cyst maturation in regions of dense E.R. Beakes (1983) suggested, as cisternae of E.R. had moderately electron dense contents and spinelike bodies characteristic of primary encystment vesicles in *Saprolegnia,* these vesicles

formed from E.R.. This author also claimed an E.R. origin for secondary encystment vesicles, as these vesicles were often observed continuous with cisternae of E.R.. Although not discussed by the authors, in the parasitic marine Oomycete, *Petersenia palmariae,* small dark vesicles, which ultimately disappear from the periphery of zoospores at encystment, can be first recognised around perinuclear cisternae of E.R. (Pueschel & van der Meer 1985).

There are some references available for peripheral vesicle formation in other zoosporic fungi. In Chytridiomycetes, Taylor and Fuller (1981) showed that both the paracrystalline body and vesicles that contained zoospore cell coat material in *Chytridium confervae* were probably Golgi-derived. In *Chytriomyces aureus* and *C. hyalinus*, the outer cell coat of zoospores is also thought to be derived from vesicles of dictyosomal origin (Dorward & Powell 1983). In *Blastocladiella,* gamma particles which are possibly involved in cell wall synthesis in encysting zoospores (Mills & Cantino 1981) were shown to be synthesised from rough E.R. (Barstow *et al.* 1985).

The unicellular chrysophycean algae are considered closely related to oomycetes (Gunderson *et al.* 1987). Members of this algal group have peripherally located organelles known as discobolocysts which are secretory in nature. In *Ochromonas tuberculatus* the discobolocysts have been shown to be derived from precursor Golgi-derived vesicles (Hibberd 1970). In other non-chrysophycean unicellular algae the Golgi apparatus has been shown to play an important role in the formation of secretory structures. The peripheral spheres of *Olisthodiscus luteus* are probably Golgi apparatus derived (Leadbeater 1969) while the cell wall scales in members of the Prasinophyceae have been shown to form in the Golgi apparatus (Brown *et al.* 1970, Domozych *et al.* 1981, McFadden *et al.* 1986) before being secreted to ornament the cyst surface.

The peripheral vesicles of *Phytophthora cinnamomi* contain glycoprotein (Gubler & Hardham 1988,1991), and they can be compared to secretory glycoproteins in other cells. In plants, secretory glycoproteins are considered rare and are confined to a few specialised systems such as digestive glands and aleurone tissues (Jones & Robinson 1989). Cell wall glycoproteins have, however, been localised to the Golgi apparatus and derived vesicles in *Chlamydomonas* (Grief & Shaw 1987) and other cell wall glycoproteins, such as extensin (Moore *et al.* 1991) and β-1,3-glucanase (Mauch & Stahelin 1989), have been localised to the Golgi apparatus. Gubler *et al.* (1986) have shown that α–amylase can be localised to the E.R. and Golgi apparatus in barley aleurone.

There are numerous accounts of the origin of secretory glycoproteins in animal cells. In canine pancreatic B cells, for example, both the regulated hormone insulin and the constitutive protein hemagglutinin, have been shown to bud away from Golgi cisternae in secretory vesicles (Orci *et al.* 1987). In human hepatoma cells, vesicular stomatitus virus glycoprotein and transferrin are shown to be present in the Golgi apparatus together and are released to the cell surface in secretory vesicles (Strous *et al.* 1983). In bovine pituitary cells, growth hormone, prolactin and secretogranin secretory granules are shown to be Golgi-derived (Hashimoto *et al.* 1987).

In the strictest sense, the contents of the large peripheral vesicles are not secretory proteins as they are not exocytosed from zoospores (see Farquhar 1985, Jones and Robinson 1989). The fact that large peripheral vesicles are probably a source of nitrogen used during germination (Gubler & Hardham 1990) makes them equivalent to the storage or vacuolar proteins of higher plants which are accumulated in preparation for vegetative growth (Wetzel *et al.* 1989, Sonnewald *et al.* 1989, Staswick 1990). Most higher plant vacuolar proteins are transported

through the Golgi apparatus before reaching their site of storage (see Chrispeels 1991 for review). Recently, however, a number of studies have suggested that some vacuolar proteins may form solely at the E.R. and may bypass the Golgi apparatus. Simon *et al.* (1990) have shown that the wheat storage proteins, α-gliadins are probably not transported through the Golgi apparatus, while the prolamin storage proteins in oats are probably transported directly from the E.R. to the vacuole and bypass the Golgi apparatus (Lending *et al.* 1989).

In the work reported in this chapter, I have used standard electron microscopy and immunoelectron microscopy to identify the point of formation of the three peripheral vesicles. The different peripheral vesicle antigens were first detected at separate positions in the secretory pathway providing further evidence about the nature of the vesicle antigens. Immunogold labelling also revealed new information about the characteristics of a possible intermediate compartment between the E.R. and the Golgi apparatus. Double labelling with antibodies to large peripheral and dorsal vesicles suggested that the vesicle contents are sorted at the level of the Golgi apparatus.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of hyphal samples

For the experiments reported in this chapter, *P. cinnamomi* 6BR hyphae were grown on miracloth on V8 agar (Chapter 2) for 5 days in the dark at 25°C. After this period the miracloth with adherant mycelia was transferred to 5% V8 broth (Chapter 2) and shaken (at 150rpm) for 24h in the light at 22°C. The miracloth samples were rinsed three times in mineral salts solution (Chapter 2) and then

shaken (150rpm) in a final volume of 100ml mineral salts solution in the light at 22°C. After 3h in the mineral salts solution there was a substantial increase in the number of large peripheral and ventral vesicles in the hyphae, and by 4h dorsal vesicles began to form (see Table 3.1). Hyphae were scraped from miracloth at 4h and fixed for 1h in 1% glutaraldehyde buffered in 100mM Pipes (pH 7.0) at room temperature.

3.2.2 Processing of tissue for electron microscopy

Tissue processing for immunocytochemistry: After a rinse in 100mM Pipes buffer the hyphae were dehydrated in an ethanol series of half hour steps (10%, 25% at 4°C, then 50%, 70%, 100% at -15°C) and infiltrated in an alcohol/Lowicryl K4M (Chemische Werke Lowi GMBH & Co., Waldkraiburg, Germany) series of 2h steps (2:1, 1:1, 1:2, ethanol:Lowicryl K4M and then pure Lowicryl K4M). After two changes over two days in pure Lowicryl K4M, the samples were embedded in an oxygen free polymerising unit with an overhead UV light at overnight -15°C, then with a further 24h at 20°C to ensure complete hardness of the blocks.

Tissue processing for conventional embedding: After rinsing in 100mM Pipes buffer and a final rinse in distilled water the samples were postfixed in a 2% solution of osmium tetroxide in distilled water for 1h at room temperature. Following three rinses in distilled water dehydration was carried out at room temperature in a graded acetone series of 30min steps (10%, 25%, 50%, 70%, and 100%). The material was then infiltrated in a graded series of acetone: Spurr's resin of 2h steps (2:1, 1:1, 1:2, acetone:Spurr's resin) and then embedded in resin at 60°C.

3.2.3 Preparation of direct gold probes

Direct gold probes were prepared by the method of Gubler and Hardham (1988). The 10nm gold particles were prepared by reducing a solution of chloroauric acid with a mixture of tannic acid and trisodium citrate (Slot & Geuze 1985), while the 18nm gold particles were prepared by reaction of chloroauric acid with trisodium citrate only (Frens 1973). Affinity purified antibodies Cpa-2 and Lpv-1 were complexed with 10 (Cpa-2-Au10) and 18nm (Lpv-1-Au18) gold particles respectively. The antibody-gold probes were purified by 10-30% glycerol density gradient centrifugation (Slot & Geuze 1985).

3.2.4 Immunogold labelling

For single labelling, sections were blocked for 10min in PBS/ 1%BSA/ 0.1%Gelatin/ 5% normal goat serum and then incubated with undiluted solutions of Cpa-2-Au10 and Lpv-1-Au18 for 1h. After washing in drops of PBS-0.2%Tween-20, the sections were post-stained in 2% aqueous uranyl acetate and lead citrate for 5min and 1min respectively.

Labelling with Vsv-1-Au10 (Chapter 2), or a two-tiered labelling system with the first antibody Vsv-1 and the second antibody S.A.M-IgAu10, gave only weak labelling of ventral vesicles. The amount of labelling with the Vsv-1 antibody was increased with a three-tiered labelling system (McCurdy & Pratt 1986). After blocking for 10min, the grids were incubated for 1h in 3µg/ml Vsv-1 antibody diluted in PBS/1% BSA/0.1% gelatin/1% normal goat serum, then, after rinsing in drops of PBS-Tween-20, incubated for 1h in rabbit-anti-mouse immunoglobulin (Silenus, Dandenong, Australia) diluted 1:50 in PBS/1% BSA/0.1% gelatin/1% normal goat serum with the addition of 2% w/v Tween-20. After rinsing in PBS-

Tween, the grids were incubated for 1h in goat anti-rabbit Au5 (Amersham, UK), diluted 1:20 in the above buffer. Following rinses in PBS-Tween-20, the grids were post-stained as above.

For double labelling, grids were blocked for 10min, then incubated for 1h with Cpa-2-Au10. After washing in drops of PBS-Tween-20, the grids were incubated 1h in Lpv-1-Au18, washed in PBS-Tween and briefly in distilled water, then post-stained as above.

For double labelling with Cpa-2-Au10 and HDEL (a marker for the E.R.), after blocking, grids were incubated in undiluted supernatant of HDEL antibody (obtained from Hugh Pelham, Medical Research Council Laboratories of Molecular Biology, Cambridge, U.K.) followed by goat anti-mouse IgAu5 (Janssen, Olen, Belgium) diluted 1:20 in PBS/ 1%BSA/ 0.1% gelatin/ 2% w/v Tween-20 plus 1% normal goat serum. After washing, the grids were blocked for 1h in 100 µg/ml non-immune mouse serum (St Louis, MO, USA) then incubated in undiluted Cpa-2-Au10.

3.3 RESULTS

All micrographs are representative examples of the labelling observed.

3.3.1 The ultrastructural formation of large peripheral vesicles

Antibodies to large peripheral vesicles labelled the *cis* (Figs 3.1a, 3.1b, 3.1c), *medial* (Figs 3.1a, 3.1b, 3.1c) and *trans* (Fig. 3.1c) cisternae of the Golgi apparatus. Characteristic electron dense, large peripheral vesicles, in both labelled (Figs 3.1a, 3.1b, 3.1c) and unlabelled (Fig. 3.1d) sections, were seen at the *trans*

part of the Golgi apparatus suggesting they had recently formed there. As well as the peripheral vesicles, vacuoles were also positive for Lpv-1-Au18 (Fig. 3.4f). Labelling with Lpv-1 occurred in the lumen of the E.R. (Figs 3.1b, 3.1c, 3.4a, 3.4b) as well as in a region between the E.R. and the Golgi apparatus (Figs 3.1a, 3.1b, 3.1c, 3.4b, 3.4e).

3.3.2 The ultrastructural formation of dorsal vesicles

Antibodies to dorsal vesicles labelled the Golgi apparatus and recently derived vesicles (Figs 3.2a, 3.2b, 3.2c), although the labelling was mostly restricted to the *cis* (Figs 3.2a, 3.2c) and *trans* side of the Golgi apparatus (Fig. 3.2b). Labelling with Cpa-2-Au10 was first seen associated with pre-Golgi regions (Figs 3.2a, 3.2c, 3.4b, 3.4c) but double labelling with an antibody to resident E.R. proteins, HDEL, which labelled the lumen of the E.R. (Fig. 3.2d), indicated that the Cpa-2 antigen was first detected in regions outside the E.R. and not the lumen of this organelle (Fig. 3.2d).

3.3.3 The ultrastructural formation of ventral vesicles

Antibodies to ventral vesicles failed to label the Golgi apparatus, but labelled vesicles were often observed near juxtanuclear Golgi apparatus and E.R. (Figs 3.3a, 3.3b). Material embedded in Spurr's resin revealed a probable Golgi origin for ventral vesicles, as vesicles with plate-like inclusions characteristic of mature vesicles (Fig. 3.3d), could be observed forming at the *trans* side of the Golgi apparatus (Fig. 3.3c).

3.3.4 Double labelling results

Double labelling with antibodies to dorsal and large peripheral vesicles revealed that these two antigens were present in the same cisternae of the Golgi apparatus (Figs 3.2a, 3.4f, 3.5). Vesicles in the process of budding (Figs 3.4b, 3.4f), or recently derived from the Golgi apparatus (Figs 3.2a, 3.4a, 3.4e) contained only one antigen type even though these antigens appeared intermixed within nearby Golgi cisternae (Fig. 3.4f). No cross reactivity occurred in mature vesicles within the hyphal cytoplasm (Figs 3.4c, 3.5). In many cases the Golgi apparatus was not easily visualised but this organelle could be recognised due to its usual proximity to E.R. and nuclei (Fig. 3.4d).

3.4 DISCUSSION

The results strongly suggest that the three peripheral vesicles are derived from the the Golgi apparatus. In their ultrastructural origin, these vesicles are thus similar to the encystment vesicles in *Lagenisma* (Schnepf *et al.* 1978a) and the fibrous vesicles of *Saprolegnia ferax* (Beakes 1983). This result further reinforces the closeness of the link between oomycetes and chrysophycean algae, as members of this algal group also contain Golgi-derived peripheral secretory organelles (Hibberd 1970). Large peripheral vesicles do not bypass the Golgi apparatus in their formation and thus, in their mode of synthesis, are like many of the storage proteins of higher plants (Chrispeels 1983, Craig and Goodchild 1984).

Secretory glycoproteins undergo *N*-linked glycosylation in the lumen of the rough E.R. but are further processed in the Golgi apparatus by *O*-glycosylation, sulphation, and phosphorylation (Farquhar 1985). The fact that large peripheral vesicle antigen can be found in both the E.R. and the Golgi apparatus suggests that the epitope for this antibody is either a protein or an *N*-linked carbohydrate.

Earlier work (Hardham unpublished) has, however, shown that binding of Lpv-1 to the large peripheral vesicle antigen is unaffected by treatment with periodate but is susceptible to pronase digestion. This suggests that the epitope to which Lpv-1 binds is a protein moiety.

The fact that vacuoles were positive for Lpv-1-Au18, suggests that these have been derived from the coalescence of mature large peripheral vesicles. The breakdown of large peripheral vesicles to large vacuoles has been shown to occur in the germinated cysts of a number of oomycetes including *Saprolegnia ferax* (Holloway & Heath 1977), *Pythium aphanidermatum* (Grove & Bracker 1978), *Phytophthora palmivora* (Bimpong & Hickman 1975), and *Phytophthora cinnamomi* (Gubler & Hardham 1990) and it is possible that this same phenomenon occurs in mature hyphae.

The presence of plate-like inclusions in vesicles at the trans side of the Golgi apparatus suggests that ventral vesicles are Golgi-derived. The lack of labelling of the Golgi apparatus with Vsv-1 antibodies, when vesicles close by are labelled, suggests that the antigen is either in too low a concentration for detection or it is not present. The Vsv-1 antibody appears to react with the plate-like inclusions characteristic of this vesicle type (Gubler & Hardham 1991) and so it is possible that these structures are not present in Golgi cisternae but are formed in vesicles that have budded away from the Golgi apparatus. If this is the case, the morphogenesis of these vesicles is similar to the formation of K2-bodies in *Saprolegnia,* where the precursors to these adhesive containing vesicles appear to form plate-like fragments after synthesis from a simplified Golgi apparatus (Lehnen & Powell 1991).

The dorsal vesicle antigen is first detected inside a structure that does not appear morphologically to be Golgi apparatus. Double labelling with an antibody that recognises E.R. resident luminal proteins with an HDEL sequence indicates that the dorsal vesicle antigen is not present in the lumen of the E.R and thus reveals that these structures are not E.R. The existence of a post-E.R.-pre-Golgi compartment has become one of the most exciting subject areas in cell biology over the last few years. In hamster BHK-21 cells, this compartment was described as a series of vacuolar elements positioned between the E.R and the *cis*-Golgi (Saraste & Kuismanen 1984). The compartment has been defined in monkey Vero cells through immunofluorescence microscopy and immunogold labelling by a 53-kDa transmembrane protein and has been shown to be an intermediate in the E.R. to Golgi apparatus protein transport pathway (Schweizer *et al.* 1990). It is possible that the intermediate E.R.-Golgi apparatus cisternae, which contain the dorsal vesicle antigen represent such a compartment in *P. cinnamomi*. The results from this study suggest that this compartment may play some role in the maturation of the dorsal vesicle antigen as the antigen is first detected in this region by the Cpa-2 antibody. The first of the two lysosomal sorting enzymes, *N*-acetylglucosaminyl-1-phosphotransferase, is believed to be localised in a post-E.R. compartment (Kornfeld & Mellman 1989) so it is possible that enzymes responsible for modifying the dorsal vesicle antigen are localised there also.

The intermixing of vesicle glycoproteins within Golgi cisternae, but the presence of only one type of antigen in forming and post-Golgi vesicles suggests that a sorting event has occurred. Protein sorting has been shown to occur in the *trans* Golgi network, the exit site from the Golgi apparatus (Griffiths & Simons 1986) and has been extensively studied in a number of systems. Orci *et al.* (1987) showed that the regulated hormone, insulin, and the constitutive protein,

hemagglutinin, are sorted at the *trans*-most Golgi cisternae in pancreatic B cells. Hemagglutinin was absent from regions engaged in the budding process and immature insulin containing vesicles, suggesting that sorting of the two proteins preceded the formation of the secretory granules. Tooze *et al.* (1987) indicated a similar situation occurs in murine pituitary tumour cells. The regulated secretory protein adrenocorticotropic hormone, and constitutive coronavirons are present in the same regions of the *trans* Golgi network but these rarely occur in the same secretory vesicle, suggesting they are sorted at this level. Sorting within the regulated secretory pathway has also been demonstrated in the bag cells of *Aplysia californica* (Fisher *et al.* 1988). These authors demonstrated distinct hormones arising from a common precursor being packaged into separate secretory granules at the *trans* Golgi network.

In contrast, however, to the above workers, von Zastrow and Castle (1987) advocated a sorting system that does not involve the Golgi apparatus. In exocrine cells, constitutive secretory proteins are sorted from regulated proteins inside secretory granules which have separated from the Golgi apparatus. Recent work with the *Aplysia* bag cell system (Sossin *et al.* 1990) also indicated there may be sorting sites for secretory proteins other than the Golgi apparatus in cells. These authors show that although sorting begins in the *trans* Golgi network, it can continue within immature granules via the activation of enzymes by acidification of the granules.

After consideration of the above two approaches to secretory protein sorting, a site of sorting in *P. cinnamomi* can be suggested. In contrast to the work of von Zastrow and Castle (1987) and Sossin *et al.* (1990), sorting of glycoproteins Lpv-1 and Cpa-2 appears to occur only at the level of the Golgi apparatus. Lack of gold labelling crossreactivity in budding and newly formed vesicles indicated that the

different glycoproteins are sorted in the Golgi apparatus before the vesicle has budded away. In this study, the poor preservation of the Golgi apparatus in Lowicryl K4M material made it difficult to tell whether or not this sorting is occurring specifically in the *trans* Golgi network.

It would be interesting to try to elucidate the mechanism behind the sorting of these two glycoproteins. Aggregation-mediated sorting of secretory proteins has been a popular concept in the last 7 years (Kelly 1985, Gerdes *et al.* 1989, Huttner & Tooze 1989). In the calcium rich conditions that are thought to be present in the *trans* Golgi, secretory proteins may form aggregates (Gerdes *et al.* 1989) and may then be enveloped by membranes to form secretory vesicles (Burgess & Kelly 1987, Pfeffer & Rothman 1987). The separation of secretory proteins at this level may reflect differences in the aggregative properties of these proteins (Huttner & Tooze 1989).

A second proposal for the sorting of secretory proteins in the *trans* Golgi is a receptor-mediated sorting event. Chung *et al.* (1989) have presented evidence that a set of proteins, known as hormone binding proteins, may recognize and concentrate peptide hormones such as prolactin, insulin and growth hormone in the *trans* Golgi of canine pancreatic cells. Receptor mediated sorting has been advocated for the sorting of lysosomal enzymes from secretory proteins (reviewed by von Figura & Hasilik 1986). A mannose-6-phosphate marker attached to the enzyme has specific receptors which facilitate the separation of lysosomal enzymes from the milieu of *trans* Golgi traffic (Griffiths *et al.* 1988).

This work has revealed the ultrastructural origin of the three peripheral vesicles in *P. cinnamomi* 6BR. In common with many other secretory systems, dorsal and ventral vesicles are shown to be derived from the Golgi apparatus. Also, like the

storage proteins of higher plants, large peripheral vesicles are shown to be similarly Golgi-derived. The Lpv-1 antigen is shown to be present in both the E.R. and the Golgi apparatus, evidence consistent with the idea that the Lpv-1 epitope is proteinaceous. Although Vsv-1 antibodies did not label the Golgi apparatus, it is considered that these vesicles are Golgi-derived, as the plate-like inclusions which are characteristic of this vesicle type, and to which the antibody binds, seem to first appear in vesicles that are closely associated with the Golgi apparatus. The presence of the dorsal vesicle and large peripheral vesicle antigens in a region between the E.R. and the Golgi apparatus is evidence for the existence of an intermediate compartment. This compartment may contain enzymes which are important in the processing of the dorsal vesicle glycoprotein, though its precise properties demand further investigation. Double labelling reveals that vesicle antigens can be present in the same Golgi cisternae, and also that a Golgi sorting mechanism exists which ensures that vesicles leave the Golgi apparatus containing only one type of antigen. Although the mechanism of sorting of peripheral vesicle antigens in the *P. cinnamomi* Golgi apparatus remains undetermined, the separation of glycoproteins at the *trans* Golgi regions enables the formation of distinct peripheral vesicles which have specific functions in the infection of hosts.

Table 3.1. Changes in peripheral vesicle populations in hyphal colonies grown on miracloth and transferred to mineral salts solution for 4h. Ventral and large peripheral vesicles were present in small numbers at the time of transfer from V8 broth to mineral salts solution to 2h, but between 2 and 3h there was a substantial increase in their numbers. Dorsal vesicles were not present in hyphae after 24h in V8 broth and first appeared between 3 and 4h in mineral salts solution. Between 3 and 4h, sporangia had begun to appear.

vesicle	0h	1h	2h	3h	4h (s)
ventral	+	+	+	++	++
dorsal	-	-	-	-	++
large	+	+	+	++	++

Table 3.1.

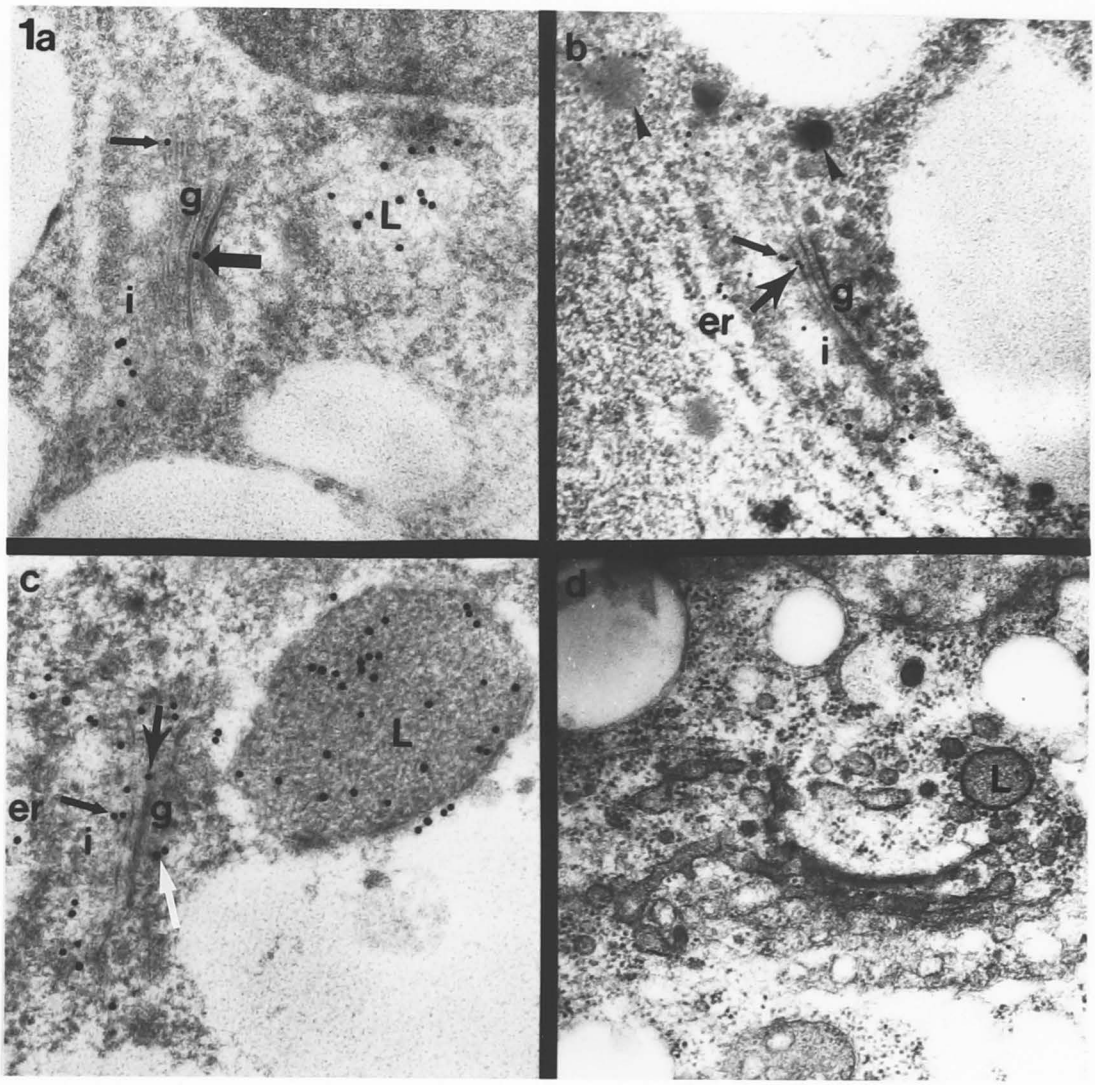
Figs 3.1a-3.1d. The ultrastructural formation of large peripheral vesicles.

