

CHAPTER 13. Ultrastructure: the Vegetative System

In spite of penetrating stains and brightfield optics of considerable resolution, definite limits of observation inevitably were reached in traditional cytology. Although the light microscope revealed basal bodies in the spores of water molds, how did they anchor successfully against the forceful beating of flagella? While the best of optical glass could reveal bivalent rings in presumed meiotic figures, were these truly bivalents, and how did they separate and migrate? These are just two of many questions that cytology with the light microscope could not resolve. At hand was the electron microscope, capable of further resolution and presumably able to suggest answers to these questions that had been so tantalizingly just out of reach. This is not to say that all traditional cytological work on the Saprolegniaceae was untrustworthy; it is to say that it had attained an inviolable limit dictated by certain physical principles of light.

The first water mold to be subjected to the preparative treatment necessary for electron microscopy was *Saprolegnia ferax*, about which Manton and her colleagues published in 1951 and 1952. It was to be some 15 years later -- beginning about 1966 -- that biologists again applied electron microscopy to the water molds. In a very real sense, this delayed application of ultrastructural methodology to these fungi proved to be advantageous. The use of the electron beam just to discover organelles and to catalogue them had flared into prominence, and while such work had by no means halted abruptly, it was giving way to interpretive, comparative ultrastructure. Indeed, this approach is encouragingly prevalent among the existing accounts of the fine structure of water molds.

It should hardly be surprising that the burst of elegant electron images published in a short span between about 1966 and 1978 should be differently interpreted on occasion or that faults in preparation should creep into methodology. As we are not experienced investigators in ultrastructure, we cannot choose confidently between what is adequate preparation and what is not. Accordingly, we do not sort out the doubtful bits and pieces, but we do erect signs along the way pointing to differences of opinion or interpretation. Perhaps at some later date, the accumulated knowledge can be analyzed and positive interpretations brought forward.

Relatively few members of the Saprolegniaceae have been probed with the electron beam and their inner structures uncovered. Chiefly these have been *Achlya ambisexualis* [studied by Tontz (Ellzey)]; *A. bisexualis* (Dargent); *A. recurva* (Steffens), *Aphanomyces euteiches* (Hoch and Mitchell); *Saprolegnia ferax*, *Dictyuchus sterile*†, *Thraustotheca clavata* (Heath, Greenwood, and Gay); *S. furcata*† (Beakes, and Beakes and Gay), (Hagedorn and Weinert), and *S. terrestris* (Howard and Moore). At least some of these species mentioned are particularly thorough review papers that serve an additional purpose of acquainting the reader with the events and theories of interpretive ultrastructure: Bracker (1967), Beckett *et al.* (1974), Kubai (1975, 1978), Bartnicki-Garcia and Hemmes (1976), Fuller (1976), and Heath (1976).

METHODS

Perfectly good accounts of ultrastructural methodology exist in a number of general references. With few exceptions, the papers cited in this and the two succeeding chapters record essentially the same standard techniques. Briefly, the usual preparation consists of fixing specimens in glutaraldehyde (2.5 or 5% Na/K phosphate buffered) followed by post fixation in buffered osmium tetroxide. Alternatively, fixation can be done by OsO₄ or potassium permanganate (2%) alone. Following dehydration in an ethanol series, the specimens are embedded and sectioned. Staining is usually accomplished by 1 or 2% aqueous uranyl acetate, sometimes followed by lead citrate. For the detection of various enzymes or other compounds associated with organelles a wealth of cytochemical methods is in existence as, for example, periodic acid-thiocarbohydrazide-protein silver (Thiéry, 1967) or periodic acid silver hexamine (PASH) for the detection of polysaccharides. These reagents detect aldehydes in the polysaccharide chain following oxidation by periodic acid.

A method to prepare specimens of water molds for freeze-etching has been described by E. A. Ellis and Mullins (1975). They developed a procedure in which the material is slowly infiltrated (during agitation) with a cryoprotectant (distilled water, 25% glycerol, and 10% ethylene glycol). This method of gradual infiltration largely circumvents severe plasmolysis.

To position hyphae for ultrathin sectioning, Dargent (1977) grew mycelium in thin layers of a suitable agar medium between two collodion sheets. The entire preparation could be fixed, dehydrated, and sectioned.