Fig. 3.1a. The Lpv-1 antibody labelled the *cis* (small arrow) and *medial* cisternae (large arrow) of the Golgi apparatus (g). Labelling also occured in an intermediate compartment (i) close to the *cis* side of the Golgi apparatus. A labelled large peripheral vesicle (L) could be seen at the *trans* side of the Golgi apparatus suggesting that it had recently formed there.
X 60,000.

Fig. 3.1b. Lpv-1 label in the lumen of the E.R. (er), inside the intermediate compartment (i) and associated with the *cis* (small arrow)and *medial* (large arrow) parts of the Golgi apparatus (g). Arrowheads indicate recently formed large peripheral vesicles. X 40,000.

Fig. 3.1c. Lpv-1 labelling associated with the E.R. (er), the intermediate compartment (i), and the *cis* (small arrow), *medial* (large arrow) and *trans* (white arrow) parts of the Golgi apparatus (g). A labelled large peripheral vesicle appeared close to the *trans* part of the Golgi apparatus (L).
X 58,700.

Fig. 3.1d. A large peripheral vesicle (L) close to the *trans* side of the Golgi apparatus. Material embedded in Spurr's resin. X 40,000.


1a
g
L
i
b
er
g
i
c
L
er
i
g
d
L

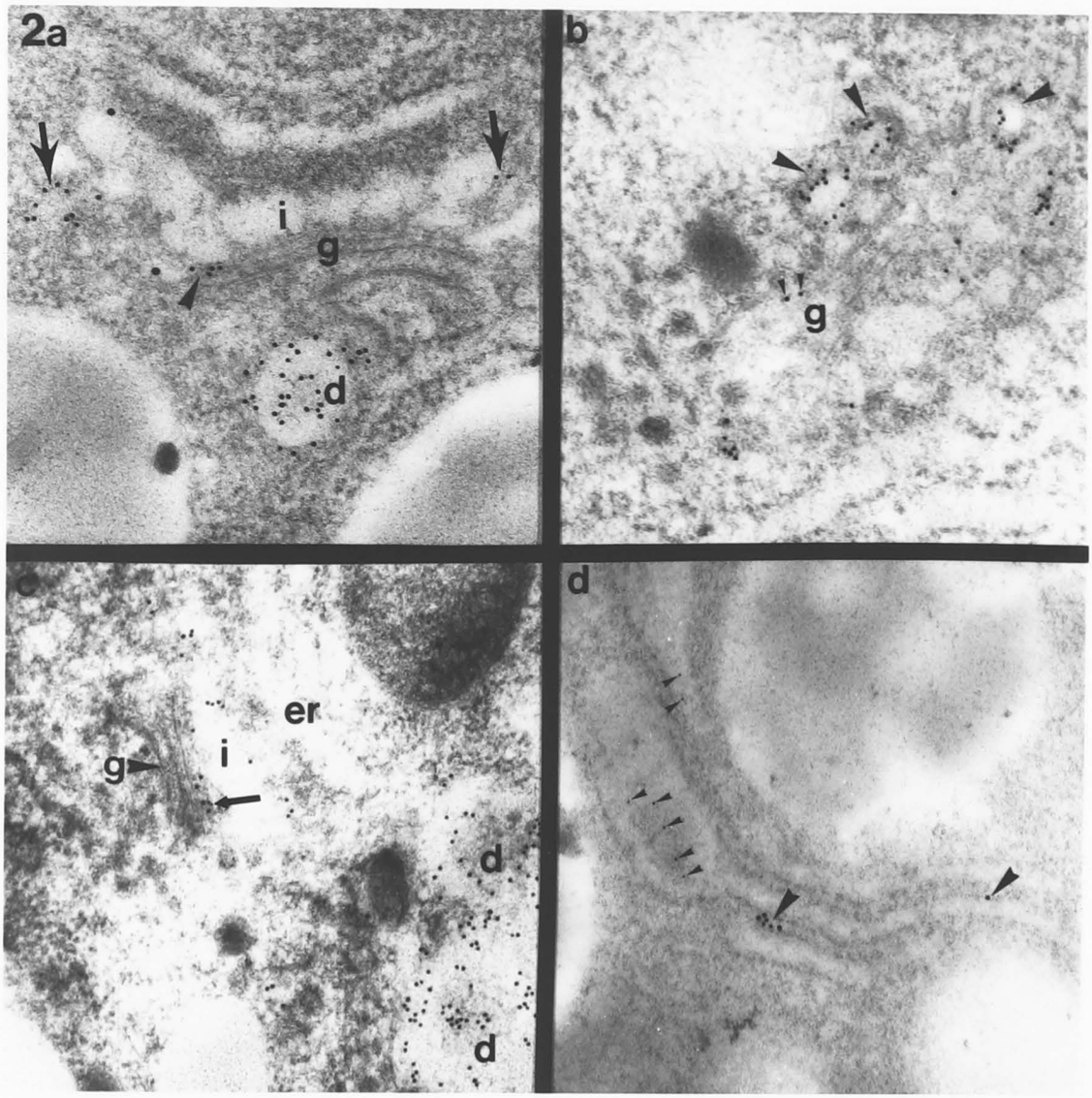
Figs 3.2a-3.2d. The ultrastructural formation of dorsal vesicles.

Fig. 3.2a. Double labelling with Cpa-2 and Lpv-1. The Cpa-2 antibody labelled *cis* Golgi regions (small arrow, 10nm gold particles) as well as an intermediate region (i, large arrows,) between the E.R. and the Golgi apparatus (g). The Lpv-1 antigen (18nm gold particle near small arrow) appeared to be present in the same Golgi cisterna as the Cpa-2 antigen. Lpv-1 antigen was also associated with the intermediate E.R./Golgi region (18nm gold particle). A dorsal vesicle (d), labelled with the Cpa-2 antibody could be seen close to the *trans* part of the Golgi apparatus suggesting that it had recently formed there. X 61,000.

Fig. 3.2b. Cpa-2 labelling in *trans* Golgi regions (g, small arrows) and in recently derived vesicles (large arrows). X 60,000.

Fig. 3.2c. Cpa-2 labelling could be observed associated with the *cis* Golgi cisternae (small arrow) as well as an intermediate Golgi-E.R. region (i). A number of labelled dorsal vesicles could be observed in the surrounding cytoplasm (d). er=E.R., g=Golgi apparatus. X 42,000.

Fig. 3.2d. Double labelling with the Cpa-2 antibody (large arrows) and the HDEL antibody (small arrows) indicated the dorsal vesicle antigen was first detected in regions outside the E.R. and not the lumen of the organelle. X 60,000.

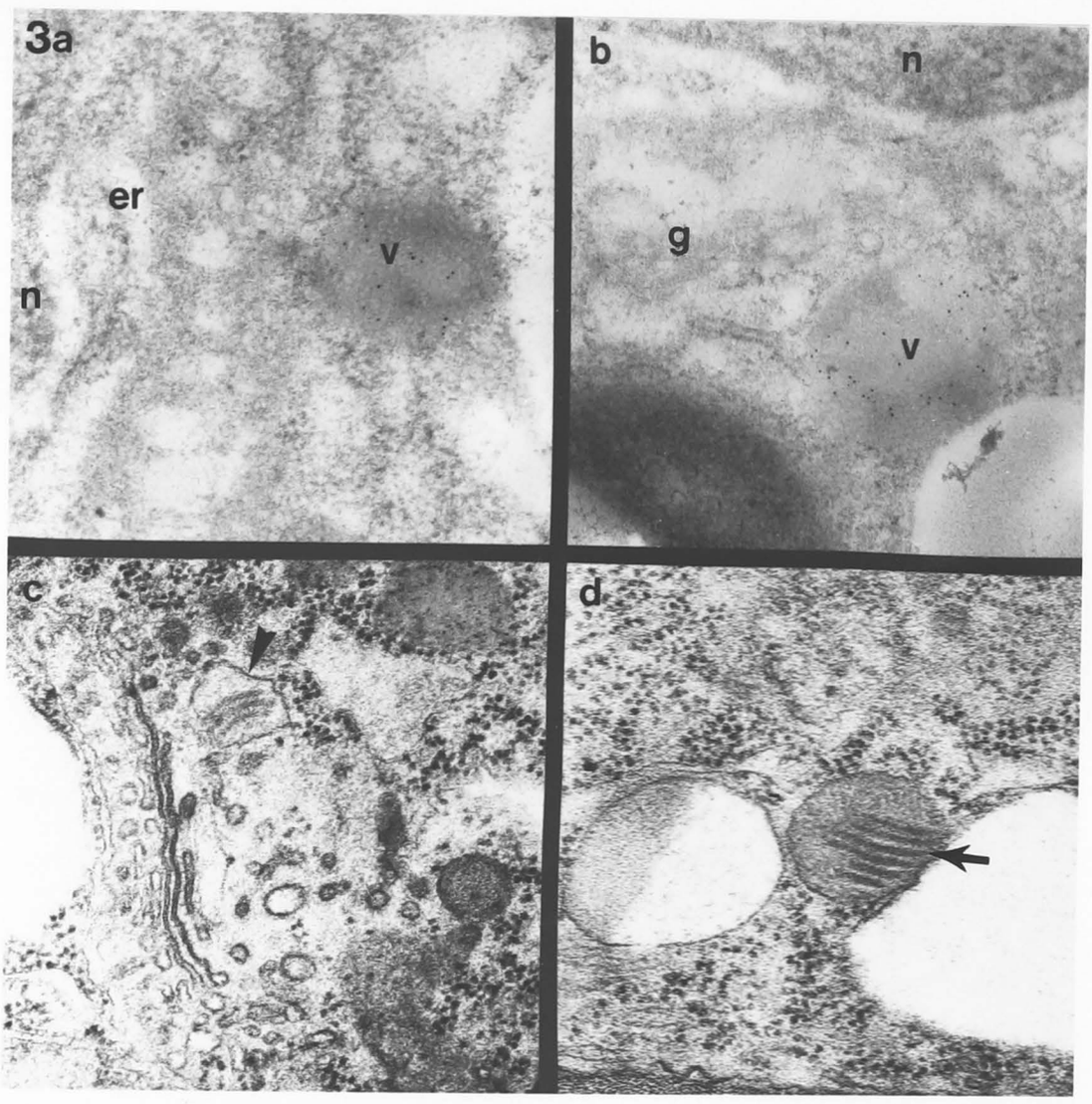

2a
i
g
d
b
g
c
er
g
i
d
d
d

Figs 3.3a-3.3d. The ultrastructural formation of ventral vesicles.

Figs 3.3a-3.3b. The Vsv-1 antibody failed to label juxtanuclear regions of E.R.(er) and Golgi apparatus (g), but labelled vesicles (v) often appeared close to these regions. n=nucleus. X 62,500.

Fig. 3.3c. A vesicle with the characteristic plate-like inclusions of mature ventral vesicles was seen at the *trans* part of the Golgi apparatus (arrow). Material embedded in Spurr's resin. X 48,000.

Fig. 3.3d. A mature ventral vesicle with characteristic plate-like inclusions (arrow). Material embedded in Spurr's resin. X 60,000.


3a
er
v
n
b
n
g
v
c
d

Figs 3.4a-3.4f. Double labelling with antibodies to large peripheral and dorsal vesicles. 10nm gold-Cpa-2. 18nm gold-Lpv-1.

Fig. 3.4a. The Lpv-1 antibody labelled the E.R. (er) and both the Lpv-1 antigen and the Cpa-2 antigen appeared intermixed within the Golgi apparatus (g). A recently formed vesicle contained only the Cpa-2 antigen (arrow). X 33,000.

Fig. 3.4b. Lpv-1 labelling occured singly in the E.R. (er) but occured with Cpa-2 labelling in an intermediate E.R.-Golgi apparatus region (i). A vesicle forming from the *trans* part of the Golgi apparatus contained only the dorsal vesicle antigen (arrow). X 51,000.

Fig. 3.4c. No crossreactivity with the two antibodies occurred in mature vesicles within the hyphal cytoplasm. (L) large peripheral vesicle; (d) dorsal vesicle. X 53,000.

Fig. 3.4d. Micrograph of hyphae demonstrating the usual proximity of the Golgi apparatus to E.R.(er arrow) and nuclei (n). Material embedded in Spurr's resin X 45,800.

Fig. 3.4e. Double labelling of the intermediate E.R.-Golgi region (i) with Lpv-1 and the Cpa-2 antibodies. A vesicle which appeared recently to have formed at the *trans* part of the Golgi apparatus, contained only the dorsal vesicle antigen (d). X 52,000.

Fig. 3.4f. The large peripheral and dorsal vesicle antigens appeared intermixed within the same cisterna of the Golgi apparatus (large arrows). A vesicle in the process of forming from the *trans* part of the Golgi apparatus contains only the dorsal vesicle antigen (small arrow). Vacuoles were also positive for the Lpv-1 antigen (vc). X 45,200.

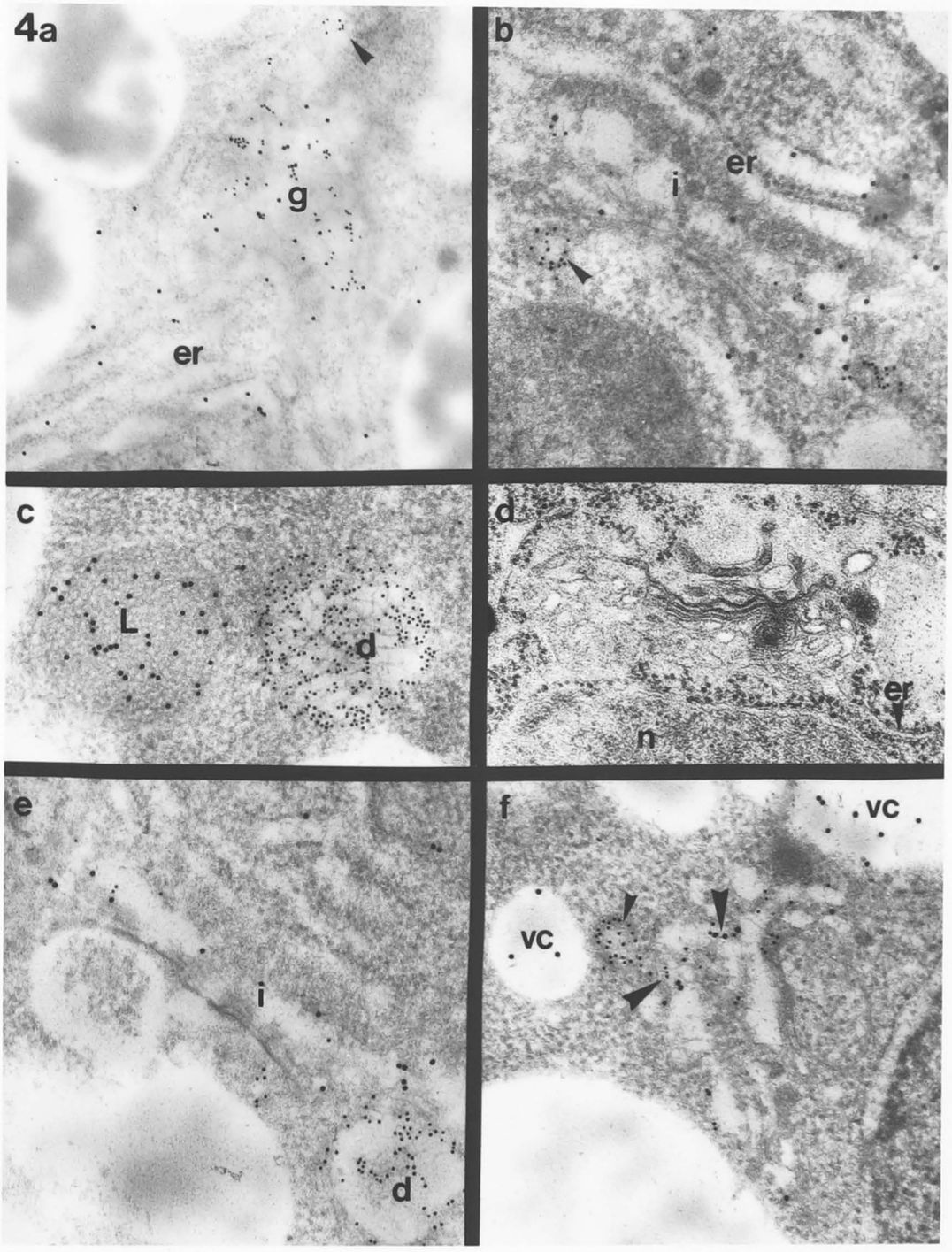
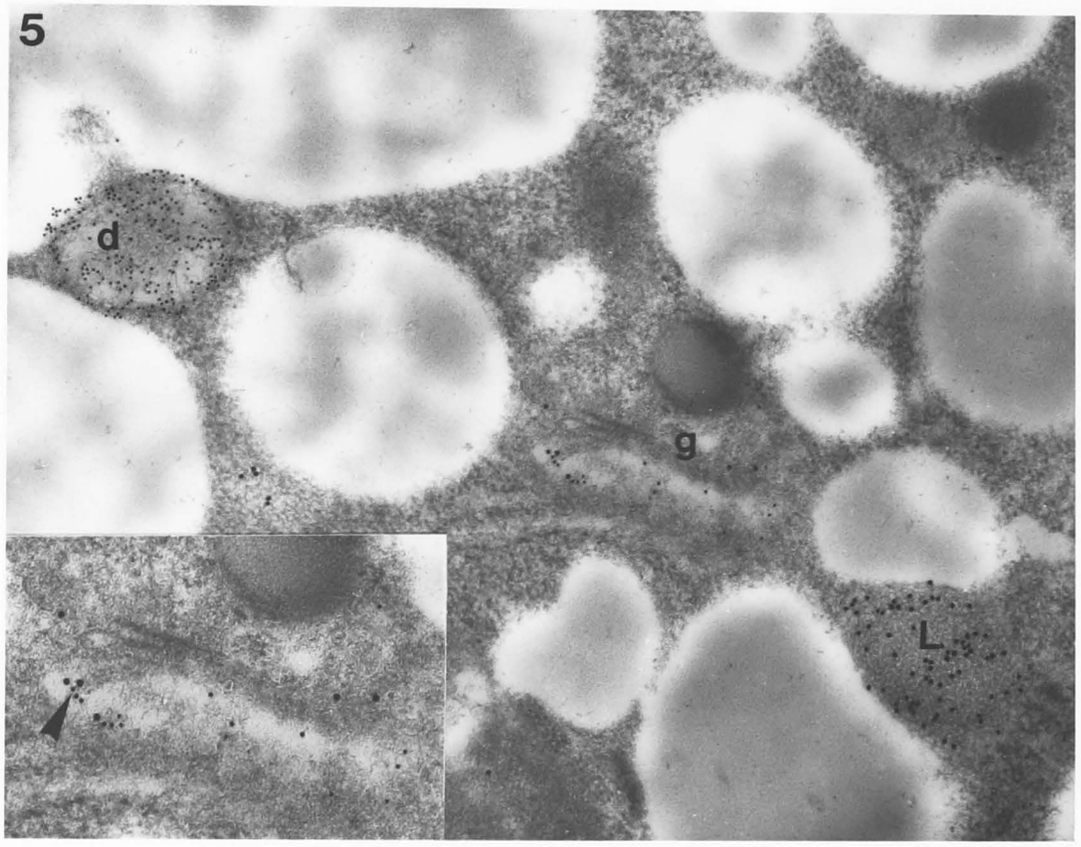

4a
g
er
b
er
i
c
L
d
d
er
n
e
i
d
f
vc
vc

Fig. 3.5. Double labelling with antibodies to large peripheral (L) and dorsal vesicles (d). There was no crossreactivity between mature vesicles in the cytoplasm. The large peripheral and dorsal vesicle antigens appeared to be present in the same Golgi cisternae (g, see inset, arrow). X 36,900, inset X 54,400.


5
d
g
L

CHAPTER FOUR: THE EFFECTS OF SECRETION INHIBITORS ON PERIPHERAL VESICLE FORMATION.

4.1 INTRODUCTION

Trends in Cell Biology, 1991 referred to brefeldin-A (BFA) as the vesicle researcher's wonder drug. Indeed, the rapid progress made with this drug in elucidating cellular pathways of protein traffic has been remarkable. BFA was initially isolated and characterised as an antiviral antibiotic (Tamura *et al.* 1968) but its potential use in cell biology was not made known until the mid 1985 when it was shown that BFA caused an intracellular accumulation of normally secreted G-protein in vesicular stomatitus virus-infected hamster kidney cells (Takatsuki & Tamura 1985). The block caused by BFA was suggested to be at the level of the E.R., as during incubation with the drug the E.R. became dilated and accumulated amorphous material (Misumi *et al.* 1986). Treatment with BFA also resulted in the breakdown of the Golgi apparatus (Fujiwara *et al.* 1988), while newly synthesised membrane proteins, retained in the E.R. in the presence of BFA, showed evidence of having been processed with Golgi specific enzymes (Lippincott-Schwartz *et al.* 1989). Further research showed that within minutes of adding BFA to cells, Golgi membranes were transported to the E.R. via long tubular processes and in the longer term, BFA did not affect the exit of proteins from the E.R. but enhanced the retrograde passage of membrane back to the E.R. from an intermediate E.R.-Golgi compartment (Lippincott-Schwartz *et al.* 1990). BFA has been shown to cause the dissociation of a 110kDa protein termed β-COP, from Golgi membranes prior to their redistribution into the E.R. (Donaldson *et al.* 1990) and, as this protein is crucial to the formation of Golgi cisternal vesicles,

there is a dysregulation of membrane traffic within the secretory pathway (Klausner *et al.* 1992). While BFA has been used almost exclusively on animal systems, a recent study documented the results of the first experiments with BFA on plant cells (Satiat-Jeunemaitre & Hawes 1992). Using BFA at 200µg/ml, 20 times higher than had been previously used in studies on animal systems, the authors showed that the drug reversibly caused the breakdown of the Golgi apparatus. In this chapter I report the effectiveness of the drug in perturbing the secretory system for the first time in a fungal system and, using morphological and immunological evidence, determine the mode of action of the drug in such a system.

The cytochalasins are some of the most potent inhibitors of the actin cytoskeleton known (Yahara *et al.* 1982). Cytochalasins are thought to inhibit actin filament elongation by binding to high affinity sites located at the polymerisation end of filaments (Flanagan & Lin 1980). In this chapter I report the effects of one of the members of this group, cytochalasin D (CD) on peripheral vesicle formation. Although CD has been shown to disrupt the actin microfilament based vesicle transport system in animal cells (Forscher & Smith 1988), fungi (Allen *et al.* 1980), algae (Kropf *et al.* 1989), bryophytes (Doonan *et al.* 1988), pteridophytes (Murata *et al.* 1987) and higher plants (Picton & Steer 1981), there is also evidence that CD may affect the rate of release of secretory vesicles from the Golgi apparatus (Shannon *et al.* 1984). The mechanism behind such an effect is not clear, but it has been suggested that CD may either inhibit the movement of vesicles between the E.R. and the Golgi apparatus or the formation of vesicles from cisternal margins.

The ionophore monensin has been used to block secretion in both plant and animal cells (see Mollenhauer *et al.* 1990 for review). By exchanging H^+ for Na^+ and

K^+, monensin induces swelling of *trans* Golgi compartments (Tartakoff 1983) and interferes with functions, such as the processing of secretory products (Orci *et al.* 1984). Though reports of experiments involving fungal cells and monensin are rare, the drug has been shown to inhibit growth, lipid and sterol biosynthesis in *Hypomyces, Neurospora, Achlya* and *Taphrina* (Weete *et al.* 1989) and to have a pronounced effect on cytoplasmic cleavage during gametogenesis and zoosporogenesis in *Allomyces* (Sewall *et al.* 1986). In this chapter I report that monensin affects peripheral vesicle formation, but, unexpectedly the site of the block in the secretory pathway caused by the drug appears not be the *trans* Golgi apparatus.

Tunicamycin has been widely used by researchers to study the behavior and function of glycoproteins in plant and animal cells. The drug blocks the glycosylation of asparagine-linked glycoproteins by inhibiting the first step in the lipid-linked saccharide pathway - the formation of *N*-acetylglucosaminyl pyrophosphorylpolyisoprenol (Struck & Lennarz 1977). Some glycoproteins have been shown to require *N*-linked glycans for transport competence in the secretory pathway (Olden *et al.* 1982). Such secretory proteins without these moieties are either retained in the E.R. (Faye & Chrispeels 1987, Rose & Doms 1988) or are more susceptible to proteolysis and are broken down (Faye & Chrispeels 1989). In this chapter I will report firstly whether tunicamycin affects peripheral vesicle formation and secondly, since the Lpv-1 antibody labels the E.R., I report what happens to the large peripheral vesicle antigen when it lacks an *N*-linked carbohydrate moiety.

In this chapter, I report the effects of BFA, CD, monensin and tunicamycin on peripheral vesicle formation. This work has enabled an assessment of the mode of action of the drugs and, has been in some cases, the first test with them on a fungal

system. Also, as these drugs affect different parts of the secretory pathway, it has been possible to reveal more information about biochemical aspects of the peripheral vesicles. The formation of peripheral vesicles during sporulation in *P. cinnamomi* is a good system to work with, as these organelles are not present in vegetatively growing cells but can be reproducibly induced to appear during the induction of sporangia. This work is greatly aided by the Lpv-1 antibody which has been shown to be an effective marker for most of the secretory pathway (see Chapter 3).

4.2 MATERIALS AND METHODS

4.2.1 Inhibitors

In experiments in which fungal hyphae were exposed to drugs for 6h, BFA (Boehringer Mannheim, Castle Hill, NSW), was made up as a stock solution of 1mg/ml in 95% ethanol, and was added to the culture medium to a final concentration of 5µg/ml. In experiments in which fungal hyphae were exposed to drugs for 1h, BFA (Sigma, St Louis, MO, USA) was made up as a stock solution of 10mg/ml in 95% ethanol with a final concentration in the culture medium of 50µg/ml. CD (Sigma) was made up as a stock solution of 5mM in dimethyl sulphoxide (DMSO) with a final concentration in the culture medium of 25µM. In the 6h drug exposure experiments, monensin (Sigma) was made up as a stock solution of 1mM in 95% ethanol with a final concentration in the culture medium of 5µM. In the 1h drug exposure experiments, monensin was made up as a stock solution of 10mM in 95% ethanol, and used at a final concentration of 10µM.

Tunicamycin (Sigma) was made up as a stock solution of 1mg/ml in 0.05% NaOH with a final concentration in the culture medium of 5μg/ml.

4.2.2 Culturing and experimental procedure

Hyphal plugs were taken from the edge of a 5 day old *P. cinnamomi* 6BR culture growing on V8 agar (Chapter 2) and placed in 4ml of 5% V8 broth (Chapter 2) in a 4cm diameter petri dish. After 24h growth in the dark at 25°C, the cultures were rinsed three times in mineral salts solution (Chapter 2) then left to gently shake at 50rpm in the light at 22°C in a final volume of 4ml mineral salts. After 4h in the mineral salts solution, BFA, CD, monensin, and tunicamycin were added and the cultures were gently shaken (50rpm) in the light at 22°C for either 6h or 1h.. The appropriate amounts of solvents were added to the controls in the 6h drug exposure experiments. In the case of BFA and monensin, ethanol to a final concentration of 0.475%, in the case of CD, DMSO to a final concentration of 0.25% and with tunicamycin, 0.25mM NaOH. The cultures were incubated in these solutions for 6h, then fixed for 30min in 4% paraformaldehyde in 50mM Pipes buffer (pH 7.0). Before fixation, small tufts of mycelium were taken from the plates and incubated for 5 days in 5% V8 broth in the dark at 25°C, to ensure that the hyphae were alive. For the experiments involving 1h incubation times, the controls were 0.475% ethanol for BFA, and 0.095% ethanol for monensin. In these experiments, cultures incubated in the drugs for 1h were rinsed three times in mineral salts and then allowed to resume growth in the absence of the drugs in a final volume of 4ml of mineral salts solution for 90min. The samples were cryosectioned and immunostained as below. All experiments were carried out three times to ensure the reproducibility of the responses.

4.2.3 Processing for immunofluorescence and electron microscopy

After rinsing in 50mM Pipes buffer (ph 7.0), the samples were processed for cryosectioning as in Chapter 2, and then immunostained with 1.5 µg/ml Vsv-1, 2µg/ml Cpa-2, and 2µg/ml Lpv-1 diluted in PBS/1% BSA. After incubation in Sheep anti-mouse FITC, diluted 1 in 60 in PBS/1% BSA, the sections were incubated in 0.1 µg/ml DAPI (4'-6'-diaminophenylindole) to assist in locating perinuclear E.R. and Golgi apparatus. The sections were then mounted in mowiol plus 0.1% PPD and examined with a Zeiss epifluorescence microscope. The same filters were used for fluoroscein conjugated antibodies as in Chapter 2, while to visualise DAPI staining an F1 filter cube with a 365nm excitation filter, a 395nm dichroic mirror and a 420nm barrier filter was used.

For electron microscopy both 6h and 1h samples were fixed at room temperature for 1h in 1% glutaraldehyde in 100mM Pipes buffer (pH 7.0), washed in buffer then postfixed in 2% osmium tetroxide in distilled water. After dehydration in an ethanol series of 30min steps (10%, 30%, 50%, 70%, 100%) the samples were infiltrated in an increasing series of LR White resin (London Resin Co., Hampshire, U.K.): acetone solutions of 2h steps (1:2, 1:1, 2:1 LR White: acetone), followed by 2 by 24h changes in pure LR White resin. The samples were embedded in LR White resin at 60°C.

4.3 RESULTS

All micrographs presented in the results section were representative of the responses observed.

4.3.1 6h drug incubation experiments-immunofluoresence results

Although it is not possible to ascertain from fluorescence microscopy whether either vesicle formation or antigenic epitopes were affected by the drugs, in experiments where hyphae were exposed to the the drugs for 6h, all four inhibitors reduced the number of *fluorescent* peripheral vesicles in hyphae (Figs 4.1-4.4). In BFA and monensin-treated hyphae, a small number of fluorescent large peripheral and ventral vesicles were present (Figs 4.1a, 4.1e, 4.2a, 4.2e). Fluorescent dorsal vesicles, however, were completely absent (Figs 4.1c, 4.2c). Larger numbers of fluorescent large peripheral and ventral vesicles were present in CD treated hyphae (Figs 4.3a, 4.3e) and in this case a small number of fluorescent dorsal vesicles was present (Fig. 4.3c). Tunicamycin almost completely inhibited the appearance of fluorescent peripheral vesicles (Figs 4.4a, 4.4c, 4.4e), though there was a very small number of fluorescent ventral vesicles present (Fig. 4.4a) and a number of regions which were positive with the Lpv-1 antibody but did not have the characteristic staining of large peripheral vesicles (Figs 4.4e, 4.5a). Double labelling with DAPI assisted in identifying these regions. In tunicamycin-treated hyphae, the Lpv-1 antigen was restricted to a narrow juxtanuclear zone (Figs 4.5a, 4.5b) while in controls the pattern of staining suggested that the antigen was confined to a much larger, wider structure though this was also near the nucleus (Figs 4.5c, 4.5d).

Figure 4.6 indicates that hyphae, previously incubated in BFA, monensin and tunicamycin were alive as they grew into colonies by 5 days. Hyphae previously incubated in CD were not used in these experiments, as after 6h incubation these hyphae had produced large numbers of peripheral vesicles-evidence that the drug had not caused hyphal death.

4.3.2 6h drug incubation experiments-results from electron microscopy

Results from electron microscopy revealed that although BFA caused distortion of the Golgi apparatus, a few vesicles had still been produced (Fig. 4.7a), though fewer than in controls (Fig. 4.7b). In CD-treated hyphae, large clusters of vesicles, that had the morphology of large peripheral vesicles could be observed adjacent to the Golgi apparatus (Fig. 4.7c). In controls these clusters were not present (Fig. 4.7d). Monensin appeared to cause swelling of the Golgi cisternae and to slow vesicle release as clusters of vesicles were closely associated with the *trans* Golgi and fewer vesicles were in the surrounding cytoplasm (Fig. 4.8a) compared to the controls (Fig. 4.8b). The Golgi apparatus in tunicamycin-treated hyphae was quiescent with no vesicles being produced and the nuclear envelope/E.R. appeared more swollen (Fig. 4.8c) compared to the control (Fig. 4.8d).

4.3.3 1h drug incubation experiments-immunofluorescence results

In the second set of experiments, the effects of BFA and monensin on peripheral vesicle formation were investigated using shorter incubation time (1h) and higher concentrations of the two drugs. The motivation for this was twofold. Firstly, in the experiments where hyphae were exposed to the drugs for 6h, each of the inhibitors had been used at the lowest possible concentration that had affected peripheral vesicle formation. In the case of BFA and monensin these had not completely inhibited vesicle formation. The second reason was that there is some evidence that both BFA and monensin lose their potency during long incubation times (Fujiwara *et al.* 1988, Mollenhauer *et al.* 1983). Only large peripheral and ventral vesicle formation was studied as these former vesicles appear together synchronously between 4 and 6h, while dorsal vesicles appear between 6 and 8h (Chapter 2) and this was temporally impossible. In these experiments, BFA at

50μg/ml and monensin at 10μM completely inhibited the appearance of fluorescent ventral (Figs 4.9c, 4.9d) and large peripheral vesicles (Figs 4.10c, 4.10d). In untreated controls there were large numbers of the two vesicle types (Figs 4.9a, 4.9b, 4.10a, 4.10b). The effects of both drugs were reversible and after washing and 90min incubation in fresh mineral salts medium the vesicles reappeared (Figs 4.9e, 4.9f, 4.10e, 4.10f). In BFA and monensin-treated hyphae the pattern of staining with Lpv-1 and Vsv-1 was very similar and was fine and fibrillar (Figs 4.9c, 4.9d, 4.10c, 4.10d). Double labelling with DAPI and Lpv-1 indicated that the Lpv-1 antigen was restricted to a narrow perinuclear region in both BFA and monensin-treated hyphae (Figs 4.11c, 4.11d, 4.12c, 4.12d). In the controls and reincubated hyphae, Lpv-1 staining was not restricted to this narrow perinuclear zone but the pattern of staining suggested that the antigen was found in larger, wider regions, though these were still associated with the nucleus (Figs 4.11a, 4.11b, 4.11e, 4.11f, 4.12 a, 4.12b, 4.12e, 4.12f).

4.3.4 1h drug incubation experiments-results from electron microscopy

Further details of the effects of these two drugs on vesicle formation during 1h experiments were obtained from ultrastructural studies. Controls and reincubation samples from both drug treatments showed fairly normal Golgi apparatus with vesicles similar to ventral vesicles budding away from *trans* regions (Figs 4.13a, 4.13b, 4.14a, 4.14b). In BFA-treated hyphae the Golgi apparatus, when present, had cisternae that were very close together (Figs 4.13c, 4.13d) and sometimes, as the vesicles were not associated with the edges of Golgi cisternae, these were possibly breaking down into small vesicles (Fig. 4.13c). In some hyphae, the Golgi apparatus was completely absent and vacuolate areas, adjacent to the nucleus (where the Golgi apparatus was normally positioned), were present instead. In monensin-treated hyphae large vacuoles appeared to be forming from

the *trans* part of the Golgi apparatus (Fig. 4.14c) and it appeared that the Golgi apparatus completely broke down around these large vacuoles (Fig. 4.14d). The nuclear envelope/E.R. appeared quite swollen in monensin treated hyphae (Fig. 4.14e).

4.4 DISCUSSION

When hyphae were incubated in BFA for 6h, peripheral vesicle formation appeared to be slowed. The distorted appearance of the Golgi apparatus in hyphae may suggest the Golgi apparatus was in the process of breaking down, perhaps because of membrane imbalance caused by the reduction of transport between the E.R. and the Golgi apparatus (Klausner *et al.* 1992, Chrispeels & Staehelin 1992). A 6h incubation period in monensin may have affected the release of peripheral vesicles away from the *trans* Golgi apparatus by impairing the final stages of maturation of the peripheral vesicles (Mollenhauer *et al.* 1990). An alternative hypothesis is that the hyphae incubated in either BFA or monensin may be in a state of recovery and it is possible that a 6h incubation time is not the ideal time period in which to assess the effects of these two inhibitors. Fujiwara *et al.* (1988) showed that when rat hepatocytes were incubated in 2.5µg/ml BFA, after 1h the Golgi apparatus broke down and secretory protein became restricted to the E.R. After 4h in the presence of the drug, however, the Golgi apparatus reconstituted from its previously dispersed form and once again contained secretory protein. This effect was attributed to the metabolization of BFA to an inert form after long incubation times. The literature on long incubation times (greater than 5h) with monensin is quite confusing. After 24h in 10µM monensin the Golgi apparatus in *Euglena gracilis* has been shown to return to normal (Mollenhauer *et al.* 1983).

Eighteen hours in 10μM monensin has, however, been shown to cause severe endomembrane disruption in maize rootcap cells (Mollenhauer *et al.* 1982), and in human fibroblasts, 7h incubation with 0.5 μM monensin causes the Golgi apparatus to swell into large refractile vacuoles and the E.R. to distend (Ledger *et al.* 1980).

The results with CD indicate that the formation of peripheral vesicles does not require actin microfilaments. Microtubules may possibly play some role in the movement of materials between the E.R. and the Golgi apparatus and the budding of peripheral vesicles from Golgi cisternal margins. Microtubules have been shown, however, not to be involved in E.R. to Golgi apparatus transport (Rogalski *et al.* 1984, Salas *et al.* 1986, Lippincott-Schwartz *et al.* 1990) and thus it appears some other yet to be described transport mechanism is in operation at this site. Some evidence exists to support the involvement of microtubules in the budding of Golgi vesicles. Microtubules, have been shown to interact with Golgi membranes *in vitro* (Karecla & Kreis 1992), and may also be involved in maintaining the organisation of the *trans* Golgi network, the exit site of casein vesicles from the Golgi apparatus (Rennison *et al.* 1992). Kreis (1990) discussed the possibility that microtubule receptors may be associated with budding secretory vesicles, a situation that may occur in the secondary cysts of *Saprolegnia ferax*, where precursor K2-bodies budding away from smooth fenestrated cisternae appear, from electron micrographs, to be transported away to the cyst periphery by microtubules (Lehnen & Powell 1991). The large clusters of vesicles adjacent to the Golgi apparatus in *P. cinnamomi* suggest, however, that the actin cytoskeleton is necessary for the transport of peripheral vesicles *away* from the Golgi apparatus. The role that the actin cytoskeleton plays in the movement of peripheral vesicles into the developing sporangium will be reported in Chapter 5.

Tunicamycin almost completely blocks peripheral vesicle formation and suggests that, like many plasma membrane and secretory proteins (Rose & Doms 1988), all three peripheral vesicles contain *N*-linked glycoproteins. The swelling of the E.R. caused by tunicamycin and the presence of the Lpv-1 antigen in nucleus-associated structures which resemble the E.R. suggest that the loss of *N*-linked glycans may prevent the entrance of the Lpv-1 antigen into the secretory system. *N*-linked glycans are important to secretory proteins as they ensure correct folding (Gibson *et al.* 1979). Misfolded proteins have been shown to form aggregates in the E.R. and may not be able to enter transport vesicles (Machamer & Rose 1988, Doms *et al.* 1988) or they may become more susceptible to proteolysis and be broken down within the secretory pathway (Faye & Chrispeels 1989). The results suggest that as the large peripheral vesicle antigen is still present within the E.R., the loss of *N*-linked glycans has not exposed it to protein degrading enzymes and that misfolding may have prevented its entrance into the secretory pathway.

Higher concentrations and shorter incubation times with BFA and monensin reveal details about the mode of action of these drugs. While much higher concentrations of BFA than those used on animal cells are necessary to inhibit peripheral vesicle formation, these concentrations were four times lower than the concentration that was shown to be effective on plant cells (Satiat-Jeunemaitre & Hawes 1992). BFA at 50 µg/ml appeared to cause the Golgi apparatus to break down, and suggests that antigens become restricted to fibrillar, perinuclear structures, most probably the E.R. Monensin caused vacuolation of *trans*-regions of the Golgi apparatus, and in some hyphae caused the entire Golgi apparatus to break down into these vacuoles. Monensin causes various morphological changes in the Golgi apparatus, such as swelling of *trans* cisternae (Mollenhauer *et al.* 1988, Moore *et al.* 1991) and curling structures or cup-shapes (Robinson 1981,

Mollenhauer *et al.* 1982) but there are a few accounts reporting the complete break down of the Golgi apparatus into large vacuoles (Ledger *et al.* 1980, Griffiths *et al.* 1983, Morre *et al.* 1983, Ellinger & Pavelka 1984). During treatment with monensin, large peripheral and ventral vesicle antigens are restricted to fibrillar, perinuclear structures, most likely the E.R., and not to the few intact Golgi apparatuses which are still present in hyphae. Although the principal site of arrest of transport in cells treated with monensin appears to be the Golgi apparatus (Tartakoff 1983), it is suggested that the drug may cause a block in transport of vesicle antigens from the E.R. to the Golgi apparatus. Ledger *et al.* (1980) suggested a similar effect of the drug in human skin fibroblasts. In these cells, normal Golgi apparatuses were not seen, while aberrant forms with large vacuoles attached were quite common. Using immunocytochemical staining, these authors revealed antigens to be present in monensin-induced vacuoles and in dilated E.R., suggesting that either the synthesized proteins exceeded the storage capacity of the Golgi-vacuoles or that monensin blocked exit from the E.R. The results from this study support the latter of these two possibilities, and therefore suggest that monensin should not necessarily be accepted as solely an inhibitor of vesicle formation at the *trans* Golgi. Indeed, a recent review on the cellular effects of monensin (Mollenhauer *et al.* 1990) documented a diversity of phenomena, including the blockage of proteins from coated pits to receptosomes, recycling of lipoprotein receptors and the inhibition of pinocytosis. It is interesting too, that a recent paper by Morre *et al.* (1992) has reported that the much documented phenomenon of monensin-induced Golgi swelling requires fixation, suggesting that the swelling of Golgi cisternae may not be the cause of the interruption of membrane flux and processing caused by the drug.

The second series of experiments with BFA and monensin reveals further information about the ventral vesicle antigen. The results reported in Chapter 3 suggested that the epitope to which the Vsv-1 antibody bound was not present until the vesicles had budded away from the Golgi apparatus. Treatment with both monensin and BFA indicates the ventral vesicle antigen may become restricted to the E.R., which should not be possible if the antigen was normally only present in budded vesicles. The lack of labelling of the Golgi apparatus with the Vsv-1 antibody may be because the antigen is in too low a concentration for detection.

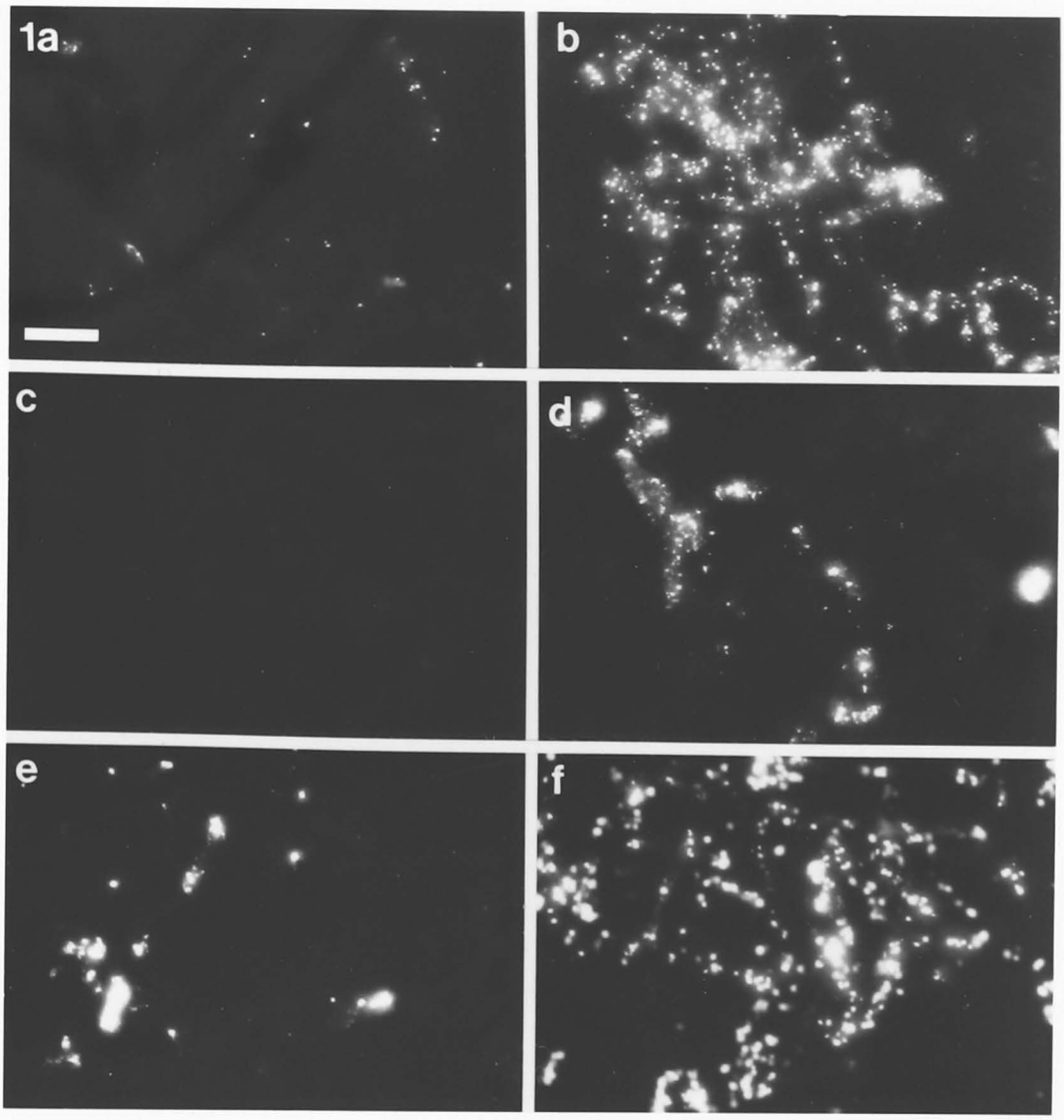
Peripheral vesicle formation is thus affected by all four inhibitors used. This study has indicated the usefulness of this system as a model in the study of secretory glycoprotein transport and in studies of the action of these drugs on the secretory system. A further study involving immunoelectron microscopy is, however, necessary to characterize fully the actions of these drugs on the *P. cinnamomi* secretory pathway. This experiments documented in this chapter have demonstrated the permeability of the *P. cinnamomi* 6BR cell wall to the four inhibitors used. This is an important point, as many studies utilising plant tissues (e.g. Moore *et al.* 1991) have been complicated by an inability of inhibitors to pass through the cell wall. These results are thus greatly encouraging with respect to the use of more specific chemicals in the control of this pathogen.

Figs 4.1a-4.1b. ***P. cinnamomi*** **hyphae incubated in 5μg/ml BFA for 6h. bar=24μm**

Figs 4.1a-4.1b. Cryosectioned hyphae immunostained with Vsv-1 followed by SAM-FITC. A small number of fluorescent ventral vesicles was present in BFA-treated hyphae (4.1a) whereas large numbers were present in controls (4.1b).

Figs 4.1c-4.1d. Cryosectioned hyphae immunostained with Cpa-2 followed by SAM-FITC. Fluorescent dorsal vesicles were absent in BFA-treated hyphae (4.1c) whereas moderate numbers were present in the controls (4.1d).

Figs 4.1e-4.1f. Cryosectioned hyphae immunostained with Lpv-1 followed by SAM-FITC. A small number of fluorescent large peripheral vesicles were present in BFA-treated hyphae (4.1e) whereas many were present in controls (4.1f).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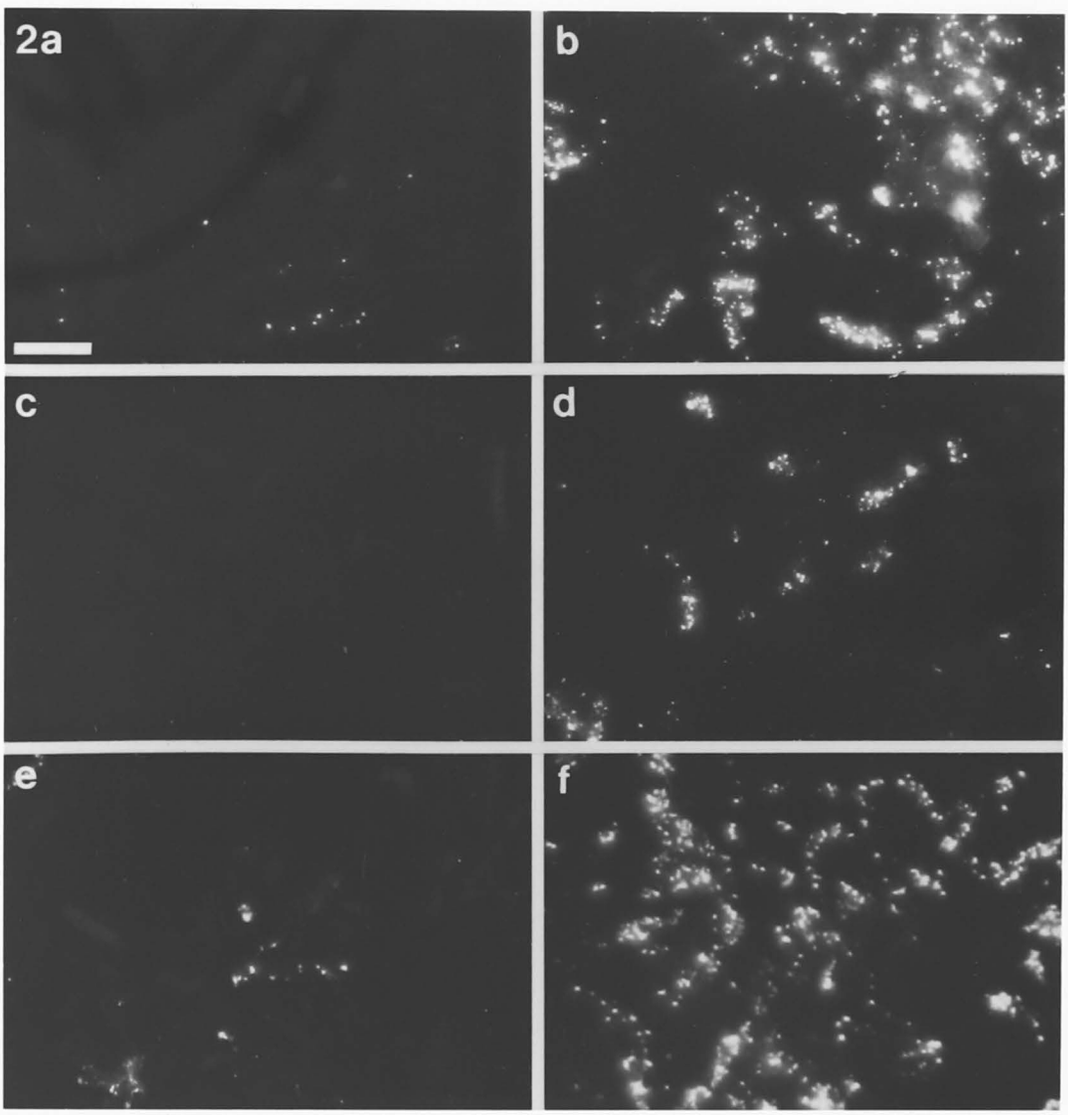
Figs 4.2a-4.2b. ***P. cinnamomi*** **hyphae incubated in 5μM monensin for 6h. bar=24μm**

Figs 4.2a-4.2b. Cryosectioned hyphae immunostained with Vsv-1 followed by SAM-FITC. A small number of fluorescent ventral vesicles were present in monensin-treated hyphae (4.2a) whereas large numbers were present in controls (4.2b).