SOMATIC HYPHAE

In their ultrastructure the somatic hyphae of water molds are basically like the cells of all other eukaryotes, having the usual organelles necessary for functioning at the subcellular level. The hyphae contain spherical or noticeably elongate nuclei (Hartog had perceived this as early as 1895), each delimited by an envelope consisting of two concentric membranes interrupted by characteristic nuclear pores. Dictyosomes¹ also are present, as are cylindrical to spherical mitochondria, endoplasmic reticulum with associated ribosomes, granules, lipid bodies, microtubular organelles, and various vesicles (Tontz, 1969: *Achlya ambisexualis*; Hagedorn and Weinert, 1971: *Saprolegnia monoica*[†]; Gay and Greenwood, 1966: *S. ferax*; Dargent, 1975, 1977, and Dargent *et al.*, 1973: *A. ambisexualis*).

According to Dargent (1977) the organelles in the hyphal apices of *Achlya*

¹Synonymous with Golgi bodies (apparatus). The term dictyosome is commonly encountered in mycological literature, but it does not enjoy universal acceptance among biologists. For the most part we use in our account the term employed in the specific paper cited; the designation "Golgi dictyosome" is redundant.

bisexualis are distributed in essentially four zones. At the extreme tip of the filament -- a segment 5-10 μm long -- vesicles are very abundant (see section following), and mitochondria are transported into the apex by cytoplasmic streaming. Dargent found three vesicle types in this summit region, namely, macrovesicles containing fibrils (or sometimes granules), and two types of microvesicles, one with a fibrillar interior and a second having an electron dense center. The second zone of the hyphal apex, extending some 10 μm behind the first, also shows vesicles, free ribosomes, and mitochondria (associated with a granular reticulum). Zone III, the nuclear region is likewise equipped with vesicles and also contains dictyosomes and a granular reticulum. A few vacuoles are visible in this region, but there are no lipid bodies. The vacuolar zone (IV), extending 40-100 μm behind the apex, is characterized by a sharply diminishing proportion of organelles in relation to the increased volume of the vacuoles. According to Dargent, then, the hypha of *A. bisexualis* has a definite polarity with respect to its organelles, and Heath *et al.*, (1971) demonstrated a similar organization in *Saprolegnia ferax*. Bracker and Grove (1971a) had reported that there was continuity between the mitochondrial surface and other endomembranes, but Dargent (1977) could not confirm this for *A. bisexualis*, and Heath (1976) did not observe such a configuration in the three water molds he examined.

Even with the limitations imposed by light microscopy and vital staining Guilliermond (Chapter 11) had noted that mitochondria in water mold hyphae (Fig. 18) exhibited a motion that was not connected with mere cytoplasmic streaming. More recent investigators, among them Heath (1976) and Heath and Greenwood (1970c), have also reported a similar kind of motility. Working with an unidentified *Achlya*, Bracker and Grove (1971b) found elongate particles arranged in two series of parallel columns along the surface of the mitochondrion. Since the core region of these particular fractions did not stain, Bracker and Grove postulated a tubular construction for them, and suggested that these might be involved in mitochondrial movement and pleomorphism. In 1970(c) Heath and Greenwood reported that in *Saprolegnia ferax* and *Thraustotheca clavata* (Heath, 1974a) microtubules, positionally coinciding with the long axis of the hypha, had mitochondria closely associated with them. They postulated that a force operating between the mitochondrion and the adjacent microtubules might be responsible for mitochondrial motility.

THE WALL AND VESICLES

The hyphal wall in species of *Saprolegnia* (Heath, 1976; a review, not original data) is constructed of three cross-linked components: fibrillar and amorphous polysaccharide, and protein. Wall thickness varies, but the outer surface shows loosely organized fibrils (osmiophilic ones occur internally as well), and polysaccharides can be detected chemically at the inner surface (Dargent, 1977). The chemistry of the wall (Sietsma *et al.*, 1969, and others) already has been discussed (Chapter 6).

One of the chief structural features of the hypha that has received abundant attention is the system of wall vesicles (Fig. 17). These organelles, apparently derived (Grove and Bracker, 1970; Heath, 1976; Heath *et al.*, 1971) from Golgi bodies are reported to fuse with the plasmalemma. It appears also that vesicles are involved in wall synthesis (see section on chemical ultrastructure), and contribute to the construction of new plasmalemma as the hyphal tip elongates (Hagedorn and Weinert, 1971, and others). Vesicles are bounded by a single membrane (Heath, 1976; Heath *et al.*, 1971) and particles similar to the ones found on the plasmalemma are present on the surface (Heath *et al.*, 1971). It has been suggested (McClure *et al.*, 1968), that the so-called "Spitzenkörper" in the hyphal tips of some fungi are aggregates of these membrane-bound vesicles. "Spitzenkörper" are reported by Hagedorn and Weinert (1971) in a *Saprolegnia*, but Grove *et al.* (1970) claimed that such elements are not found in Oomycetes.

Although it is logical to suppose from physical evidence that the fibrillar components of the hyphal walls of the saprolegniaceous fungi are assembled external to the plasmalemma, some polymerization may take place enroute.

For wall synthesis to occur, hods to carry the bricks, as it were, must be available. There is certainly abundant circumstantial (if not direct) evidence that wall vesicles may be those carriers, as is seen in vesicle fusion with the plasmalemma, and presence of these bodies only during the periods of active wall synthesis (Heath, 1976; Heath *et al.*, 1971).

There is good evidence in some organisms that there is an endomembrane system (Bracker and Grove, 1971a) involving a functional continuity between dictyosome and the endoplasmic reticulum. From a study of *Achlya bisexualis* and other fungi, Dargent (1977) postulated a similar spatial and functional continuum between the nuclear membrane and the plasmalemma (Fig. 19). The basic thread of his proposal with respect to the water molds is that the dictyosome is a transitional organelle to which the ER contributes from a smooth forming surface. In turn, the dictyosome (Gay and Greenwood, 1966; Heath and Greenwood, 1971) buds off at the margins of its cisternae vesicles that migrate to the plasmalemma and there fuse with that membrane. Heath and Greenwood (1971) and Heath (1976) have demonstrated convincingly that in *Saprolegnia ferax* hyphae, there can be tripartite associations in the endomembrane system. They found individual Golgi bodies associated with a cisternum of endoplasmic reticulum and a mitochondrion. When the visual evidence of organelle formation and continuity is combined with biochemical identity of compounds associated with those organelles, the conclusion that the vesicles function simultaneously in the formation of plasmalemma and assembly of the hyphal wall is a logical one. This biochemical evidence will be examined in a subsequent section.

MICROTUBULES

By the use of chemical agents known to disrupt microtubules, Heath (1975a, d) showed that the somatic and nuclear-associated microtubules of *Saprolegnia ferax* were

noticeably stable. Somatic tubules were resistant to colchicine, colcemid, *d*-camphor, griseofulvin, veratrine sulfate (alkaloids), and vinblastine sulfate, even when these were added to preparations at lethal levels or in amounts known to inhibit growth dramatically (hydrostatic pressure of 14,000 psi was disruptive). Thus, the chemical agents were not microtubule-specific in *S. ferax*. Membrane impermeability did not seem to be the cause of failure of the agents to act on the microtubules. Heath suggested that some non-tubulin compounds associated with the microtubules in this species, or with the intracellular environment surrounding them, might have conferred resistance. These are but hypotheses, however.

Papers by Heath (1975d) and Holloway and Heath (1977a) comment on the function of microtubules in the hyphae and spores of water molds. Within the hypha, extranuclear organelles (for example, wall vesicles) are associated with track-like aggregations of microtubules, and these latter structures have a major role in the events of nuclear division (*see* also, section on nuclear ultrastructure). In spores, microtubules are intimately associated with the flagellar rootlet system.

MICROBODIES AND LOMASOMES

Three types of microbodies have been recognized (Tolbert, 1973): those with a specific function in supplying such enzymes as catalase or uricase, nonspecific glyoxysomes (providing glyoxylate shunt enzymes), and leaf peroxisomes (sources of enzymes for photorespiration and glucanogenesis). In their study of *Achlya ambisexualis*, Chionski and Mullins (1977) found spherical or ovoid microbodies, 0.2-0.4 μm in diameter, associated with lipid accumulations, mitochondria, or rough ER. Another report of microbodies in the water molds is that by Heath (1976). He detected pleomorphic organelles similar to microbodies in *Thraustotheca clavata* and *Saprolegnia ferax*; these bodies contained tubular structures of an unknown nature.

Investigating the subcellular structure of hyphae, R. T. Moore and McAlear (1961) described "sponge-like intumescences" associated with the plasmalemma but external to it. They named these structures lomasomes. The paper by these two authors is highly speculative -- being the first report of lomasomes -- and later accounts (Heath and Green, 1970b, for example) supplant theirs. However, it is at least of historical interest to note that Moore and McAlear (1961: fig. 11) show (diagrammatically) the migration of a vesicle through the cytoplasmic matrix, fusing with the plasmalemma, and there opening toward the wall as it becomes a part of the membrane. This may well be viewed as an early report of what Dargent (1977) was to later postulate on the physical disposition of wall vesicles in water molds.