Figs 4.2c-4.2d. Cryosectioned hyphae immunostained with Cpa-2 followed by SAM-FITC. Fluorescent dorsal vesicles were absent in monensin-treated hyphae (4.2c) whereas moderate numbers were present in the controls (4.2d).

Figs 4.1e-4.1f. Cryosectioned hyphae immunostained with Lpv-1 followed by SAM-FITC. A small number of fluorescent large peripheral vesicles were present in monensin-treated hyphae (4.2e) whereas large numbers were present in controls (4.2f).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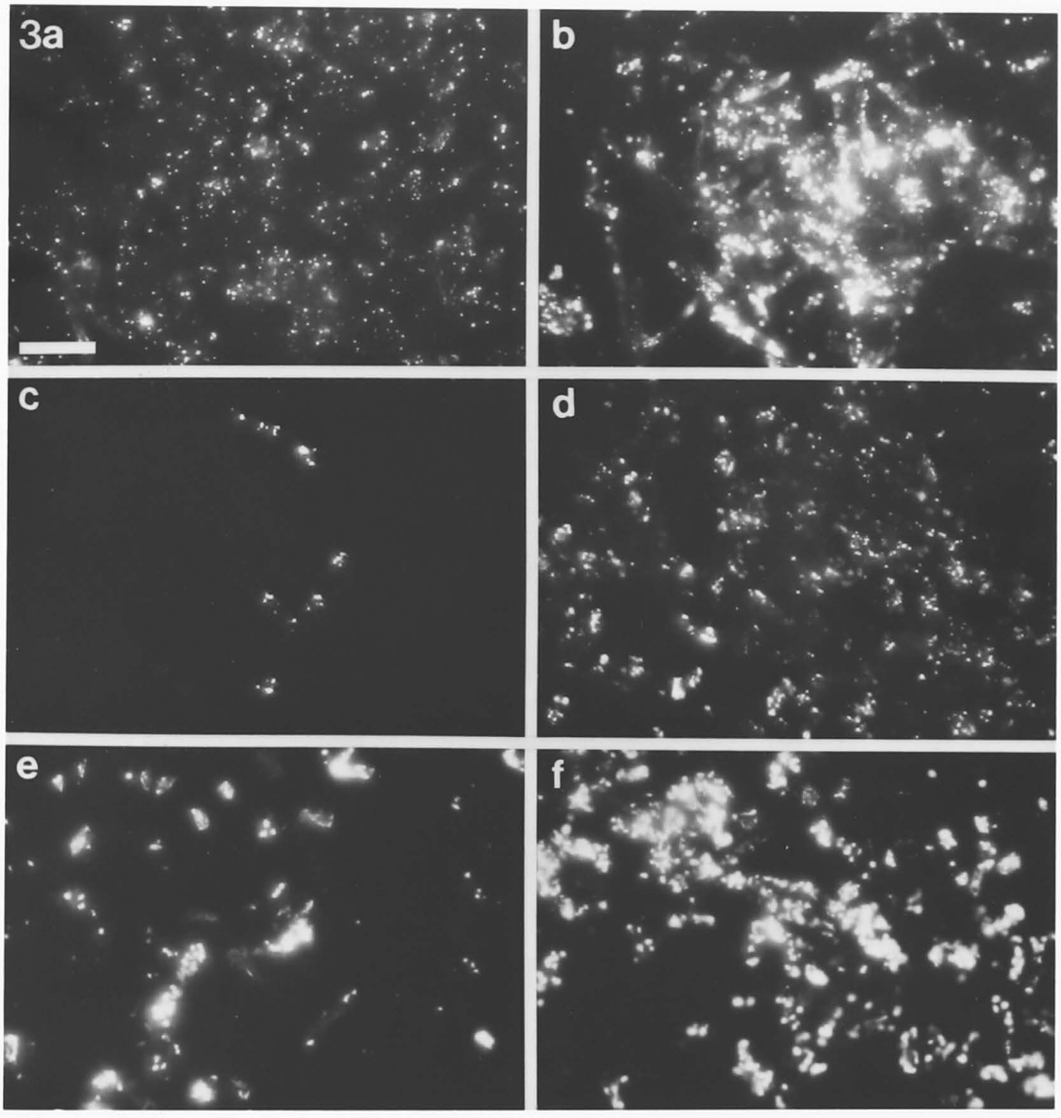
Figs 4.3a-4.3b. *P. cinnamomi* hyphae incubated in 25μM cytochalasin D for 6h. bar=24μm

Figs 4.3a-4.3b. Cryosectioned hyphae immunostained with Vsv-1 followed by SAM-FITC. Moderate numbers of fluorescent ventral vesicles were present in CD-treated hyphae (4.3a). In controls, more fluorescent ventral vesicles were present (4.3b).

Figs 4.3c-4.3d. Cryosectioned hyphae immunostained with Cpa-2 followed by SAM-FITC. A small number of fluorescent dorsal vesicles were present in CD-treated hyphae (4.3c). In controls there were more fluorescent dorsal vesicles present (4.3d).

Figs 4.3e-4.3f. Cryosectioned hyphae immunostained with Lpv-1 followed by SAM-FITC. Moderate numbers of fluorescent large peripheral vesicles were present in CD-treated hyphae (4.3e). In controls more fluorescent large peripheral vesicles were present (4.3f).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Figs 4.4a-4.4b. *P. cinnamomi* hyphae incubated in 5μg/ml tunicamycin for 6h. bar=24μm

Figs 4.4a-4.4b. Cryosectioned hyphae immunostained with Vsv-1 followed by SAM-FITC. A very small number of fluorescent ventral vesicles were present in tunicamycin-treated hyphae (4.4a). Large numbers of fluorescent ventral vesicles were present in controls (4.4b).

Figs 4.4c-4.4d. Cryosectioned hyphae immunostained with Cpa-2 followed by SAM-FITC. Fluorescent dorsal vesicles were absent from tunicamycin-treated hyphae (4.4c). Moderate numbers of fluorescent dorsal vesicles were present in controls (4.4d).

Figs 4.4e-4.4f. Cryosectioned hyphae immunostained with Lpv-1 followed by SAM-FITC. Fluoresent large peripheral vesicles were absent from tunicamycin-treated hyphae (4.4e). A number of regions were positive for the Lpv-1 antigen, but these did not have the characteristic staining pattern of large peripheral vesicles (4.4e, see 4.5a). Large numbers of fluorescent large peripheral vesicles were present in controls (4.4f).

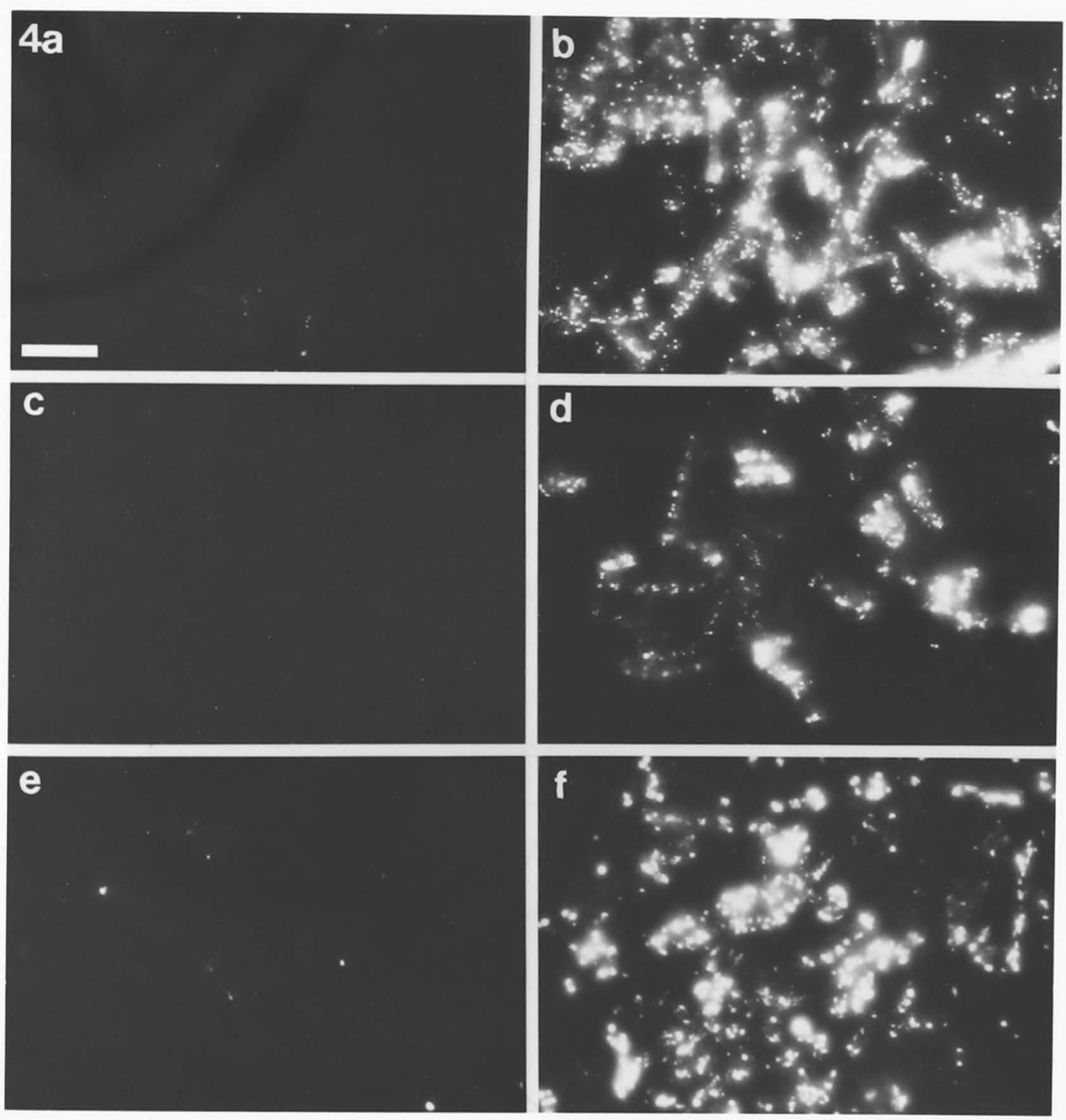
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Fig 4.5a-4.5d. Higher magnification of tunicamycin-treated hyphae double stained with Lpv-1 followed by SAM-FITC (Figs 4.5a, 4.5c) and DAPI (Figs 4.5b, 4.5d). bar=1.9μm.

Figs 4.5a-4.5b. In tunicamycin-treated hyphae the Lpv-1 antigen was restricted to a narrow juxtanuclear region.

Figs 4.5c-4.5d. In controls, the pattern of staining suggested that the Lpv-1 antigen was confined to a much larger, wider structure though this was also near the nucleus.

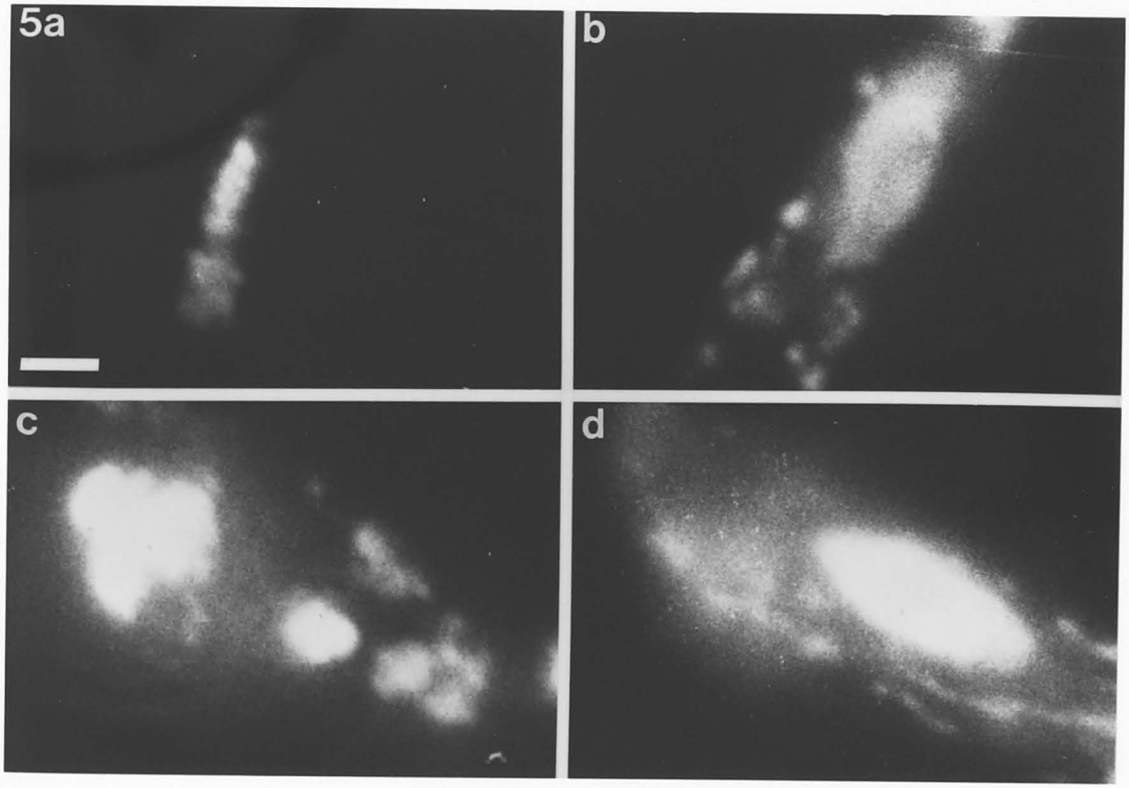
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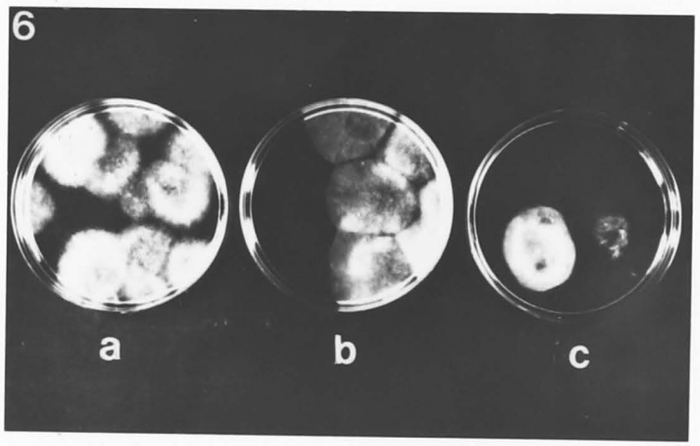
Figure 4.6. Hyphae previously incubated in BFA (a), monensin (b), and tunicamycin (c) for 6h, grew into colonies after reincubation in mineral salts solution in which the drugs were absent.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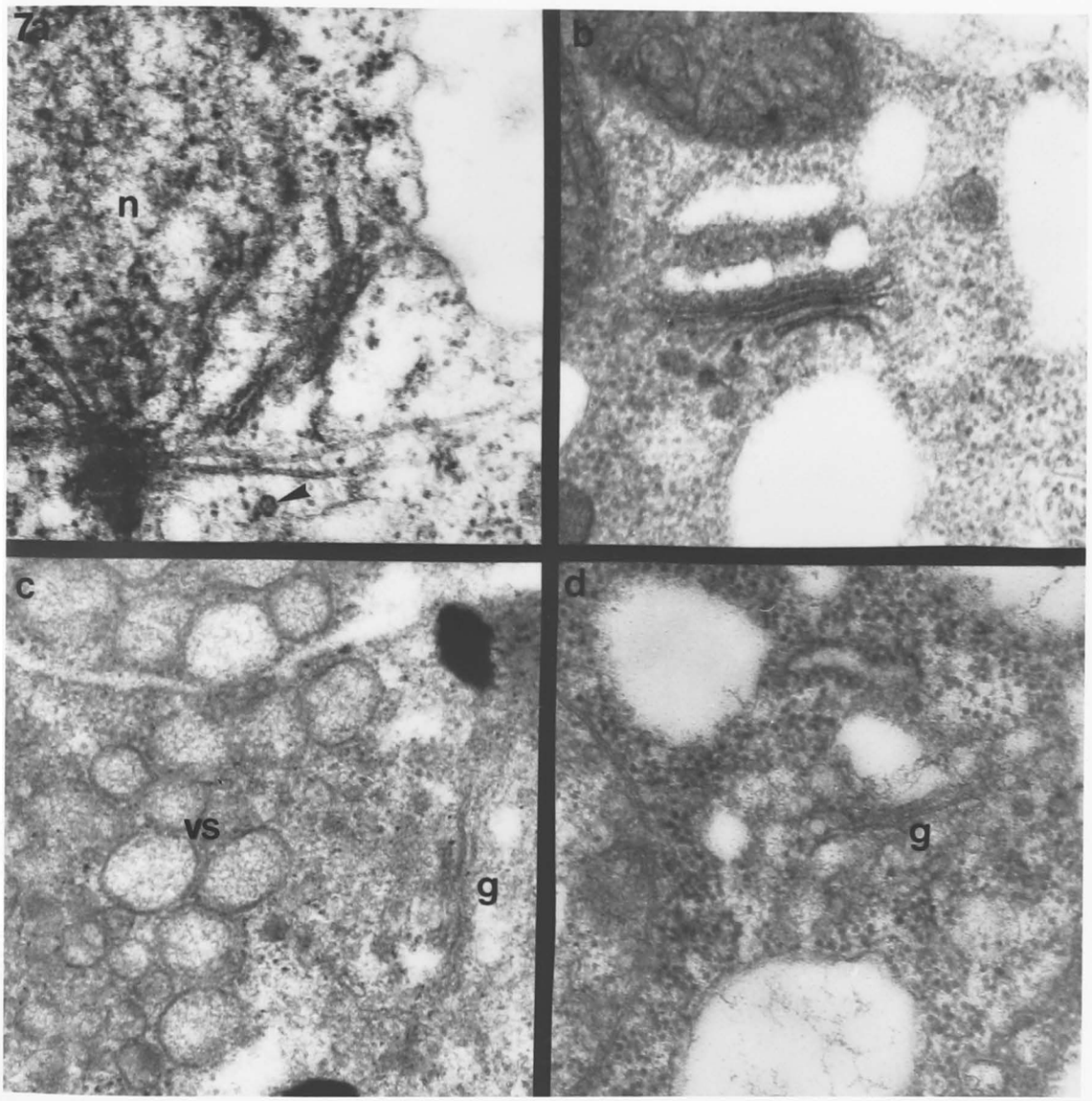
Figs 4.7a-4.7d. Electron microscopy of hyphae incubated in 5μg/ml BFA (4.7a-4.7b) and 25μM CD (4.7c-4.7d) for 6h.

Figs 4.7a-4.7b. Although the Golgi apparatus in BFA-treated hyphae appeared distorted, a few vesicles had still been produced (arrow, 4.7a), though in fewer numbers than the controls (4.7b). n=nucleus. 4.7a X 60,000, 4.7b X 62,500.

Figs 4.7c-4.7d. In CD-treated hyphae (4.7c), large clusters of vesicles (vs) with a similar morphology to large peripheral vesicles, could be observed adjacent to the Golgi apparatus (g). In controls these clusters were not present (4.7d). 4.7c X 65,000, 4.7d X 60,000.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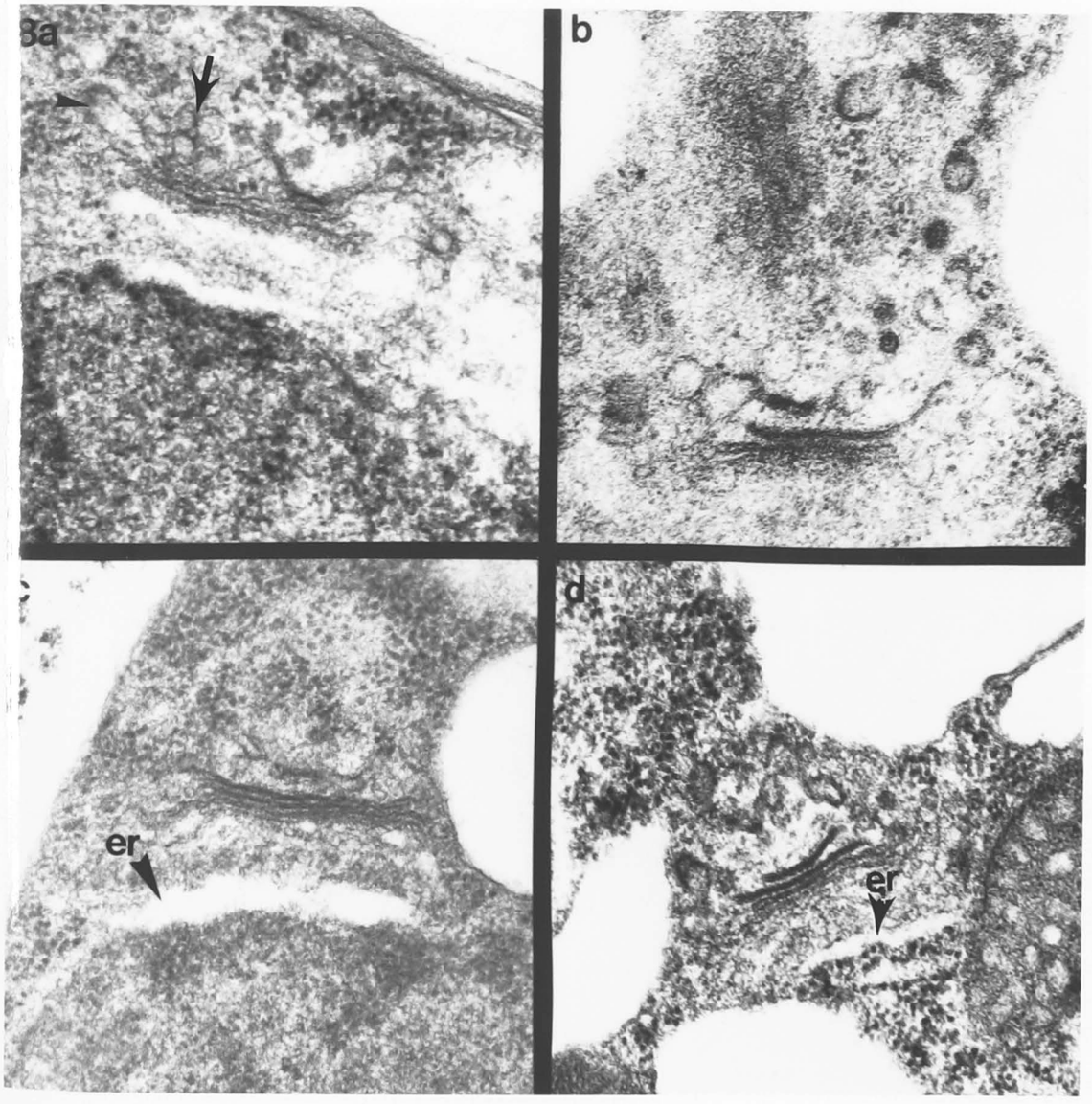
Figs 4.8a-4.8d. Electron microscopy of hyphae incubated in 5μM monensin (4.8a-4.8b) and 5μg/ml tunicamycin (4.8c-4.8d) for 6h.

Figs 4.8a-4.8b. Monensin appeared to cause swelling of *trans* Golgi cisternae (small arrow, 4.8a) and to impede vesicle release as clusters of vesicles were closely associated with the *trans* Golgi apparatus (large arrow, 4.8a) and fewer vesicles were in the surrounding cytoplasm (4.8a) compared to controls (4.8b). 4.8a X 60,000, 4.8b X 60,000.

Figs 4.8c-4.8d. In tunicamycin-treated hyphae the Golgi apparatus was quiescent with no vesicles being produced and the nuclear envelope/E.R. appeared more swollen (4.8c, arrow er) than the controls (4.8d, arrow er). 4.8c X 55,000, 4.8d X 52,000.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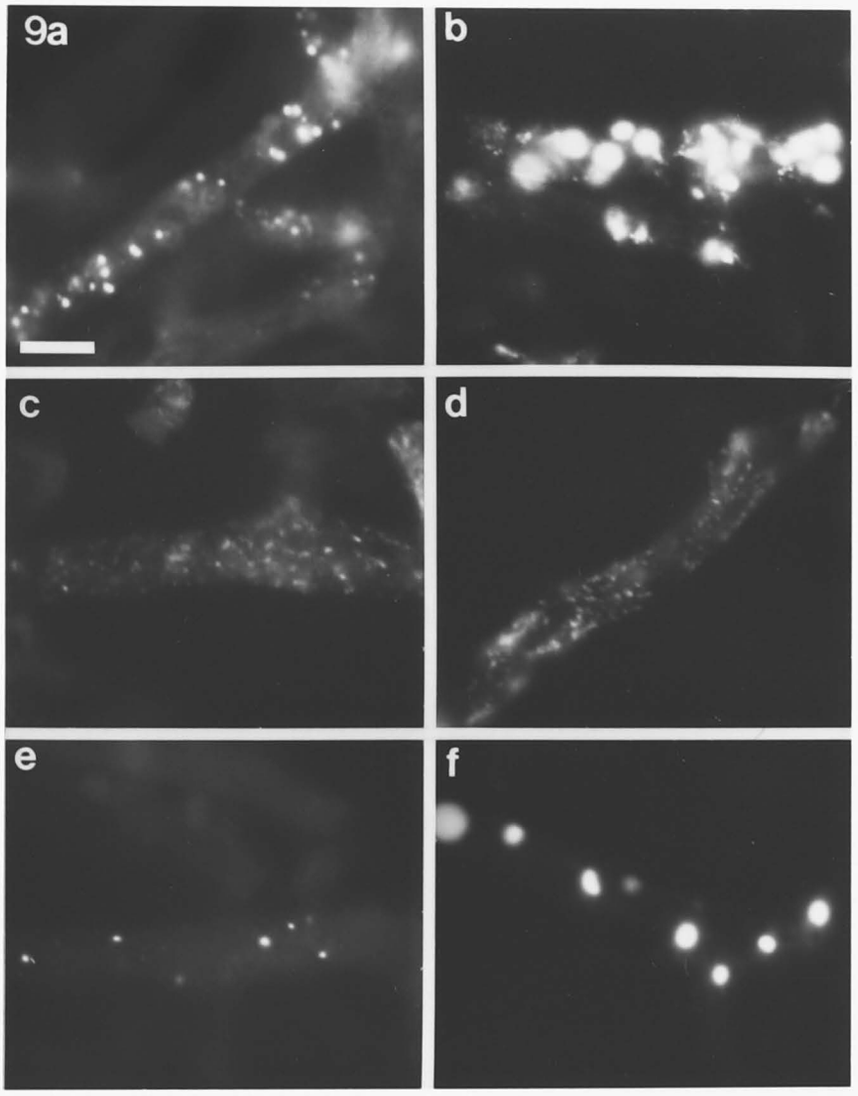
Figs 4.9a-4.9f. Hyphae incubated in 50μg/ml BFA for 1h and reincubated for 90 min in fresh mineral salts solution. Figs 4.9a, 4.9c and 4.9e-immunostained with Vsv-1 followed by SAM-FITC. Figs 4.9b, 4.9d, 4.9f-immunostained with Lpv-1 followed by SAM-FITC. bar=6.45μm.

Figs 4.9a, 4.9c and 4.9e. Whereas controls had large numbers of fluorescent ventral vesicles (4.9a), ventral vesicles were absent from BFA-treated hyphae and the cytoplasm contained fine, fibrillar fluorescent material (4.9c). In hyphae that were reincubated in fresh mineral salts solution, fluorescent ventral vesicles were once again present (4.9e).

Figs 4.9b, 4.9d and 4.9f. Whereas controls had large numbers of fluorescent large peripheral vesicles (4.9b), these vesicles were absent from BFA-treated hyphae and instead the cytoplasm contained fine, fibrillar fluorescent material (4.9d). In hyphae that were reincubated in fresh mineral salts solution, fluorescent large peripheral vesicles were once again present (4.9f).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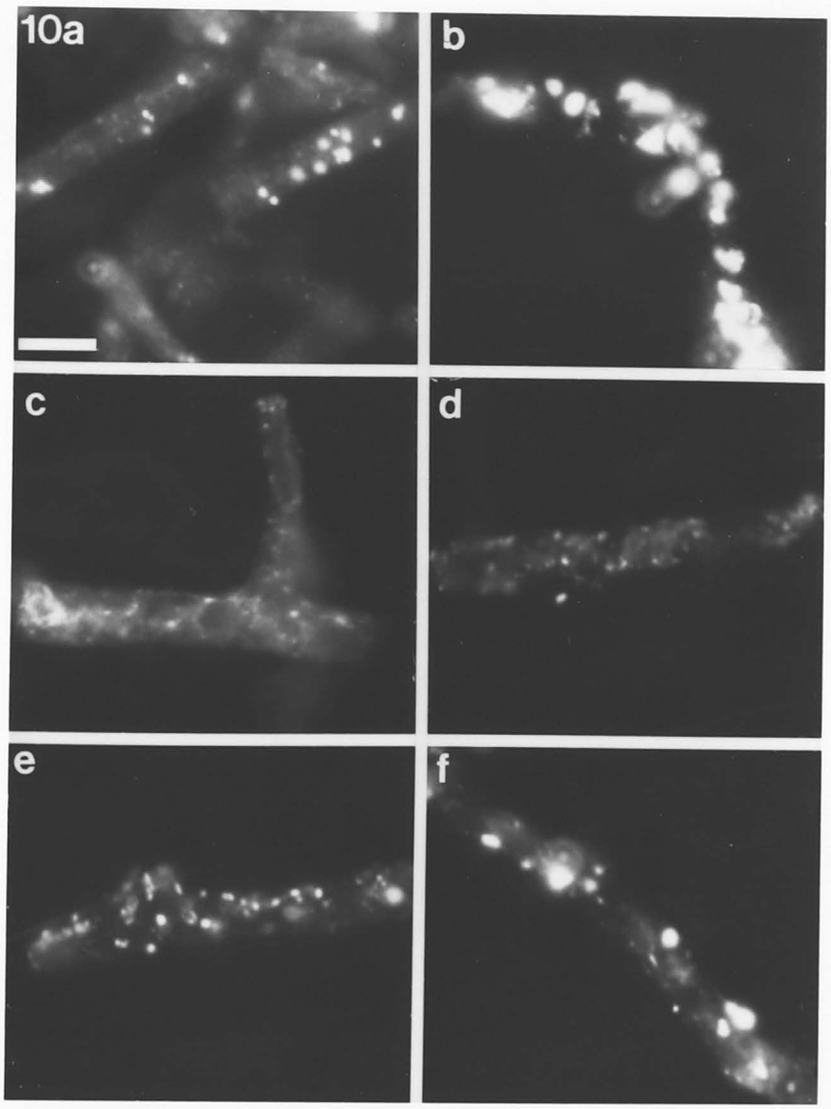
Figs 4.10a-4.10f. Hyphae incubated in 10μM monensin for 1h and reincubated for 90 min in fresh mineral salts solution. Figs 4.10a, 4.10c and 4.10e-immunostained with Vsv-1 followed by SAM-FITC. Figs 4.10b, 4.10d, 4.10f-immunostained with Lpv-1 followed by SAM-FITC. bar=6.45μm.

Figs 4.10a, 4.10c and 4.10e. Whereas controls had large numbers of fluorescent ventral vesicles (4.10a), ventral vesicles were absent from monensin-treated hyphae and the cytoplasm contained fine, fibrillar fluorescent material (4.10c) similar to that seen in BFA-treated hyphae. In hyphae that were reincubated in fresh mineral salts solution, fluorescent ventral vesicles were once again present (4.10e).

Figs 4.10b, 4.10d and 4.10f. Whereas controls had large numbers of fluorescent large peripheral vesicles (4.10b), these vesicles were absent from monensin-treated hyphae and instead the cytoplasm contained fine, fibrillar fluorescent material (4.10d) similar to that seen in BFA-treated hyphae. In hyphae that were reincubated in fresh mineral salts solution, fluorescent large peripheral vesicles were once again present (4.10f).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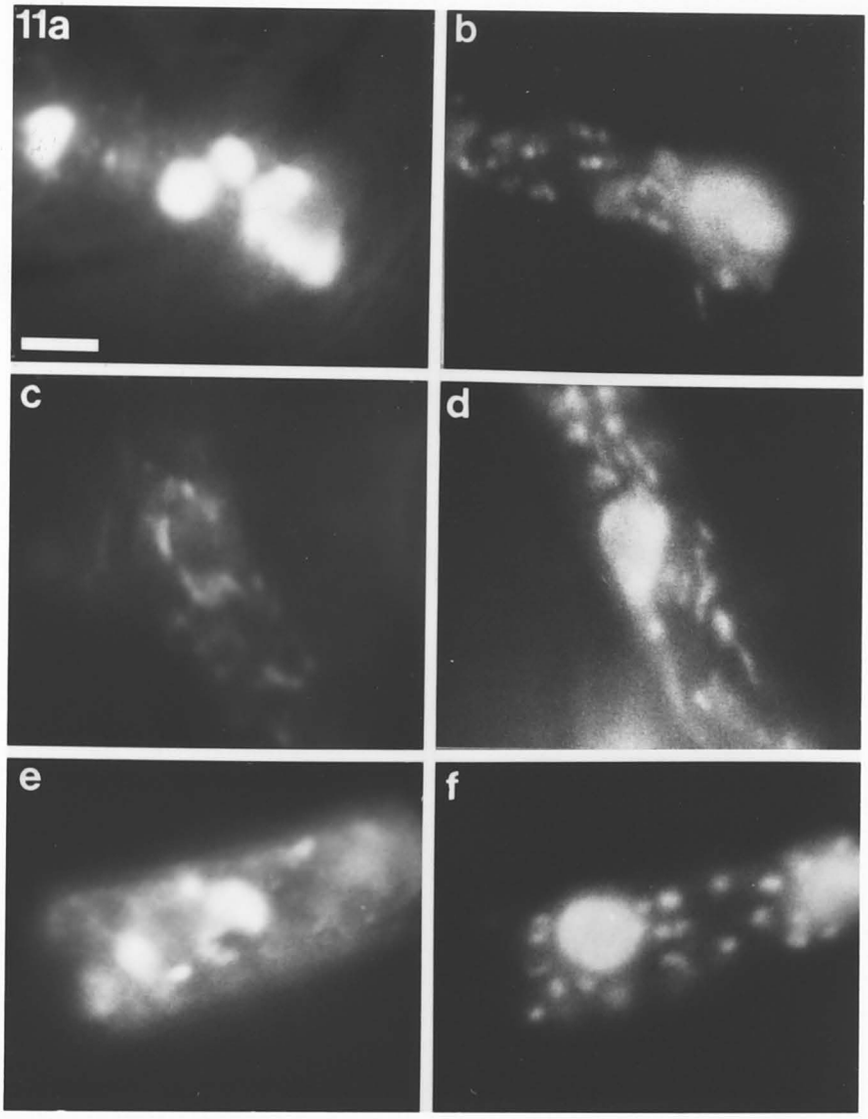
Figs 4.11a-4.11f. Higher magnification of 1h BFA-treated and reincubated hyphae, double stained with Lpv-1 (4.11a, 4.11c, 4.11e) and DAPI (4.11b, 4.11d, 4.11f). bar=2.9μm.

Double staining with Lpv-1 and DAPI indicated that the Lpv-1 antigen was restricted to a narrow perinuclear zone in BFA-treated hyphae (Figs 4.11c, 4.11d). In the controls (Figs 4.11a, 4.11b) and reincubated hyphae (Figs 4.11e, 4.11f), the pattern of staining suggested that the Lpv-1 antigen was found in larger, wider regions though these were still associated with the nucleus.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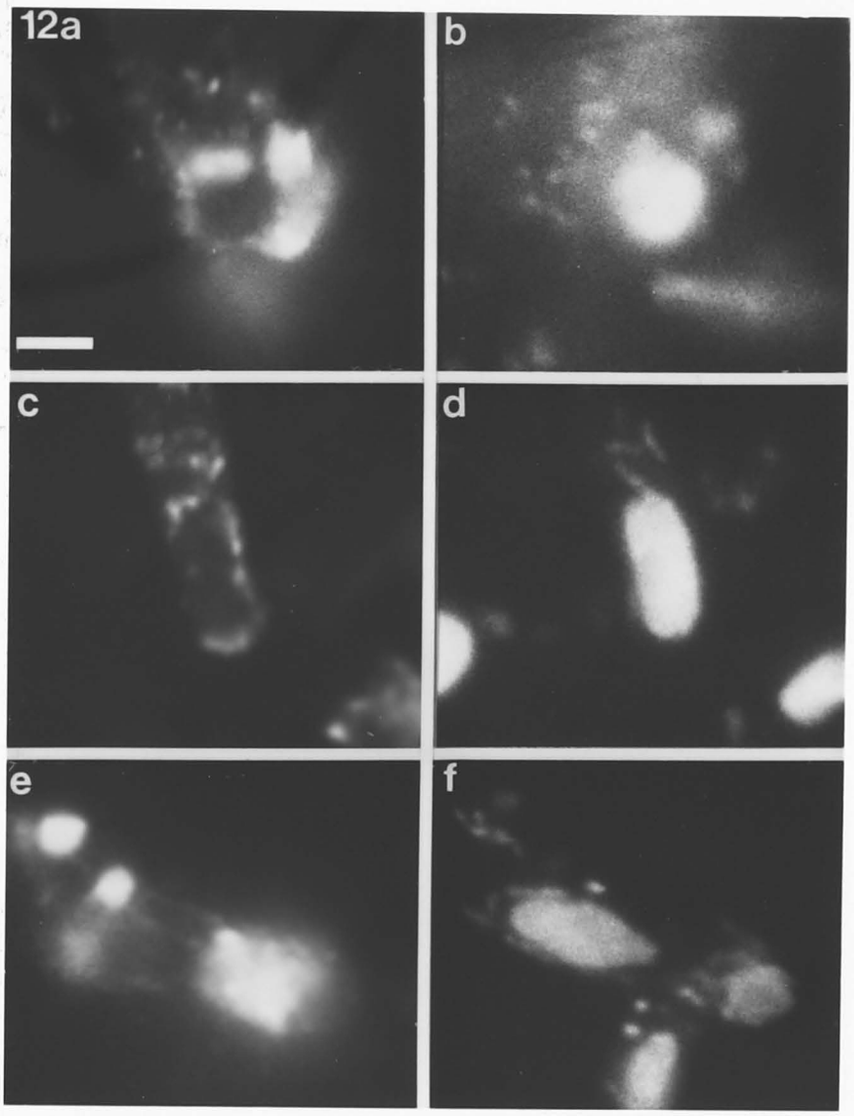
Figs 4.12a-4.12f. Higher magnification of 1h monensin-treated and reincubated hyphae, double stained with Lpv-1 (4.12a, 4.12c, 4.12e) and DAPI (4.12b, 4.12d, 4.12f). bar=2.9μm. Double staining with Lpv-1 and DAPI indicated that the Lpv-1 antigen was restricted to a narrow perinuclear zone in monensin-treated hyphae (Figs 4.12c, 4.12d). In the controls (Figs 4.12a, 4.12b) and reincubated hyphae (Figs 4.12e, 4.12f), the pattern of staining suggested that the Lpv-1 antigen was found in larger, wider regions, though these were still associated with the nucleus.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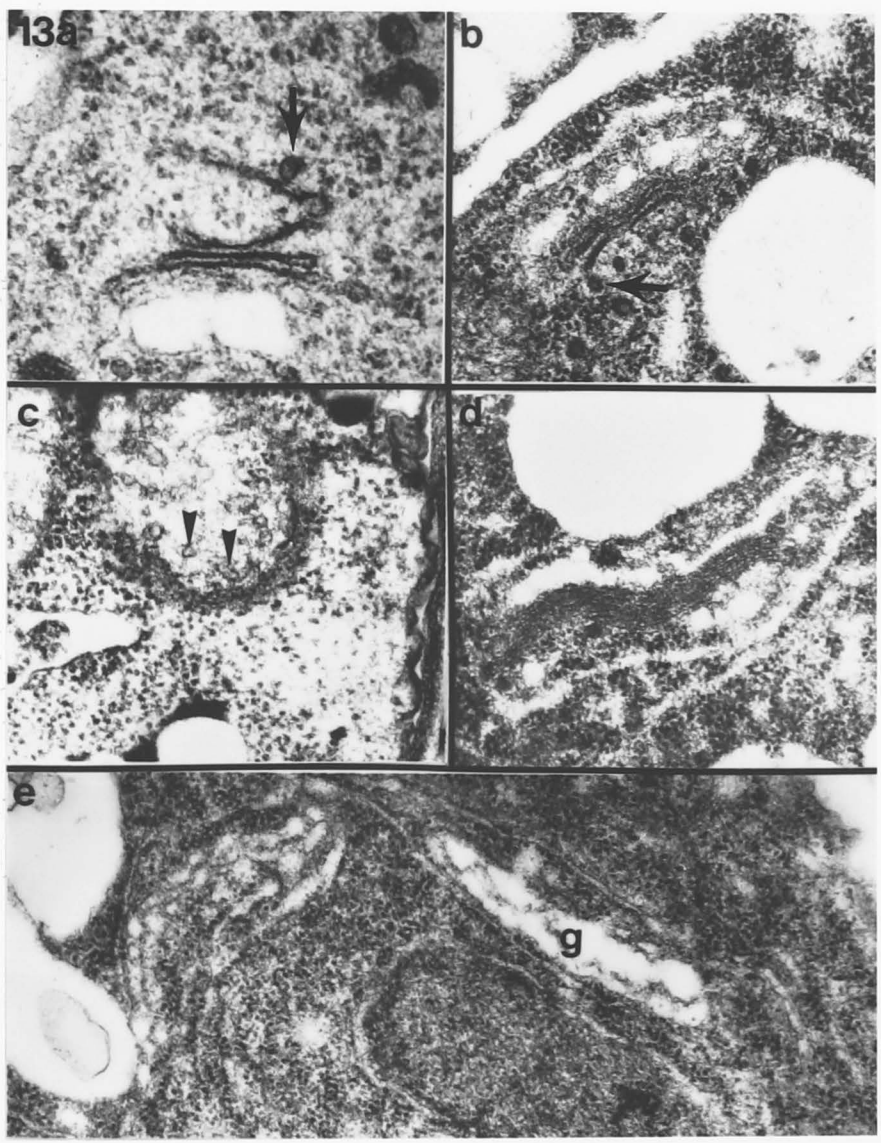
Figs 4.13a-4.13e. Electron microscopy of hyphae incubated in 50 μg/ml BFA for 1h.

Fig. 4.13a. Golgi apparatus from control hyphae, showing vesicles budding away from *trans* regions (arrow). X 55,000.

Fig. 4.13b. Golgi apparatus from reincubated hyphae, showing vesicles budding away from *trans* regions (arrow). X 41,000.

Fig. 4.13c-4.13d. The Golgi apparatus from BFA-treated hyphae had cisternae which were very close together (4.13c, 4.13d) and sometimes these cisternae appeared to be breaking down into small vesicles (4.13c arrows). 4.13c-X 43,000. 4.13d-X 41,000.

Fig. 4.13e. In some BFA-treated hyphae, the Golgi apparatus was absent and vacuolate areas, adjacent to the nucleus where the Golgi apparatus was normally positioned, were present instead (g). X 41,000.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Figs 4.14a-4.14e. Electron microscopy of hyphae incubated in 10 μM monensin for 1h.

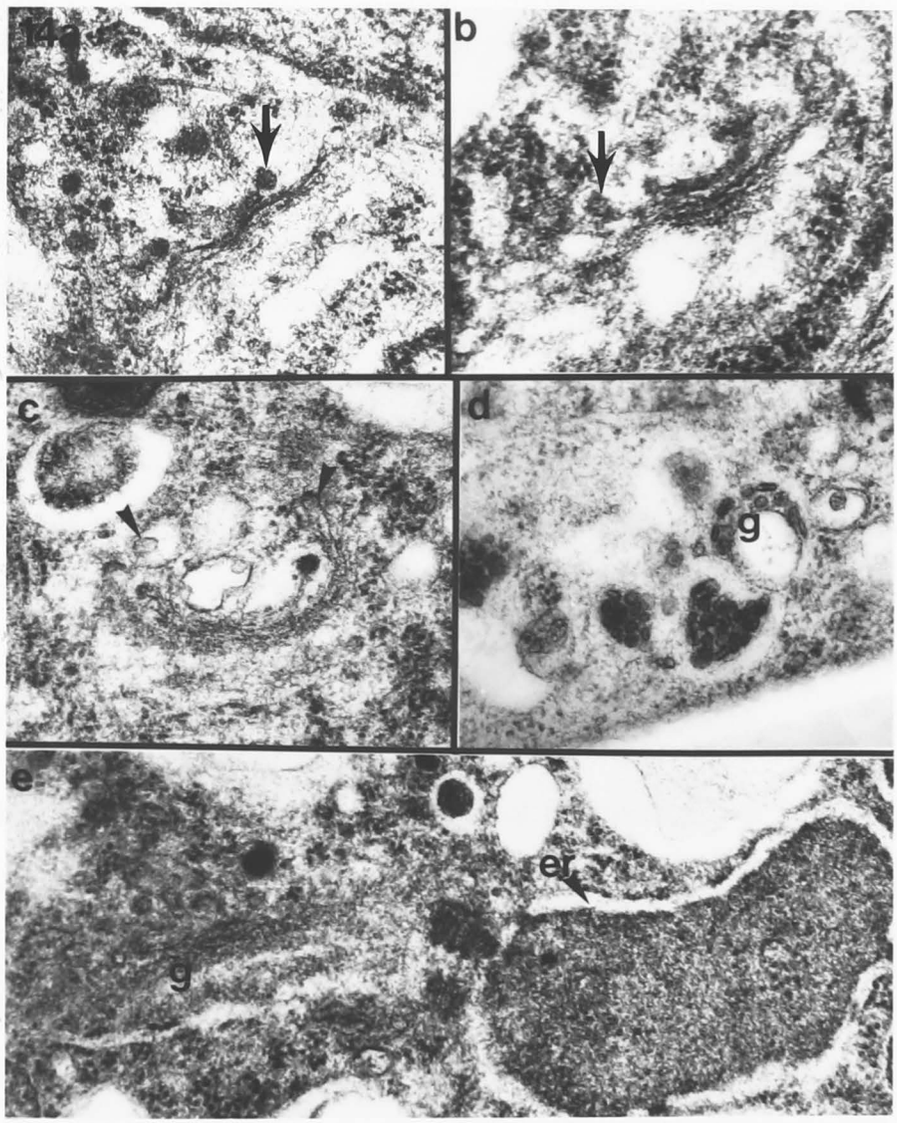
Fig. 4.14a. Golgi apparatus from control hyphae, showing vesicles budding away from *trans* regions (arrow). X 42,000.

Fig. 4.14b. Golgi apparatus from reincubated hyphae, showing vesicles budding away from *trans* regions (arrow). X 48,000.

Fig. 4.14c. Large vacuoles appeared to be forming from the *trans* part of the Golgi apparatus in monensin-treated hyphae (arrows). X 42,000.

Fig. 4.14d. The Golgi apparatus (g) appeared to be breaking down around the *trans* Golgi vacuoles in monensin-treated hyphae. X 37,500.

Fig. 4.14e. The nuclear envelope/E.R. (er) appeared quite swollen in monensin-treated hyphae. g=Golgi apparatus. X 41,000.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CHAPTER FIVE: THE INVOLVEMENT OF THE CYTOSKELETON IN THE TRANSPORT OF PERIPHERAL VESICLES INTO THE DEVELOPING SPORANGIUM.

5.1 INTRODUCTION

The cytoskeleton in fungi consists of mostly actin and tubulin (McKerracher & Heath 1987) and is involved in organelle motility. In this chapter an investigation into the arrangement of microtubules and actin microfilaments in vegetative and sporulating hyphae is reported. From these results the possible mechanism by which peripheral vesicles move from their site of formation and into the developing sporangium is examined.

There is good evidence that microtubules mediate secretory vesicle transport in a number of systems. For example, drugs that depolymerise microtubules inhibit wall vesicle transport in *Funaria* protonemata (Howard & Aist 1980) and apical membrane protein and casein vesicle transport in epithelial cells (Achler *et al.* 1989, Eilers *et al.* 1989, Rennison *et al.* 1992).