Bracker (1967) regarded the lomasomes as artifacts generated during specimen preparation, but others held that these were actually structural features. In a paper published in 1968 Marchant and Robards referred to paramutual bodies: any group of membrane-bound subcellular elements positioned between the plasmalemma and the cell wall. Two types existed, they believed. Lomasomes were structures in which the constitutive elements were derived from cytoplasmic membranes, transported across

the plasmalemma, and deposited external to it. Plasmalemmasomes were formed as foldings of the plasmalemma alone without any cytoplasmic contribution. Working with *Saprolegnia terrestris* Marchand and Moore (1973) found lomasome vesicles -- these appeared to be accumulations of ejected material -- in the hyphae. Hagedorn and Weinert (1971) also reported lomasomes in another species (*S. monoica*[†]) and attributed to these units a function in the synthesis of wall material. At the extreme apex (and subapically) of growing hyphae of *Achlya bisexualis* Dargent and associates (1973) detected lomasome vesicles in pockets between the plasmalemma and the hyphal wall. Dargent (1977) found lomasomes in the mycelium of this same *Achlya* but “preferentially”, in the subapical region of the hyphae.

The most definitive work to date on lomasomes is that of Heath and Greenwood (1970b) with *Saprolegnia ferax* and *Dictyuchus sterile*[†]. They defined organelles external to the membrane in very precise terms, and their observations are chronicled here in this light. A lomasome was defined as membranous, vesicular material external to the plasmalemma, and embedded in the confining wall (Figs. 20, 21). Plasmalemmasomes, Heath and Greenwood specified, are also membranous configurations external to the cytoplasmic membrane, but are often positioned in a pocket projecting into the cytoplasm and are not noticeably embedded in the wall (Fig. 20). They saw intergrades between these extremes as well. Lomasomes in *S. ferax* are present in hyphal apices (but not adjacent to older portions of the wall), at points along the lateral walls of developing and discharging sporangia, and in developing apical papillae of sporangia. These conglomerates also are found in the walls of encysted spores. In *D. sterile*[†], lomasomes are present in regions of active wall synthesis (Heath and Greenwood, 1970b), and in both this species and *S. ferax*, membranous lomasomes (of a special type) are associated with the basal septum of the sporangium. In hyphae of *D. sterile*[†] and *S. ferax* the plasmalemmasomes are located at points similar to those occupied by lomasomes. The former, however, are more variable in shape than the latter, being both tubular and vesicular. After proper staining, hyphae of these water molds exhibit areas of intense fluorescence in the walls, due, Heath and Greenwood thought, to membrane fragments in the wall itself. The possibility of fixation artifacts was ruled out.

Heath and Greenwood (1970b) attempted to explain the origin of the membrane conglomerates deposited external to the plasmalemma. There is, in the two species of water mold with which they worked, intense vesicular activity at the sites where lomasomes occur, and cell wall synthesis likely occurs at these points as well. These authors hypothesize that when more plasmalemma is formed by vesicles than is needed for hyphal growth plasmalemmasomes result. These subsequently are sequestered in the wall where they are then recognizable as lomasomes. The spores of *Saprolegnia ferax* and *Dictyuchus sterile*[†] do not increase in size as they encyst, hence they cannot accommodate additional plasmalemma (Heath and Greenwood, 1970b). Accordingly, lomasomes and plasmalemmasomes are evident in the spores of these species as well.

SOMATIC ULTRASTRUCTURE IN THE PATHOGENIC SPECIES

Subcellular features of hyphae of the parasitic species of *Aphanomyces* are only known in scant detail. The vegetative mycelium of *Aphanomyces euteiches* contains the usual organelles associated with eukaryotic cells (Shatla *et al.*, 1966; Hoch and Mitchell, 1972a). The ribosome-studded endoplasmic reticulum is uniformly distributed in the cytoplasm, and typical dictyosomes are present; some ribosomes are free in the cytoplasmic matrix. Shatla and his collaborators (1966) also reported that lomasomes are present in the hyphae of *A. euteiches*, and some bodies which they were unable to identify were later (Hoch and Mitchell, 1972a) assumed to be plasmalemmasomes. Microtubules also were found by Shatla and his associates, and theirs appears to be the first report of these structures in the pathogenic water molds. They described an amorphous layer of material lining the inner peripheral surface of the cytoplasm in vegetative hyphae of *A. euteiches*, but Hoch and Mitchell (1972a) later remarked that such a layer was not present along the inner wall surface of sporangia in this species.