The strongest evidence for the role of microtubules in vesicle movement, however, comes from direct observation mostly provided by studies involving a few specialised cell types. Video-enhanced light microscopy has enabled the direct visualisation of organelles and vesicles being transported along microtubules in frog corneal keratocytes (Hayden *et al.* 1983) and suggested that in the transport of neurotransmitter vesicles from the cell body to the synapse in giant squid axons, microtubules act as guiding rails (Allen *et al.* 1985, Schnapp *et al.* 1985). Video microscopy and electron microscopy of the isolated feeding network of the freshwater amoeba *Reticulomyxa*, reveals that the movement of

organelles such as vesicles is along microtubules within the network (Koonce & Schliwa 1986).

One system that has been used extensively to investigate the involvement of microtubules in secretory vesicle transport is At-T20 pituitary tumor cells (Tooze & Burke 1987, Kreis *et al.* 1989, Rivas & Moore 1989). In such cells, microtubules have been shown to transport regulated ACTH containing secretory vesicles away from their site of formation and to maintain them in clusters at the outer processes of these cells (Tooze & Burke 1987). These authors indicated that during metaphase and anaphase, when microtubules were depolymerised, the secretory vesicles were no longer held in position and became uniformly distributed throughout the cytoplasm. Further work with this system has shown, however, that in the secretion of constitutive cell proteins, microtubules may not be necessary, as secretion of this latter vesicle type is unaltered by microtubule inhibitors (Rivas & Moore 1989).

The evidence for the involvement of the actin cytoskeleton in secretory vesicle transport is equally extensive. Most support comes from work involving cytochalasins, some of the most potent inhibitors of the actin cytoskeleton. Picton and Steer (1981) showed that cytochalasin D inhibited secretory vesicle transport in pollen tubes while Harold and Harold (1986) suggested that the cytochalasin-induced branching in *Achlya bisexualis* was caused by the diversion of wall vesicles to sites other than the apex.

Further evidence is provided by the observations of Heath and Kaminskyj (1989) who showed a correlation between wall vesicles in the growing tip of *Saprolegnia* hyphae and the presence of actin microfilaments and Kobori *et al.* (1992) who recently localised actin to growing buds and hyphal tips in different forms of

yeast. These strongly suggest that actin microfilaments play an important role in transport of fungal cell wall vesicles. The most convincing evidence for the involvement of the actin cytoskeleton in wall vesicle transport comes, though, from work using the giant internodal cells of Characean algae (Kohno and Shimmen 1988). These authors showed through video microscopy that wall vesicles isolated from pollen tubes could be transported along actin bundles in algal cells. Myosin associated with the vesicles or the bundles themselves appeared to provide the motive force for vesicle movement (Grolig *et al.* 1988).

Although there have been few studies of cytoskeleton-mediated organelle movements in *Phytophthora*, there has been a considerable amount of work carried out on the cytoskeleton of the related oomycete, *Saprolegnia ferax.* Microtubules have been shown to be too short to act as tracks for wall vesicle movement in vegetative hyphae of *Saprolegnia* (Heath & Kaminskyj 1989), but because of the correlation between microtubules and recently formed K2-bodies in primary cysts, are likely to be involved in the transport of these organelles to the cell periphery (Lehnen & Powell 1989). The peripheral location and longitudinal arrangement of actin microfilaments in vegetative hyphae tends to suggest that, in addition to maintaining the longitudinal shape of nuclei (Heath & Harold 1992), actin microfilaments are involved in the transport of vesicles containing wall precursors to the growing apex (Heath & Kaminskyj 1989). Actin microfilaments are not involved in the transport of saltatory vesicles of unknown function in growing hyphae (Heath 1988) and, as actin microfilaments break down in hyphal tips during sporulation (Heath & Harold 1992), it is probable they are not involved in the transport of encystment vesicles which form in hyphae at this stage (Beakes 1983).

Although Chapter 4 reported that the movement of peripheral vesicles away from the Golgi apparatus was sensitive to CD, the above evidence suggest, that either or both microtubules and actin microfilaments could transport peripheral vesicles into the developing sporangium. In this chapter, evidence is reported for the cytoskeletal mechanism behind such transport.

5.2 MATERIALS AND METHODS

5.2.1 Culture

For vegetative hyphae, small plugs of *P. cinnamomi* 6BR hyphae taken from the edge of a colony growing on V8 nutrient agar (Chapter 2) were placed in 5ml of 5% V8 broth (Chapter 2) and kept at 25°C, in the dark for 24h. Colonies previously grown in V8 broth, were rinsed three times in mineral salts solution (Chapter 2) and then shaken gently at 50rpm for 8h in the light at 22°C, to obtain sporulating hyphae.

5.2.2 Actin staining in hyphae

Hyphal tufts of both vegetative (i.e. after 24h growth in V8 broth) and sporulating stages were fixed for 15min in 4% paraformaldehyde in 50 mM Pipes buffer (pH 7.0). After a 10min rinse in 50mM Pipes, the tufts were incubated in Rhodamine Phalloidin (Molecular Probes, Inc, Eugene, USA) diluted 2:3 in methanol, in the dark for 15min. Tufts were double stained for 15min with DAPI diluted in PBS/1% BSA to a concentration of 0.1µg/ml, after a brief rinse in PBS. Samples were mounted in mowiol with 0.1% PPD. Actin was visualised with

epifluorescence optics with an F1 filter cube (excitation 546nm; dichroic mirror 395nm, barrier 420nm) on a Zeiss axioplan microscope.

5.2.3 Immunostaining microtubules in hyphae

Hyphal tufts were fixed for 20min in 4% paraformaldehyde in 50 mM Pipes buffer (pH 7.0). After freezing in Tissuetek and cryosectioning (see Chapter 2), twelve and fourteen micrometer thick cryosections of hyphae of both stages were immunostained to reveal microtubules. After drying down for 5min onto poly-l-lysine coated slides, the sections were extracted with 1% Triton X-100 (Pierce Co., Rockford, Illinois, USA), rinsed 5min in PBS then incubated for 40min in anti-β tubulin (Amersham Australia, Sydney, Australia) diluted 1:500 with PBS/1% BSA. The sections were rinsed in PBS for 5min then incubated in 1:60 dilution of SAM-FITC. After rinsing in PBS the sections were double stained with 50 µg/ml soybean agglutinin (SBA)-rhodamine (E. Y. Laboratories Inc., San Mateo, CA) in PBS, for 1h in the dark. SBA-rhodamine was used to identify the association between microtubules and peripheral vesicles in sporulating hyphae as past research has shown that the dorsal vesicle antigens are the only cell components labelled by SBA (Gubler & Hardham 1988). Tubulin immunostained hyphae were also double stained with 0.1 µg/ml DAPI for 15min. Following a rinse in PBS and a quick 5s rinse in distilled water, the sections were mounted in mowiol with 0.1% PPD and examined in a Zeiss Axioplan microscope equipped with epifluorescence optics.

5.3 RESULTS

5.3.1 Actin in hyphae

Some differences in the arrangement of actin in vegetative and sporulating hyphae were evident. Actin plaques were absent from apical regions of vegetative hyphae (Fig. 5.1a) but in sporulating hyphae the plaques extended into the tip (Fig. 5.1c). Actin microfilaments were present in both stages and usually had plaques interspersed among them (Figs 5.1a, 5.1c). In both vegetative and sporulating hyphae actin microfilaments extended into the tip of the hypha (Figs 5.1a, 5.1c). To assist further in investigating the role that actin microfilaments play in maintaining nuclear shape, double labelling was carried out with DAPI. Nuclear shape changed during the transition of vegetative hyphae to sporulating hyphae (compare Figs 5.1b & 5.1d). Nuclei from sporangiophores were significantly shorter ($P< 0.025$) and smaller ($P< 0.01$) than nuclei found in vegetatively growing tips (Table 5.1). Nuclei in developing sporangia were always short and small (Fig. 5.1d).

5.3.2 Microtubules in hyphae

In cryosectioned vegetatively growing cultures, extensive microtubules were present in most hyphae (Fig. 5.3a). In cryosectioned sporulating cultures, there was a mixture of hyphae with microtubules and hyphae in which microtubules were absent (Fig. 5.3c). Double labelling with anti-tubulin and SBA-rhodamine helped ascertain whether microtubules had a direct association with peripheral vesicles in sporulating hyphae. In cryosectioned sporulating hyphae which contained dorsal vesicles, microtubules were always absent (Figs 5.2a, 5.2b). Conversely, the cryosectioned sporulating hyphae where dorsal vesicles were

absent always contained microtubules (similar to those seen in most vegetative hyphae, see Fig. 5.3a), (Figs 5.2c, 5.2d).

Double labelling with anti-tubulin and DAPI gave an insight into the association between microtubules and nuclear shape. In all cryosectioned vegetative hyphae, long, parallel microtubules traversed the cytoplasm and were positioned around the nuclei, which were large and long (Figs 5.3a, 5.3b). In sporulating hyphae, many hyphae did not have microtubules and in these, the nuclei were small and round (Fig. 5.3d), typical of those seen in sporangiophores. In such hyphae, although microtubules were not present, very fine, tubulin-positive staining was observed (Fig. 5.3c).

5.4 DISCUSSION

This work has shown that there is no direct association between dorsal vesicles and microtubules in sporulating hyphae and thus it seems unlikely that peripheral vesicle transport is microtubule mediated. Since microtubules do not appear to be responsible for the translocation of peripheral vesicles into the developing sporangium, it is possible that vesicle movement is mediated by the actin cytoskeleton. Although this study provides no direct evidence that actin microfilaments are involved in the transport of peripheral vesicles, it shows that actin microfilaments are *available* for transport into the developing sporangium. It is suggested that peripheral vesicles are transported away from the Golgi apparatus and into the developing sporangium via actin microfilaments. This is presumably similar to the system of organelle transport operating in vegetative hyphae of *Saprolegnia* (Heath & Kaminskyj 1989) and the actin-myosin based

system in Characean algae (Kohno & Shimmen 1988, Grolig *et al.* 1988). The system of peripheral vesicle transport in *P. cinnamomi* 6BR appears to be different to that in *Saprolegnia.* Although K1-bodies in *Saprolegnia* are probably synthesised in the developing sporangium (Holloway & Heath 1977) and therefore do not require extensive transport, encystment vesicles which originate in zoosporangial initials (Beakes 1983) must be moved from their site of synthesis and into the developing sporangium by some mechanism. The absence of actin microfilaments from sporangial tips in *Saprolegnia* (Heath & Harold 1992) suggests that another cellular transport mechanism, possibly microtubules, which may be longer than in vegetative hyphae (Heath & Kaminskyj 1989), is at work. This seems possible, as Armbruster (1982) has identified long microtubules in pre-sporangial hyphae of members of the Saprolegniaceae.

Although the most striking change in the actin cytoskeleton between vegetative and sporulating hyphae is in the positioning of actin plaques in the hyphal apex (developing sporangium), it is unlikely that these structures play a role in intracellular transport of organelles. Heath and Harold (1992) suggest that plaques are involved in the positioning of organelles by facilitating adhesion to the plasma membrane and cell wall so it is possible actin plaques in *P. cinnamomi* 6BR are involved in the adhesion of the peripheral vesicles to specific regions of the sporangium wall. Evidence contradicting this exists, however, in research conducted by Hyde (1992). This worker found that peripheral vesicle location in sporangia was cytochalasin-insensitive and that microtubules maintained the position of peripheral vesicles at the plasma membrane. The recent study of Heath & Harold (1992) has shown that actin plaques persist in the presence of cytochalasins. It is possible then, that peripheral vesicles are maintained at the zoospore plasmamembrane by a combination of both microtubules and actin

plaques. This is not unexpected, as in many systems microtubules and actin are known to interact to maintain the properties of the cytoskeleton (McKerracher & Heath 1986, Doonan *et al.* 1988, Heath & Kaminskyj 1989).

The apparent absence of microtubules from sporulating hyphae suggests that microtubules are not involved in peripheral vesicle transport. While there is good evidence that microtubules are involved in secretory vesicle transport in some systems, there are accounts of systems where there is a lack of involvement of microtubules in this process. The transport of cell wall vesicles for example, appears not to be mediated by microtubules. Hyphal growth has been shown to continue in the absence of microtubules (Herr & Heath 1982, Hoch *et al.* 1987) and microtubules do not extend into the apical zones of tip-growing cells (Pierson *et al.* 1986, Heath & Kaminskyj 1989) making them unavailable for wall vesicle transport. Transport of invertase vesicles in yeast has been shown not to require microtubules (Huffaker *et al.* 1988) and microtubule disruption does not affect intracellular transport of secretory vesicles in cultured fibroblasts (Virtannen & Vartio 1986).

The results from this study also suggest that nuclear movement in sporulating hyphae is independent of microtubules. This has proved to be the case in other tip growing cells such as *Basidiobolus* hyphae (McKerracher and Heath 1985), and pollen tubes (Heslop-Harrison *et al.* 1988). In pollen tubes, the actin cytoskeleton appears to be involved in nuclear migration (Heslop-Harrison & Heslop-Harrison 1989) - this lends further weight to the hypothesis that nuclear movement is motivated by the actin cytoskeleton in sporulating hyphae of *P. cinnamomi.*

In a recent study on *Achlya* and *Saprolegnia* actin microfilaments were shown to be lost with the changing of nuclear shape at sporulation (Heath & Harold 1992).

In *P. cinnamomi* 6BR, a role for actin microfilaments in maintaining nucleus shape can be discounted as microfilaments are retained in sporulating hyphae. Since actin microfilaments do not appear to be involved in maintaining nuclear shape, it is possible that microtubules perform this role in vegetative hyphae. These results show microtubules enclosing and in parallel to elongated nuclei in vegetative hyphae. Microtubules are likely to be responsible for the pyriform shape of nuclei found in zoospores (Heath & Greenwood 1971) so it is possible they perform a similar morphogenic role in vegetative hyphae.

The decrease in nuclear size in sporulating hyphae may be due to nuclear division. The development of infection structures in *Uromyces* has been shown normally to accompany nuclear division (Staples *et al.* 1975). Further to this, Staples and Hoch (1982) have suggested the induction of nuclear division in germ tubes of *Uromyces* was caused by the depolymerisation of cytoplasmic microtubules and microfilaments. Salo *et al.* (1989) showed that during nuclear division in filamentous fungi, cytoplasmic microtubules disassembled to provide a pool of tubulin subunits used in the formation of the nuclear spindle. No such spindles were seen in sporulating hyphae in this study but it is possible that microtubules do break down in response to, or in preparation for nuclear division. Why should nuclei be rounder and smaller in sporulating hyphae? It is likely that nuclei have to be smaller and more uniform in size as they will ultimately become part of an evenly partitioned sporangium where each zoospore contains approximately the same number of cellular organelles (such as peripheral vesicles and mitochondria) and a single pyriform nucleus (Hyde 1992, Hyde & Hardham 1992). A further question arises out of this, and that is why do the nuclei not undergo mitosis within the developing sporangium? Although Heath and Greenwood (1970b) have claimed that nuclei in sporangia of *Saprolegnia ferax* undergo mitosis after

septum formation, mitosis has not been observed in septate sporangia of *Brevilegnia minutandra* (Armbruster 1982) and during sporangiogenesis in *Isoachlya* (Bhargava 1950). It is possible that the change in nuclear shape, like peripheral vesicle formation (see Chapter 2), occurs early in sporulation because the necessary cellular machinery in sporangial development (in the case of nuclei - sporangial microtubules) are taken up with other functions such as organelle positioning (Hyde 1992).

The actin cytoskeleton probably plays a major role in the transport of peripheral vesicles from their point of synthesis at the Golgi apparatus into the developing sporangium. Results from this study suggest that peripheral vesicles and other cellular organelles such as nuclei may be transported along actin microfilaments, possibly in a manner similar to tip growth in *Saprolegnia* and to cytoplasmic streaming in green algae. Microtubules are not associated with peripheral vesicles in sporulating hyphae and are, therefore, not involved in the movement of peripheral vesicles into developing sporangia. It is suggested that microtubules may maintain the longitudinal shape of nuclei as this is lost at the same time microtubules are lost from sporulating hyphae.

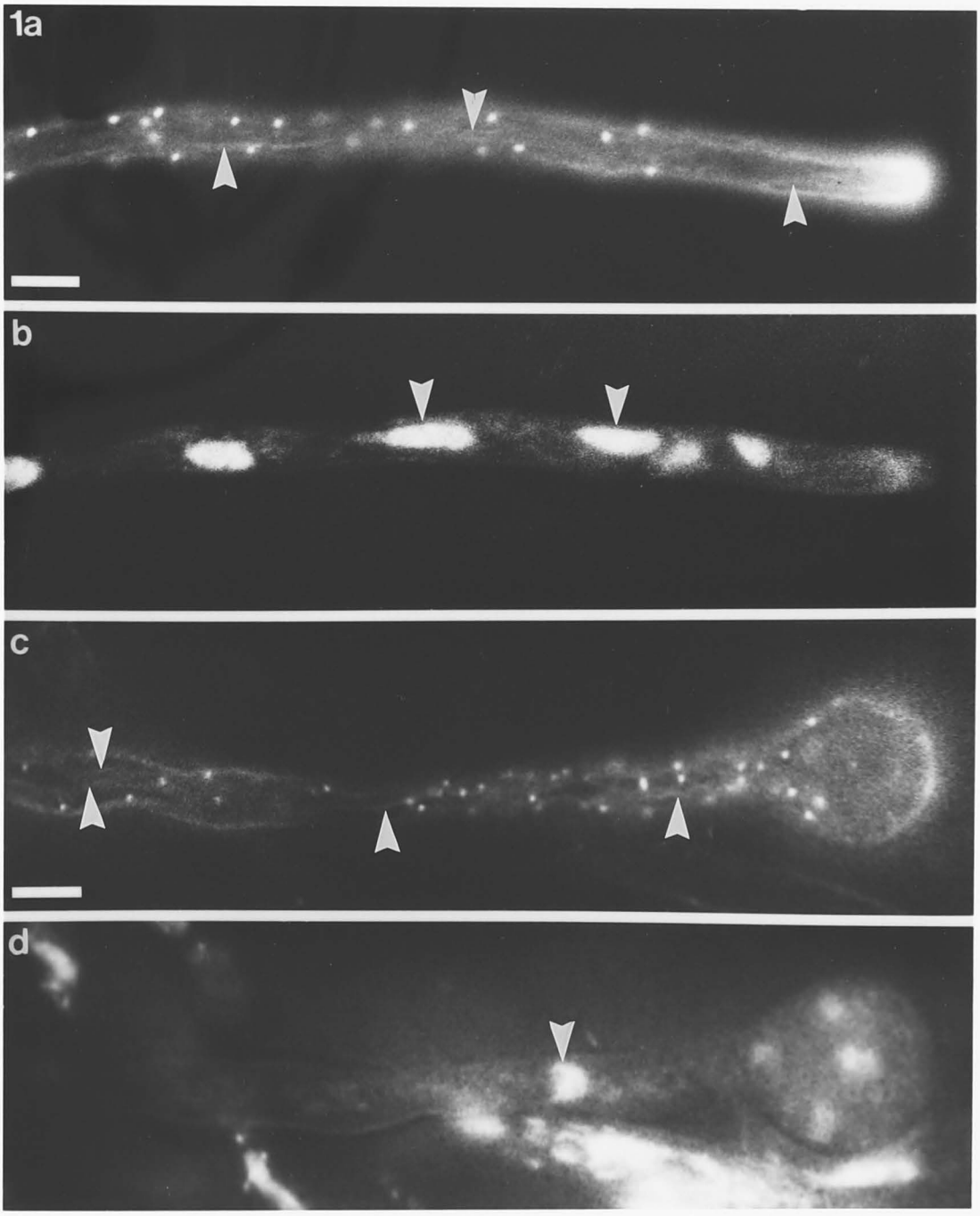
Figs 5.1a-5.1d. Rhodamine phalloidin (5.1a, 5.1c) and DAPI staining (5.1b, 5.1d) of vegetative and sporulating hyphae.

Fig. 5.1a. Actin plaques were absent from apical regions of vegetatively-growing hyphae while actin microfilaments extended into these regions. Arrows indicate actin microfilaments. bar= 5.36μm.

Fig. 5.1b. Nuclei from same hypha as above. In vegetatively-growing hyphae nuclei tended to be long and narrow (arrows).

Fig. 5.1c. Actin plaques and microfilaments extended into the tips of sporulating hyphae. Arrows indicate actin microfilaments. bar=4.51μm.

Fig. 5.1d. Nuclei in same sporulating hypha as above. The nucleus in the sporangial stalk is characteristically short and small (arrow)-similar to those in the developing sporangium.


1a
b
c
d

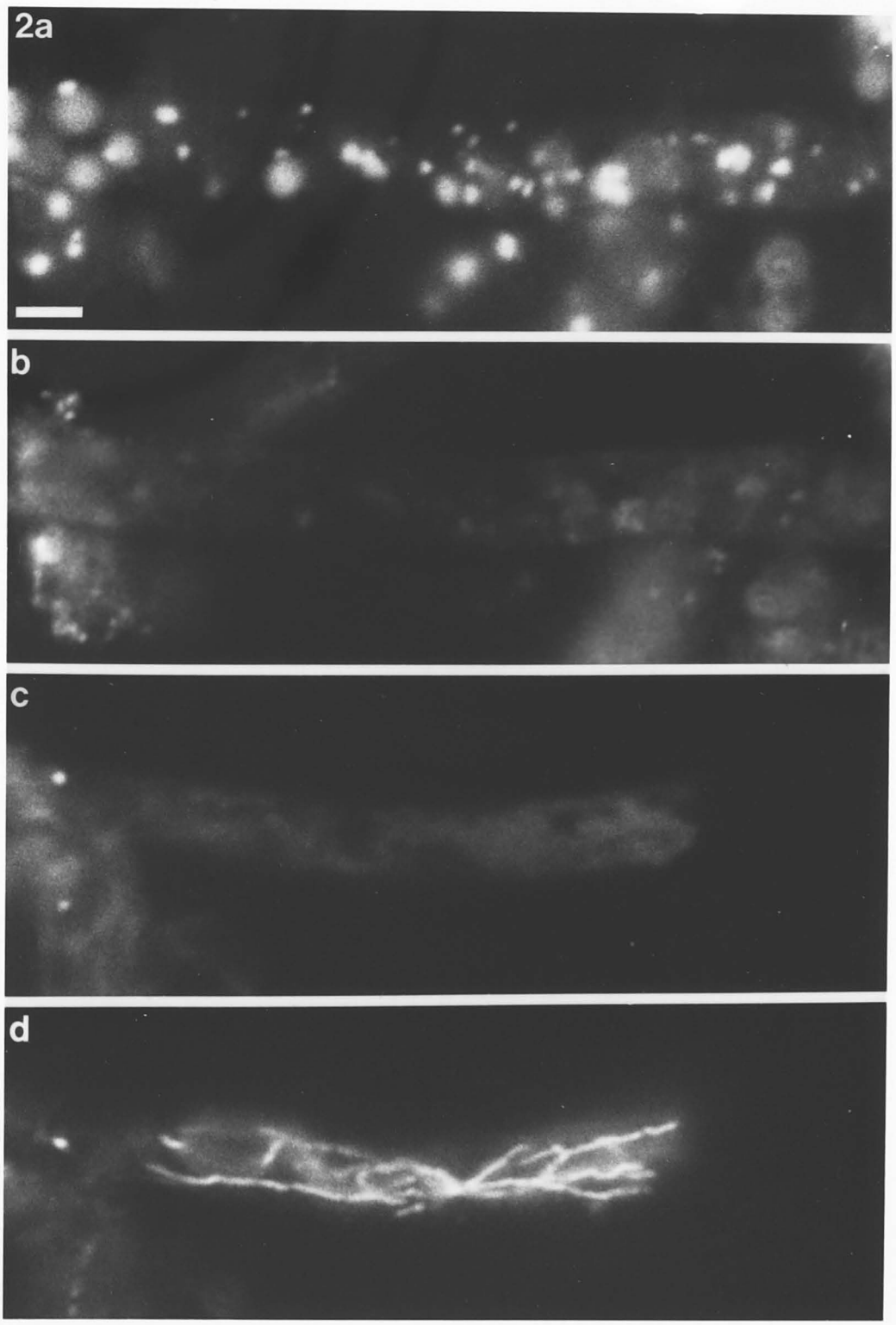
Figs 5.2a-5.2. Double labelling of cryosectioned sporulating hyphae with SBA-rhodamine and anti-tubulin. bar=3.15μm.

Fig. 5.2a. Cryosectioned sporulating hypha showing SBA-rhodamine stained dorsal vesicles.

Fig. 5.2b. Same hypha as above immunostained with anti-tubulin. Note the lack of microtubules.

Fig. 5.2c. Cryosectioned sporulating hypha stained with SBA-rhodamine. Note the absence of dorsal vesicles.

Fig. 5.2d. Same hypha as above immunostained with anti-tubulin showing extensive microtubules.


2a
b
c
d

Figs 5.3a-5.3d. The association between microtubules and nuclear shape in vegetative and sporulating hyphae. bar=4.9μm. Hyphae double stained with anti-tubulin (5.3a, 5.3c) and DAPI (5.3b, 5.3d).

Fig. 5.3a. Microtubules in a cryosectioned vegetative hypha.

Fig. 5.3b. Large and long nuclei in same hypha as above.

Fig. 5.3c. Cryosectioned sporulating hyphae in which microtubules were absent. Very fine tubulin positive staining could be observed in such hyphae (arrow).

Fig. 5.3d. Small and round nuclei from above hypha.

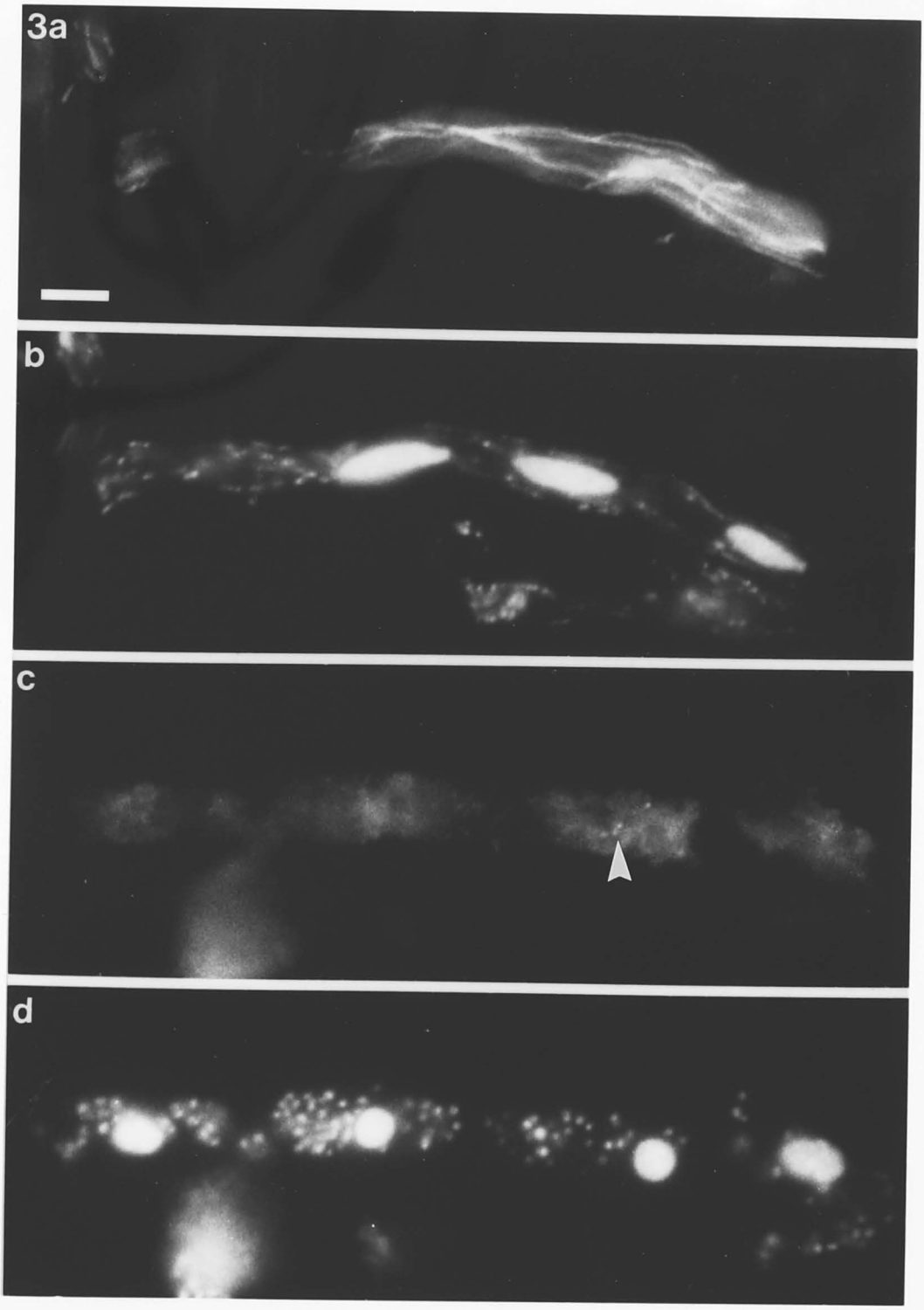

3a
b
c
d

Table 5.1. Comparison of mean nuclear diameter and mean nuclear volume between hyphal tips in the vegetative and sporulating state.

* mean nuclear diameter significantly different ([P< 0.025] paired sample t-test. Sokal & Rohlf (1981)).

** mean nuclear volume significantly different ([P< 0.01] paired sample t-test. Sokal & Rohlf (1981)).

	Vegetative	Sporulating
Nuclear diameter (μm)	**5.22 ±1.3**	**3.44 ±1.02***
Nuclear volume (μm^3)	**353.75 ±235.41**	**109.7 ±108.19****

Table 5.1.

CHAPTER SIX

GENERAL CONCLUSION

Phytophthora cinnamomi is a serious problem to vegetation in many areas of Australia (Weste & Marks 1987). Much has been done in an attempt to control the spread of the pathogen over the last 20 years. In understanding more about the cell biology of the infection of hosts by the species, great strides have been made with the advent of monoclonal antibody technology. For example, since the first ultrastructural descriptions of oomycetes, vesicles located to the periphery of zoospores had been assigned roles (mostly erroneous) in infection based purely on morphological observation. It was not till the work of Gubler and Hardham (1988, 1990) that we gained a more complete insight into the crucial roles that these immunologically distinct peripheral vesicles play in the infection of hosts.

Peripheral vesicles in *P. cinnamomi* are sporulation specific, in that they are synthesised only during sporangium and chlamydospore formation. Their role in the infection of hosts is reflected by the timing of their appearance. In addition to the adhesive role that ventral vesicles play in the attachment to hosts, both ventral and large peripheral vesicles can presumably be broken down in periods of vegetative growth. These two vesicle types form earlier in sporulation than dorsal vesicles, whereas dorsal vesicles appear to form only when the mycelium is committed to sporangium formation. The reason for this could be that dorsal vesicles play solely a role in zoospore encystment.

The process of peripheral vesicle formation occurs in pre-sporangial hyphae and not in the developing sporangium, perhaps because if conditions that favor vegetative growth return, before sporangia have formed, hyphae can recycle large peripheral and ventral vesicles during such growth. Alternatively, peripheral vesicle formation may not occur in the developing sporangium because the Golgi apparatus in developing sporangia may be taken up with other major functions, for example the massive production of vesicles needed to produce the sporangial wall.

Peripheral vesicles are present in chlamydospores because these structures can germinate directly to form sporangia. In this way, chlamydospores can be considered analogous to a sporulating hypha - containing all of the requisite components for sporangial formation.

Ventral and dorsal vesicles, like many vesicles containing secretory glycoproteins in both animal and plant systems, both probably form from the Golgi apparatus. Likewise, in their mode of formation from the Golgi apparatus, large peripheral vesicles are analogous to higher plant storage proteins, which are mostly derived from the Golgi apparatus. The specificity of the labelling of the peripheral vesicle antibodies has made this system an ideal one in which the phenomenon of secretory and storage protein formation could be investigated.

Secretory proteins in plant and animal cells undergo processing in the E.R. and the Golgi apparatus (Farquhar 1985, Moore *et al.* 1991) but are then sorted into distinct vesicles at the *trans* Golgi apparatus (Griffiths and Simons 1986, Staehelin *et al.* 1991). Work reported in this thesis indicates that the Golgi apparatus in *P. cinnamomi* 6BR is capable of processing at least two distinct proteins at a time and that it can, synchronously, sort a storage protein from a secretory protein. Evidence suggests that this sorting event occurs in *trans* Golgi regions, though

further work, utilizing such techniques as rapid freezing and freeze substitution, which have been shown to be superior to chemical fixation as a method for preserving the *trans* Golgi network in *P. cinnamomi* (Hyde et al. 1991b), may be necessary to confirm this.

The process of peripheral vesicle formation in *P. cinnamomi* has been shown to be susceptible to secretion inhibitors. Such work has been valuable in studying the behavior of secretory glycoproteins and further elucidating parts of the secretory pathway affected by the drugs. The permeability of the hyphal wall to these inhibitors is greatly encouraging of the use of chemical agents in the control of the fungus.

Peripheral vesicle transport into the developing sporangium is not dependent on microtubules and may be mediated by the actin cytoskeleton. The sensitivity of vesicle transport to cytochalasin D and the presence of extensive actin cables in sporangiophores, suggest that the actin cytoskeleton of sporulating hyphae would be an excellent system in which the process of actin-mediated organelle transport could be further studied.

The investigations reported in this thesis have gained further information about these important cellular organelles. The formation of distinct vesicles at sporulation and their transport from the point of synthesis into the developing sporangium are vital to the infective capabilities of the fungus. Further studies of peripheral vesicles in *P. cinnamomi*, are demanded, especially those that examine the molecular nature of these organelles. This may provide us with the vital knowledge in combating this destructive pathogen.

REFERENCES

Achler, C., D. Filmer, C. Merte, and D. Drenckhahn. (1989). Role of microtubules in polarized delivery of apical membrane proteins to the brush border of the intestinal epithelium. *J. Cell Biol.* 109:179-189.

Allen, E. D., R. Aiuto, and A. S. Sussman. (1980). Effects of cytochalasins on *Neurospora crassa.* Growth and ultrastructure. *Protoplasma.* 102:63-75.

Allen, R. D., D. G. Weiss, J. H. Hayden, D. T. Brown, H. Fujiwake, and M. Simpson. (1985). Gliding movement of and bidirectional transport along native microtubules from squid axoplasm evidence for an active role of microtubules in cytoplasmic transport. *J. Cell. Biol.* 100:1736-1752.

Armbruster, B. L. (1982). Sporangiogenesis in three genera of the Saprolegniaceae. II Primary spore initial to secondary spore inital stage. *Mycologia* 74:975-999.

Barstow, W. E., and J. S. Lovett. (1975). Formation of gamma particles during zoosporogenesis in *Blastocladiella emersonii. Mycologia.* 67:518-529.

Barstow, W. E., W. L. Lingle, and J. S. Lovett. (1985). Observations on tubular endoplasmic reticulum in the aquatic fungi *Blastocladiella emersonii, Blastocladiella britannica, and Catenaria anguillulae. J. Ultrast. Res.* 93:168-178.

Bartnicki-Garcia, S., and D.E. Hemmes. (1976). Some aspects of the form and function of Oomycete spores. In: The Fungal Spore. D.J. Weber, and W.M. Hess, editors. John Wiley & Sons, New York. 593-639.

Beakes, G. W. (1983). A comparative account of cyst coat ontogeny in saprophytic and fish-lesion (pathogenic) isolates of the *Saprolegnia diclina-parasitica* complex. *Can. J. Bot.* 61:603-625.

Bessey, E. A. (1950). Morphology and taxonomy of fungi. (Hafner, New York). cited in Powell *et al.* (1985).

Bhargava, K. S. (1950). Formation and liberation of zoospores of *Isoachlya anispora* var. *indica. Bot. Gaz.* (Crawfordsville). 112:24-31. cited in Armbruster (1982).

Bimpong, C. E., and C. J. Hickman. (1975). Ultrastructural and cytochemical studies of zoospores, cysts, and germinating cysts of *Phytophthora palmivora. Can. J. Bot.* 53:1310-1327.

Bland, C. E. and H. V. Amerson. (1973). Electron microscopy of zoosporogenesis in the marine phycomycete *Lagenidium callinectes* Couch. *Arch. Mikrobiol.* 94:47-64.

Bortnick, R. N., M. J. Powell, and T. N. Bangert. (1985). Zoospore fine structure in the mycoparasite *Olpidiopsis saprolegniae* var. *saprolegniae* (oomycetes, Lagenidiales). *Mycologia.* 77:861-879.

Brown, R. M. Jr., W. W. Franke, H. Kleinig, H. Falk, and P. Sitte. (1970). Scale formation in Chrysophycean algae. I. Cellulosic and noncellulosic wall components made by the Golgi apparatus. *J. Cell Biol.* 45:246-271.

Broxholme, S. J., N. D. Read, and D. J. Bond. (1991). Developmental regulation of proteins during fruit-body morphogenesis in *Sordaria brevicollis*. *Mycol. Res.* 95:958-969.

Burgess, T. L., and R. B. Kelly. (1987). Constitutive and regulated secretion of proteins. *Annu. Rev. Cell Biol.* 3:243-293.

Champe, S.P., M.B. Kurtz, L.N. Yager, N.J. Butnick, and D.E. Axelrod. (1981). Spore formation in *Aspergillus nidulans* competence and other developmental processes. In: The Fungal Spore morphogenetic controls. G. Turian, and H.R. Hohl, editors. Academic Press, London. 255-276.

Chrispeels, M. J. (1983). The Golgi apparatus mediates the transport of phytohemagglutinen to the protein bodies of bean cotyledons. *Planta.* 158:140-151.

Chrispeels, M. J. (1991). Sorting of proteins in the secretory system. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 42:21-53.

Chrispeels, M. J., and L. A. Staehelin. (1992). Budding, fission, transport, targeting, fusion-frontiers in secretion research. *Plant Cell.* 4:1008-1015.

Christen, J., and H. R. Hohl. (1972). Growth and ultrstructural differentiation of sporangia of *P. palmivora. Can. J. Microbiol.* 18:1959-1964.

Chung, K-N., P. Walter, G. W. Aponte, and H.-P. H. Moore. (1989). Molecular sorting in the secretory pathway. *Science.* 243:192-197.

Cope, M., and A. R. Hardham. (1992). Immunochemical and morphological comparison of peripheral vesicles in zoospores of *Phytophthora cinnamomi* and *Pythium aphanidermatum. Abstracts of the joint meeting of the American Phytopathological society and the Mycological Society of America. Portland, Oregon.* M100.

Craig, S., and D. J. Goodchild. (1984). Periodate-acid treatment of sections permits on-grid immunogold localization of pea seed vicilin in ER and Golgi. *Protoplasma.* 122:35-44.

Derksen, J., E. S. Pierson, and J. A. Traas. (1985). Microtubules in vegetative and generative cells of pollen tubes. *Eur. J. Cell. Biol.* 38:142-148.

Domozych, D. S., K. D. Stewart., K. R. Mattox. (1981). Development of the cell wall in *Tetraselmis* role of the Golgi apparatus and extracellular wall assembly. *J. Cell Sci.* 52:351-371.

Doms, R. W., A. Ruusala, C. Machamer, J. Helenius, A. Helenius, and J. K. Rose. (1988). Differential effects of mutations in three domains on folding, quaternary structure, and intracellular transport of VSV G protein. *J. Cell Biol.* 107:89-99.

Donaldson, J. G., J. Lippincott-Schwartz, G. S. Bloom, T. E. Kreis, and R. D. Klausner. (1990). Dissociation of a 110-kD peripheral membrane protein from the Golgi apparatus is an early event in brefeldin A action. *J. Cell Biol.* 111:2295-2306.

Doonan, J. H., D. J. Cove, and C. W. Lloyd. (1985). Immunofluorescence microscopy of microtubules in intact cell lineages of the moss, *Physcomitrella patens. J. Cell Sci.* 75:131-147.

Doonan, J. H., D. J. Cove, and C. W. Lloyd. (1988). Microtubules and microfilaments in tip growth Evidence that microtubules impose polarity on protonemal growth in *Physcomitrella patens*. *J. Cell Sci.* 89:533-540.

Dorward, D. W., and M. J. Powell. (1983). Cytochemical detection of polysaccharides and the ultrastructure of the cell coat of zoospores of *Chytriomyces aureus* and *Chytriomyces hyalinus*. *Mycologia*. 75:209-220.

Eilers, U., J. Klumperman, and H-P. Hauri. (1989). Nocodazole, a microtubule-active drug, interferes with apical protein delivery in cultured intestinal epithelial cells. (Caco-2). *J. Cell Biol.* 108:13-22.

Ellinger, A., and M. Pavelka. (1984). Effects of monensin on the Golgi apparatus of absorptive cells in small intestine of rat. Morphological and cytochemical study. *Cell Tissue Res.* 235:187-194.

Englander, L., and W. Turbitt. (1979). Increased chlamydospore production by *Phytophthora cinnamomi* using sterols and near-ultraviolet light. *Phytopathology*. 69:813-817.

Estrada-Garcia, M. T., J. A. Callow, and J. R. Green. (1990). Monoclonal antibodies to the adhesive cell coat secreted by *Pythium aphanidermatum* zoospores recognise 200 x 103 Mr glycoproteins stored within large peripheral vesicles. *J. Cell Sci.* 95:199-206.

Farquhar, M. G. (1985). Progress in unraveling pathways of Golgi traffic. *Ann. Rev. Cell Biol.* 1:447-488.

Faye, L., and M. J. Chrispeels. (1987). Transport and processing of the glycosylated precursor of concanavalin A in jackbean. *Planta*. 170:217-224.

Faye, L., and M. J. Chrispeels. (1989). Apparent inhibition of β-fructosidase secretion by tunicamycin may be explained by breakdown of the unglycosylated protein during secretion. *Plant Physiol.* 89:845-851.

Fisher, J. M., W. Sossin, R. Newcomb, and R. H. Scheller. (1988). Multiple neuropeptides derived from a common precursor are differentially packaged and transported. *Cell*. 54:813-822.

Flanagan, M. D., and S. Lin. (1980). Cytochalasins block actin filament elongation by binding to high affinity sites associated with F-actin. *J. Biol. Chem*. 255:835-838.

Forscher, P., and S. J. Smith. (1988). Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone. *J. Cell Biol*. 107:1505-1516.

Frens, G. (1973). Controlled nucleation for the regulation of the particle size in monodisperse gold suspensions. *Nature, phys. Sci.* 241:20-22.

Fujiwara, T., K. Oda, S. Yokota, A. Takatsuki, and Y. Ikehara. (1988). Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. *J. Cell Chem.* 263:18545-18552.

Gay, J.L., and A.D. Greenwood. (1966). Structural aspects of zoospore production in *Saprolegnia ferax* with particular reference to the cell and vacuolar membranes. In: The Fungal Spore. M.F. Madelin, editor. Butterworths, London. 95-108.

Gerdes, H-H., P. Rosa, E. Phillips, P. A. Baeuerle, R. Frank, P. Argos, and W. B. Huttner. (1989). The primary structure of human secretogranin II, a widespread tyrosine-sulphated secretory granule protein that exhibits low pH - and calcium-induced aggregation. *J. Biol. Chem.* 264:12009-12015.

Gibson, R., S. Schlesinger, and S. Kornfeld. (1979). The nonglycosylated glycoprotein of vesicular stomatitus virus is temperature sensitive and undergoes intracellular aggregation at elevated temperatures. *J. Biol. Chem.* 254:3600-3607.

Gotelli, D. (1974). The morphology of Lagenidium callinectes. II. Sporogenesis. *Mycologia.* 66:846-858.

Gotelli, D., and L. C. Hanson. (1987). An ultrastructural investigation of the zoospore of *Sapromyces androgynus,* (oomycetes, Leptomitales). *Mycologia.* 79:745-752.

Grief, C., and P. J. Shaw. (1987). Assembly of cell-wall glycoproteins of *Chlamydomonas reinhardii* oligosaccharides are added in medial and trans Golgi compartments. *Planta.* 171:302-312.

Griffiths, G., P. Quinn, and G. Warren. (1983). Dissection of the Golgi complex. I. Monensin inhibits the transport of viral proteins from medial to trans Golgi cisternae in baby hamster kidney cells infected with Semliki Forest Virus. *J. Cell Biol.* 96:835-850.

Griffiths, G., and K. Simons. (1986). The *trans* Golgi network sorting at the exit site of the Golgi complex. *Science.* 234:430-443.

Griffiths, G., B. Hoflack, K. Simons, I. Mellman, and S. Kornfeld. (1988). The mannose 6-phosphate receptor and the biogenesis of lysosomes. *Cell.* 52:329-341.

Grolig, F., R. E. Williamson, J. Parke, C. Miller, and B. H. Anderton. (1988). Myosin and Ca^{2+}-sensitive streaming in the alga Chara detection of two polypeptides reacting with a monoclonal anti-myosin and their localization in the streaming endoplasm. *Eur. J. Cell Biol.* 47:22-31.

Grove, S.N. (1970). Fine structure of zoospore encystment and germination in *Pythium aphanidermatum. Amer. J. Bot.* 57:745-746.

Grove, S. N., and C. E. Bracker. (1978). Protoplasmic changes during zoospore encystment and cyst germination in *Pythium aphanidermatum. Expt. Mycol.* 2:51-98.

Gubler, F., J. V. Jacobsen, and A. E. Ashford. (1986). Involvement of the Golgi apparatus in the secretion of α– amylase from gibberelin-treated barley aleurone cells. *Planta.* 168:447-452.

Gubler, F., and A. R. Hardham. (1988). Secretion of adhesive material during encystment of *Phytophthora cinnamomi* zoospores, characterized by immunogold labelling with monoclonal antibodies to components of peripheral vesicles. *J. Cell Sci.* 90:225-235.

Gubler, F., and A. R. Hardham. (1990). Protein storage in large peripheral vesicles in *Phytophthora* zoospores and its breakdown after cyst germination. *Expt. Mycol.* 14:393-404.

Gubler, F., and A.R. Hardham. (1991). The fate of peripheral vesicles in zoospores of *Phytophthora cinnamomi* during infection of plants. In: Electron Microscopy of Plant Pathogens. K. Mendgen and D.-E. Lesemann, editors. Springer-Verlag, Berlin. 197-210.

Gunderson, J. H., H. Elwood, A. Ingold, K. Kindle, and M. L. Sogin. (1987). Phylogenetic relationships between chlorophytes, chrysophytes, and oomycetes. *Proc. Nat. Acad. Sci. USA.* 84:5823-5827.

Gwynne, D. I., and B. P. Brandhorst. (1982). Changes in gene expression during sporangium formation in *Achlya ambisexualis*. *Dev. Biol.* 91:263-277.

Hardham, A. R., E. Suzaki, and J. L. Perkin. (1986). Monoclonal antibodies to isolate-, species-, and genus-specific components on the surface of zoospores and cysts of the fungus *Phytophthora cinnamomi*. *Can J. Bot.* 64:311-321.

Hardham, A. R. (1987). Ultrastructure and serial section reconstruction of zoospores of the fungus *Phytophthora cinnamomi*. *Expt. Mycol.* 11:297-306.

Hardham, A. R. (1989). Lectin and antibody labelling of surface components of spores of *Phytophthora cinnamomi*. *Aust. J. Plant Physiol.* 16:19-32.

Hardham, A. R., and F. Gubler. (1990). Polarity of attachment of zoospores of a root pathogen and pre-alignment of the emerging germ tube. *Cell Biol. Int. Rep.* 14:947-956.

Hardham, A.R., F. Gubler, and J. Duniec. (1991a). Ultrastructural and immunological studies of zoospores of *Phytophthora*. In: Phytophthora. J.A. Lucas, R.C. Shattock, D.S. Shaw, and L.R. Cooke, editors. Cambridge University Press, Cambridge. 50-69.

Hardham, A. R., F. Gubler, J. Duniec, and J. Elliott. (1991b). A review of methods for the production and use of monoclonal antibodies to study zoosporic plant pathogens. *J. Microsc.* 162:305-318.

Harold, R. L., and F. M. Harold. (1986). Ionophores and cytochalasins modulate branching in *Achlya bisexualis*. *J. Gen. Microbiol.* 132:213-219.

Hashimoto, S., G. Fumagalli, A. Zanini, and J. Meldolesi. (1987). Sorting of three secretory proteins to distinct secretory granules in acidotrophilic cells of cow anterior pituitary. *J. Cell Biol.* 105:1579-1586.

Hayden, J. H., R. D. Allen, and R. D. Goldman. (1983). Cytoplasmic transport in keratocytes direct visualization of particle translocation along microtubules. *Cell Motility*. 3:1-19.

Heath, I. B., and A. D. Greenwood. (1970a). Wall formation in the Saprolegniales. II. Formation of cysts by the zoospores of *Saprolegnia* and *Dictyuchus*. *Arch. Mikrobiol.* 75:67-79.

Heath, I. B., and A. D. Greenwood. (1970b). Centriole replication and nuclear division in *Saprolegnia*. *J. Gen. Microbiol.* 62:139-148.

Heath, I. B., and A. D. Greenwood. (1971). Ultrastructural observations on the kinetosomes and Golgi bodies during the asexual life cycle of *Saprolegnia*. *Z. Zellforsch.* 11:2371-2389.

Heath, I. B. (1988). Evidence against a direct role for cortical actin arrays in saltatory organelle motility in hyphae of the fungus *Saprolegnia ferax*. *J. Cell Sci.* 91:41-47.

Heath, I. B., and S. G. W. Kaminskyj. (1989). The organization of tip-growth-related organelles and microtubules revealed by quantitative analysis of freeze-substituted oomycete hyphae. *J. Cell Sci.* 93:41-52.

Heath, I. B., and R. Harold. (1992). Actin has multiple roles in the formation and architecture of zoospores of the oomycetes, *Saprolegnia ferax* and *Achlya bisexualis. J. Cell Sci.*102:611-627.

Hemmes, D. E., and H. R. Hohl. (1971). Ultrastructural aspects of encystation and cyst-germination in *Phytophthora parasitica. J. Cell Sci.* 9:175-191.

Hemmes, D. E., and L. D. S. Wong. (1975). Ultrastructure of chlamydospores of *Phytophthora cinnamomi* during development and germination. *Can. J. Bot.* 53:2945-2957.

Hemmes, D.E. (1983). Cytology of *Phytophthora.* In: Phytophthora. Its Biology, Taxonomy, Ecology, and Pathology. D.C. Erwin, S. Bartnicki-Garcia, and P.H. Tsao, editors. American Phytopathological Society, St Paul, Minnesota. 9-40.

Herr, F. B., and M. C. Heath. (1982). The effects of antimicrotubule agents on organelle positioning in the cowpea rust fungus, *Uromyces phaseoli* var. *vignae. Expt. Mycol.* 6:15-24.

Heslop-Harrison, J.,Y. Heslop-Harrison, M. Cresti, A. Tiezzi, and A. Moscatelli. (1988). Cytoskeletal elements, cell shaping and movement in the angiosperm pollen tube. *J. Cell Sci.* 91:49-60.

Heslop-Harrison, J., and Y. Heslop-Harrison. (1989). Conformation and movement of the vegetative nucleus of the angiosperm pollen tube: association with the actin cytoskeleton. *J. Cell Sci.* 93:299-308.

Hibberd, D.J. (1970). Observations on the cytology and ultrastructure of *Ochromonas tuberculatus* sp. Nov. (Chrysophyceae), with special reference to the discobolocysts. *Brit. Phycol. J.* 5:119-143.

Ho, H. H., K. Zachariah, and C. J. Hickman. (1968). The ultrastructure of zoospores of *Phytophthora megasperma* var. *sojae. Can. J. Bot.* 46:37-41.

Hoch, H. C., and J. E. Mitchell. (1972a). The ultrastructure of *Aphanomyces euteiches* during asexual spore formation. *Phytopathology.* 62:149-160.

Hoch, H. C., and J. E. Mitchell. (1972b). The ultrastructure of zoospores of *Aphanomyces euteiches* and of their encystment and subsequent germination. *Protoplasma.* 75:113-138.

Hoch, H. C., B. E. Tucker, and R. C. Staples. (1987). An intact microtubule cytoskeleton is necessary for mediation of the signal for cell differentiation in *Uromyces. Eur. J. Cell Biol.* 45:209-218.

Holloway, S. A., and I. B. Heath. (1977). An ultrastructural analysis of the changes in organelle arrangement and structure between the various spore types of *Saprolegnia. Can. J. Bot.* 55:1328-1339.

Howard, R. J., and Aist, J. R. (1980). Cytoplasmic microtubules and fungal morphogenesis ultrastructural effects of methyl benzimdazole-2-ylcarbamate determined by freeze-substitution of hyphal tip cells. *J. Cell Biol.* 87:55-64.

Howlett, B. (1989). An electrophoretic karyotype for *Phytophthora megasperma. Expt. Mycol.* 13:199-202.

Huang, B., and R. C. Staples. (1982). Synthesis of proteins during differentiation of the bean rust fungus. *Expt. Mycol.* 6:7-14.

Huffaker, T. C., J. H. Thomas, and D. Botstein. (1988). Diverse effects of β-tubulin mutations on microtubule formation and function. *J. Cell Biol.* 106:1997-2010.

Huttner, W. B., and S. A. Tooze. (1989). Biosynthetic protein transport in the secretory pathway. *Curr. Opin. in Cell Biol.* 1:648-654.

Hyde, G. J., F. Gubler, and A. R. Hardham. (1991a). Ultrastructure of zoosporogenesis in *Phytophthora cinnamomi. Mycol. Res.* 95:577-591.

Hyde, G. J., S. Lancelle, P. K. Hepler, and A. R. Hardham. (1991b). Freeze substitution reveals a new model for sporangial cleavage in *Phytophthora*, a result with implications for cytokinesis in other eukaryotes. *J. Cell Sci.* 100:735-748.

Hyde, G. J. (1992). The structural basis of zoospore formation in *Phytophthora cinnamomi.* PhD thesis, Australian National University, Canberra, Australia.

Hyde, G. J., and A. R. Hardham. (1992). Confocal microscopy of microtubule arrays in cryosectioned sporangia of *Phytophthora cinnamomi. Expt. Mycol.* 16:207-218.

Jones, R. L., and D. G. Robinson. (1989). Protein secretion in plants. *New. Phytol.* 111:567-597.

Karecla, P. I., and T. E. Kreis. (1992). Interaction of membranes of the Golgi complex with microtubules in vitro. *Eur. J. Cell Biol.* 57:139-146.

Kelly, R. B. (1985). Pathways of protein secretion in eucaryotes. *Science.* 230:25-32.

Klausner, R. D., J. G. Donaldson, and J. C. Lippincott-Schwartz. (1992). Brefeldin A: Insights into the control of membrane traffic and organelle structure. *J. Cell Biol.* 116:1071-1080.

Kobori, H., M. Sato, and M. Osumi. (1992). Relationship of actin organization to growth in the two forms of the dimorphic yeast *Candida tropicalis. Protoplasma.* 167:193-204.

Kohno, T., and T. Shimmen. (1988). Accelerated sliding of pollen tube organelles along characeae actin bundles regulated by Ca^{+}. *J. Cell. Biol.* 106:1539-1543.

Koonce, M. P., and M. Schliwa. (1986). Reactivation of organelle movements along the cytoskeletal framework of a giant freshwater amoeba. *J Cell. Biol.* 103:605-612.

Kornfeld, S. and I. Mellman. (1989). The biogenesis of lysosomes. *Ann. Rev. Cell Biol.* 5:483-525.

Kreis, T. E., R. Matteoni, M. Hollinshead, and J. Tooze. (1989). Secretory granules and endosomes show saltatory movement biased to the anterograde and retrograde directions, respectively, along microtubules in AtT20 cells. *Eur. J. Cell Biol.* 49:128-139.

Kreis, T. E. (1990). Role of microtubules in the organisation of the Golgi apparatus. *Cell Motil. Cytoskel.* 15:67-70.

Kropf, D. L., S. K. Berge, and R. S. Quatrano. (1989). Actin localisation during *Fucus* embryogenesis. *Plant Cell.* 1:191-200.

Leadbeater, B. S. C. (1969). A fine structural study of *Olisthodiscus luteus* Carter. *Br. J. Phycol.* 4:3-17.

Ledger, P. W., N. Uchida, and M. L. Tanzer. (1980). Immunocytochemical localization of procollagen and fibronectin in human fibroplasts effects of the monovalent ionophore, monensin. *J. Cell Biol.* 87:663-671.

Lehnen, L. P. Jr., and M. J. Powell. (1988). Cytochemical localization of carbohydrates in zoospores of *Saprolegnia ferax. Mycologia.* 80:423-432.

Lehnen, L. P. Jr. and M. J. Powell. (1989). The role of kinetosome-associated organelles in the attachment of encysting secondary zoospores of *Saprolegnia ferax* to substrates. *Protoplasma.* 149:163-174.

Lehnen, L. P. Jr. and M. J. Powell. (1991). Formation of K-2 bodies in primary cysts of *Saprolegnia ferax. Mycologia.* 83:163-179.

Lending, C.R., R. S. Chesnut, K. L. Shaw, and B.A. Larkins. (1989). Immunolocalization of avenin and globulin storage proteins in developing endosperm of *Avena sativa* L. *Planta.* 178:315-324.

Lippincott-Schwartz, J., C. Yuan, J. S. Bonifacino, and R. D. Klausner. (1989). Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A evidence for membrane cycling from Golgi to ER. *Cell.* 56:801-813.

Lippincott-Schwartz, J., J. G. Donaldson, A. Schweizer, E. G. Berger, H. P. Hauri, L. C. Yuan, and R. D. Klausner. (1990). Microtubule-dependent retrograde transport of proteins into the ER in the presence of brefeldin A suggests an ER recycling pathway. *Cell.* 60:821-836.

Lodi, W. R., and D. R. Sonneborn. (1974). Protein degradation and protease activity during the life cycle of *Blastocladiella emersonii. J. Bacteriol.* 117:1035-1042.

Lunney, C. Z., and C. E. Bland. (1976). An ultrastructural study of zoosporogenesis in *Pythium proliferum* de Bary. *Protoplasma.* 88:85-100.

Machamer, C. E., and J. K. Rose. (1988). Vesicular stomatitus virus G proteins with altered glycosylation sites display temperature-sensitive intracellular transport and are subject to aberrant intermolecular disulfide bonding. *J. Biol. Chem.* 263:5955-5960.

Mauch, F., and L. A. Staehelin. (1989). Functional implications of the subcellular localisation of ethylene-induced chitinase and β-1,3 glucanase in bean leaves. *Plant Cell.* 1:447-457.

McCurdy, D. W., and L. H. Pratt. (1986). Immunogold electron microscopy of phytochrome in Avena identification of intracellular sites responsible for phytochrome sequestering and enhanced pelletability. *J. Cell Biol.* 103:2541-2550.

McFadden, G. I., H. R. Preisig, and M. Melkonian. (1986). Golgi apparatus activity and membrane flow during scale biogenesis in the green flagellate *Scherffelia dubia* (Prasinophyceae). II. Cell wall secretion and assembly. *Protoplasma.* 131:174-184.

McKerracher, L. J., and I. B. Heath. (1985). Microtubules around migrating nuclei in conventionally fixed and freeze-substituted cells. *Protoplasma* 125:162-172.

McKerracher, L. J., and I. B. Heath. (1986). Polarized cytoplasmic movement and inhibition of saltations induced by calcium-mediated effects of microbeams in fungal hyphae. *Cell Motil. Cytoskel.* 6:136-145.

McKerracher, L. J., and I. B. Heath. (1987). Cytoplasmic migration and intracellular organelle movements during tip growth of fungal hyphae. *Expt. Mycol.* 11:79-100.

Mills, G. L., and E. C. Cantino. (1981). Chitosome-like vesicles from gamma-particles of *Blastocladiella emersonii* synthesize chitin. *Arch. Mikrobiol.* 130:72-77.

Mircetich, S. M., and G. A. Zentmyer. (1966). Production of oospores and chlamydospores of *Phytophthora cinnamomi* in roots and soil. *Phytopathology.* 56:1076-1078.

Misumi, Y., Y. Misumi, K. Miki, A. Takatsuki, G. Tamura, and Y. Ikehara. (1986). Novel blockade by brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes. *J. Biol. Chem.* 261:11398-11403.

Mollenhauer, H. M., D. J. Morre, and J. O. Norman. (1982). Ultrastructural observations of maize root tips following exposure to monensin. *Protoplasma.* 112:117-126.

Mollenhauer, H. M., D. J. Morre, and R. Droleskey. (1983). Monensin affects the trans half of *Euglena* dictyosomes. *Protoplasma.* 114:119-124.

Mollenhauer, H. M., D. J. Morre, and C. E. Bracker. (1988). Swelling of Golgi apparatus cisternae in monensin-treated tissues is modulated by fixation conditions. *Protoplasma.* 145:66-69.

Mollenhauer, H. M., D. J. Morre, and L. D. Rowe. (1990). Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity. *Biochim. et Biophysica Acta.* 1031:225-246.

Moore, P.J., K. M. M. Swords, M. A. Lynch, and L. A. Staehelin. (1991). Spatial reorganisation of the assembly pathways of glycoproteins and complex polysaccharides in the Golgi apparatus of plants. *J. Cell Biol.* 112:589-602.

Morre, D. J., W. F. Boss, H. Grimes, and H. H. Mollenhauer. (1983). Kinetics of Golgi apparatus membrane flux following monensin treatment of embryogenic carrot cells. *Eur. J. Cell Biol.* 30:25-32.

Morre, D. J., H. H. Mollenhauer, H. Spring, M. Trendelenberg, M. Montag, B. A. Mollenhauer, and D. M. Morre. (1992). Swelling of Golgi apparatus of bovine mammary epithelial cells in response to monensin treatment requires fixation. *Eur. J. Cell Biol.* 57:321-324.

Murata, T., A. Kadota, T. Hogetsu, and M. Wada. (1987). Circular arrangement of cortical microtubules around the subapical part of a tip-growing fern protonema. *Protoplasma.* 141:135-138.

Nasrallah, J. B., and A. M. Srb. (1977). Occurrence of a major protein associated with fruiting body development in *Neurospora* and related Ascomycetes. *Proc. Nat. Acad. Sci. USA.* 74:3831-3834.

Nasrallah, J. B., and A. M. Srb. (1978). Immunofluorescent localization of a phase-specific protein in *Neurospora tetrasperma* perithecia. *Exp. Mycol.* 2:211-215.

Olden, K., J. B. Parent, and S. L. White. (1982). Carbohydrate moieties of glycoproteins a re-evaluation of their function. *Biochim. et Biophys. Acta.* 650:209-232.

Orci, L., P. Halban, M. Amherdt, M. Ravazzolla, J. D. Vassalli, and A. Perrelet. (1984). A clathrin-coated, Golgi related compartment of the insulin secreting cell accumulated proinsulin in the presence of monensin. *Cell.* 39:39-47.

Orci, L., M. Ravazzolla, M. Amherdt, A. Perrelet, S. K. Powell, D. L. Quinn, and H-P. H. Moore. (1987). The *trans*-most cisternae of the Golgi complex a compartment for sorting of secretory and plasma membrane proteins. *Cell.* 51:1039-1051.

Overton, S. V., T. P. Tharp, and C. E. Bland. (1983). Fine structure of swimming, encysting and germinating spores of *Haliphthoros milfordensis. Can. J. Bot.* 61:1165-1177.

Pfeffer, S. R., and J. E. Rothman. (1987). Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. *Ann. Rev. Biochem.* 56:829-852.

Philippi, M.L., R. W. Parish, and H. R. Hohl. (1975). Histochemical and biochemical evidence for the presence of microbodies in *Phytophthora palmivora. Arch Mikrobiol.* 103:127-132.

Picton, J. M., and M. W. Steer. (1981). Determination of secretory vesicle production rates by dictyosomes in pollen tubes of Tradescantia using cytochalasin D. *J. Cell Sci.* 49:261-272.

Pierson, E. S., J. Derksen, and J. A. Traas. (1986). Organization of microfilaments and microtubules in pollen tubes grown in vitro or in vivo in various angiosperms. *Eur. J. Cell Biol.* 41:14-18.

Pinto da Silva, P., and L. M. Nogueira. (1977). Membrane fusion during secretion. A hypothesis based on electron microscope observation of *Phytophthora palmivora* zoospores during encystment. *J. Cell Biol.* 73:161-181.

Powell, M. J., L. P. Lehnen, Jr., and R. N. Bortnick. (1985). Microbody-like organelles as taxonomic markers among oomycetes. *Biosystems.* 18:331-334.

Powell, M. J., and C. E. Bracker. (1986). Distribution of the diaminobenzidene reaction products in zoospores of *Phytophthora palmivora. Mycologia.* 78:892-900.

Pueschel, C. M., and J. P. van der Meer. (1985). Ultrastructure of the fungus *Petersenia palmariae* (oomycetes) parastic on the alga *Palmaria mollis* (Rhodophyceae). *Can. J. Bot.* 63:409-418.

Quader, H., W. Herth, U. Ryser and E. Schnepf. (1987). Cytoskeletal elements in cotton seed hair development *in vitro* their possible regulatory role in cell wall organisation. *Protoplasma.* 137:56-62.

Reichle, R. E. (1969). Fine structure of *Phytophthora parasitica* zoospores. *Mycologia.* 61:30-51.

Rennison, M. E., S. E. Handel, C. J. Wilde, and R. D. Burgoyne. (1992). Investigation of the role of microtubules in protein secretion from lactating mouse mammary epithelial cells. *J. Cell Sci.* 102:239-247.

Rivas, R. J., and H-P. H. Moore. (1989). Spatial segregation of the regulated and constitutive secretory pathways. *J. Cell Biol.* 109:51-60.

Robb, J., and B. Lee. (1986). Developmental sequence of the attack apparatus of *Haptoglossa mirabilis*. *Protoplasma.* 135:102-111.

Robinson, D. G. (1981). The ionic sensitivity of secretion-associated organelles in root cap cells of maize. *Eur. J. Cell Biol.* 23:267-272.

Rogalski, A. A., J. E. Bergmann, and S. J. Singer. (1984). Effect of microtubule assembly status on the intracellular processing and surface expression of an integral protein of the plasma membrane. *J. Cell Biol.* 99:1101-1109.

Rose, D. K., and R. W. Doms. (1988). Regulation of protein export from the endoplasmic. *Annu. Rev. Cell Biol.* 4:257-288.

Runeberg, P., M. Raudaskoski, and I. Virtanen. (1986). Cytoskeletal elements in the hyphae of the Homobasidiomycete *Schizophyllum commune* visualized with indirect immunofluorescence and NBP-phallacidin. *Eur. J. Cell Biol.* 41:25-32.

Salas, P. J. I., D. E. Misek, D. E. Vega-Salas, D. Gundersen, M. Cereijido, and E. Rodriguez-Boulan. (1986). Microtubules and actin filaments are not critically involved in the biogenesis of epithelial cell surface polarity. *J. Cell Biol.* 102:1853-1867.

Salo, V., S. S. Niini, I. Virtanen, and M. Raudaskoski. (1989). Comparative immunocytochemistry of the cytoskeleton in filamentous fungi with dikaryotic and multinucleate hyphae. *J. Cell Sci.* 94:11-24.

Saraste, J. E., and E. Kuismanen. (1984). Pre- and post-Golgi vacuoles operate in the transport of Semliki forest virus membrane glycoproteins to the cell surface. *Cell.* 38:535-549.

Satiat-Jeunemaitre B., and C. Hawes. (1992). Reversible dissociation of the plant Golgi apparatus by brefeldin A. *Biol. Cell.* 74:325-328.

Schnapp, B. J., R. D. Vale, N. P. Sheetz, and T. S. Reese. (1985). Single microtubules from squid axon support bidirectional movement of organelles. *Cell.* 40:455-462.

Schnepf, E., G. Deichgraber, and G. Drebes. (1978a). Development and ultrastructure of the marine, parasitic Oomycete, *Lagenisma coscinodisci* Drebes (Lagenidiales): Formation of the primary zoospores and their release. *Protoplasma.* 94:263-280.

Schnepf, E., G. Deichgraber, and G. Drebes. (1978b). Development and ultrastructure of the marine parasitic oomycete, *Lagenisma coscinodisci* (Lagenidiales): encystment of primary zoospores. *Can. J. Bot.* 56:1309-1314.

Schweizer, A., J. A. M. Fransen, K. Matter, T. E. Kreis, L. Ginsel, and H-P. Hauri. (1990). Identification of an intermediate compartment involved in protein transport from endoplasmic reticulum to Golgi apparatus. *Eur. J. Cell Biology.* 53:185-196.

Sewall, T., L. Olson, L. Lange, and J. Pommerville. (1986). The effect of monensin on gametogenesis and zoosporogenesis in the aquatic fungus, *Allomyces macrogynus. Protoplasma.* 133:129-139.

Shannon, T. M., J. M. Picton, and M. W. Steer. (1984). The inhibition of dictyosome vesicle formation in higher plant cells by cytochalasin D. *Eur. J. Cell Biol.* 33:144-147.

Simon, R., Y. Altschuler, R. Rubin, and G, Galili. (1990). Two closely related wheat storage proteins follow a markedly different subcellular route in *Xenopus laevis* oocytes. *Proc. Nat. Acad. Sci. USA.* 88:834-838.

Sing, V. O., and S. Bartnicki-Garcia. (1975a). Adhesion of *Phytophthora palmivora* zoospores electron microscopy of cell attachment and cyst wall fibril formation. *J. Cell Sci.* 18:123-132.

Sing, V. O., and S. Bartnicki-Garcia. (1975b). Adhesion of *Phytophthora palmivora* zoospores detection and ultrastructural visualization of concanavalin A receptor sites appearing during encystment. *J. Cell Sci.* 19:11-20.

Slot, J. W. and H. J. Geuze. (1985). A new method of preparing gold probes for multiple-labeling cytochemistry. *Eur. J. Cell Biol.* 38:87-93.

Sokal, R. R., and F. J. Rohlf. (1981). Biometry: The principles and practice of statistics in biological research. 2nd Edition. W.H. Freeman and Co. New York.

Sonnewald, U., D. Studer, M. Rocha-Sosa, and L. Willmitzer. (1989). Immunocytochemical localization of patatin, the major glycoprotein in potato (*Solanum tuberosum* L.). *Planta.* 178:176-183.

Sossin, W. S., J. M. Fisher, and R. H. Scheller. (1990). Sorting within the regulated secretory pathway occurs in the *trans*-Golgi network. *J. Cell Biol.* 110:1-12.

Springer, M. L., K. M. Hager, C. Garrett-Engele, and C. Yanofsky. (1992). Timing of synthesis and cellular localization of two conidiation-specific proteins of *Neurospora crassa. Dev. Biol.* 152:255-262.

Staehelin, L.A., T.H. Giddings, S. Levy, M.A. Lynch, P.J. Moore, and K.M.M. Swords. (1991). Organisation of the secretory pathway of cell wall glycoproteins and complex polysaccharides in plant cells. In: Endocytosis, Exocytosis and Vesicle Traffic in Plants. C.R. Hawes, J.O.D. Coleman, and D.E. Evans, editors. Cambridge University Press, Cambridge. 183-198.

Staples, R. C., A. A. App, and P. Ricci. (1975). DNA synthesis and nuclear division during formation of infection structures by bean rust uredospore germlings. *Arch. Microbiol.* 104:123-127.

Staples, R. C., and H. C. Hoch. (1982). A possible role for microtubules and microfilaments in the induction of nuclear division in bean rust uredospore germlings. *Expt. Mycol.* 6:293-302.

Staswick, P. E. (1990). Novel regulation of vegetative storage protein genes. *Plant Cell.* 2:1-6.

Strous, G. J. A. M., R. Willemsen, P. van Kerkhof, J. W. Slot, H. J. Geuze, and H. F. Lodish. (1983). Vesicular stomatitus virus glycoprotein, albumin, and transferrin are transported to the cell surface via the same Golgi vesicles. *J. Cell Biol.* 97:1815-1822.

Struck, D. K., and W. J. Lennarz. (1977). Evidence for the participation of saccharide-lipids in the synthesis of the oligosaccharide chain of ovalbumin. *J. Biol. Chem.* 252:1007-1013.

Takatsuki, A., and G. Tamura. (1985). Brefeldin A, a specific inhibitor of intracellular translocation of vesicular stomatitus virus G protein: intracellular accumulation of high mannose type G protein and inhibition of its cell surface expression. *Agric. Biol. Chem.* 49:899-902. cited in Misumi *et al.* (1986).

Tamura, G., K. Ando, S. Suzuki, A. Takatsuki, and K. Arima. (1968). Antiviral activity of brefeldin A and Verrucarin A. *J. Antibiotics.* 21:160-161. cited in Klausner *et al.* (1992).

Tartakoff, A. M. (1983). Perturbation of vesicular traffic with the carboxylic ionophore monensin. *Cell.* 32:1026-1028.

Taylor, J. W., and M. S. Fuller. (1981). The Golgi apparatus, zoosporogenesis, and development of the zoospore discharge apparatus of *Chytridium confervae. Expt. Mycol.* 5:35-59.

Tooze, J., and B. Burke. (1987). Accumulation of adrenocorticotropin secretory granules in the midbody of teleophase AtT20 cells evidence that secretory granules move anterogradely along microtubules. *J. Cell Biol.* 104:1047-1057.

Tooze, J., S.A. Tooze, and S. D. Fuller. (1987). Sorting of progeny coronavirus from condensed secretory proteins at the exit from the *trans*-Golgi network of AtT20 cells. *J. Cell Biol.* 105:1215-1226.

Traas, J. A., P. Braat, A. M. C. Emons, H. Meekes, and J. Derksen. (1985). Microtubules in root hairs. *J. Cell Sci.* 76:303-320.

Truesdell, L. C., and E. C. Cantino. (1970). Decay of gamma particles on germinating zoospores of *Blastocladiella emmesonii. Arch. Mikrobiol.* 70:378-392.

van Etten, J. L., S. N. Freer, and McCune. (1979). Presence of a major (storage?) protein in dormant spores of the fungus *Botryodiplodia theobromae. J. Bact.* 138:650-652.

Virtanen, I., and T. Vartio. (1986). Microtubule disruption does not prevent intracellular transport and secretory processes of cultured fibroplasts. *Eur. J. Cell Biol.* 42:281-287.

von Figura, K., and A. Hasilik. (1986). Lysosomal enzymes and their receptors. *Annu. Rev. Biochem.* 55:167-193.

von Zastrow, M., and J. D. Castle. (1987). Protein sorting among two distinct export pathways occurs from the content of maturing exocrine storage granules. *J. Cell Biol.* 105:2675-2684.

Weete, J. D., A. El Mougith, and J.-M. Touze-Soulet. (1989). Inhibition of growth, lipid, and sterol biosynthesis by monensin in fungi. *Expt. Mycol.* 13:85-94

Weste, G. (1983). Population dynamics and survival of *Phytophthora.* In: Phytophthora. Its Biology, Taxonomy, Ecology, and Pathology. D.C. Erwin, S. Bartnicki-Garcia, and P. Tsao, editors. American Phytopathological Society, St Paul Minnesota. 237-257.

Weste, G., and G. C. Marks. (1987). The biology of *Phytophthora cinnamomi* in Australasian forests. *Annu. Rev. Phytopath.* 25:207-229.

Wetzel, S., C. Demmers, and J. S. Greenwood. (1989). Seasonally fluctuating bark proteins are a potential form of nitrogen in three temperate hardwoods. *Planta.* 178:275-281.

Williams, W. T., and R. K. Webster. (1970). Electron microscopy of the sporangium of *Phytophthora capsici. Can. J. Bot.* 48:221-227.

Yahara, I., F. Harada, S. Sekita, K. Yoshihira, and S. Natori. (1982). Correlation between effects of 24 different cytochalasins on cellular structures and cellular events and those on actin in vitro. *J. Cell Biol.* 92:69-78.

Zentmyer, G. A. (1980). *Phytophthora cinnamomi* and the Diseases it Causes. The American Phytopathological Society, St Paul, Minnesota. 1 pp.