In the hyphae of a nonsporulating strain of *Aphanomyces astaci* Heath and Unestam (1974) found membrane-bound bodies (some indented at various points) associated with a matrix of osmiophilic granular material. They were not certain that these "organelles" were mycoplasmas, although the structure of these inclusions was suggestive of such protoplasmic units.

SOMATIC NUCLEI

It is quite evident from the account of the cytology of water molds (Chapter 11) that nuclei have not been interpreted or described uniformly through the years, simply because classical methodology was inadequate for the task. Accordingly, the structure and behavior of the nucleus in these fungi remained obscure until the electron microscope was employed as an analytical tool. Almost all of what is known of the fine structure of the nuclei in vegetative hyphae of the water molds comes from the exceptionally informative work published by I. B. Heath and his associates. Kubai (1975, 1978) and Fuller (1976) have reviewed instructively the ultrastructural aspects of vegetative nuclear division, and Heath (1978a) has provided a particularly clear presentation of the behavior of nuclei and nucleus-associated organelles during the various mitotic events. It is apparent from these summary accounts that nuclear reproduction in the water molds does not adhere to the usual pattern of mitosis. Certainly in specimens of *Saprolegnia* somatic nuclei can undergo replication without apparent accompanying chromatin condensation.

THE ULTRASTRUCTURE OF MITOSIS

Slifkin (1967a) described some features of the subcellular nature of mitosis in a water mold *Saprolegnia delicat*, based on observations of the encysted spores. Heath and Greenwood (1968) recorded the initial description of the fine structure of nuclear division in the hyphae. They at first described mitosis in general terms as it was

observed in two species of *Saprolegnia*. (The doctoral thesis by Heath, 1969, contains much of what was subsequently published in his early papers.)

Heath and Greenwood (1968) found that in a typical mitotic division of nuclei in the mycelium of *Saprolegnia ferax* and *S. furcata* there was an intranuclear spindle converging toward the poles, but ending near the nuclear envelope and not penetrating it. At each pole, in a well-defined pocket of the nuclear membrane, lay an exogenous pair of centrioles. Microtubules (probably the astral ray configurations seen in many of the classical cytological preparations) radiated outward from each centriole. Throughout mitosis the nuclear envelope remained intact, and the nucleus and spindle elongated progressively. Accounts of more detailed observations of the mitotic process in somatic nuclei were to follow.

Heath and Greenwood in 1970(c), and Heath in 1974(a), published meticulous records of the events taking place at the subcellular level during the division of nuclei in the hyphae (and young sporangia) of *Saprolegnia ferax*, *Dictyuchus sterile*[†] and *Thraustotheca clavata*. In these papers the particulars of centriole replication emerged. As Heath (1974a) cogently pointed out, mitosis in these fungi is a two-phase event, a progression of intranuclear changes occurring coordinately with extranuclear ones. The interphase nucleus is associated with a pair of centrioles lying external to the nuclear envelope in adjacent pocket-like indentations of the nuclear profile (Fig. 22). The centrioles oriented at 180° to one another, subsequently duplicate. As mitosis progresses, the pair of centrioles migrate away from each other, but are still retained within pockets in the nuclear envelope. In time a few intranuclear microtubules appear, one group radiating from each of the infoldings of the nuclear envelope (Fig. 22). When the centrioles have moved for some distance, a spindle-like array of microtubules (Fig. 22) is seen to traverse the intranuclear matrix (Heath, 1974c). As the centrioles continue their migration, the spindle apparatus coordinately elongates (Heath, 1976). Accompanying spindle development is the appearance of kinetochore-like organelles (first discovered in water molds by Heath and Greenwood, 1968, 1970a). Eventually, kinetochores are visible near the poles as telophase progresses (Fig. 22).

The observations by Heath (1980b) on kinetochore microtubules in *Saprolegnia ferax* trace the behavior of these organelles during hyphal mitoses. In prophase the kinetochores appear as a single, hemispherical array adjacent to the centrioles. After the centrioles replicate, this apparently single group of tubules separates into two like assemblages. Heath proposes that kinetochore replication occurs at the onset of prophase, but the duplicated elements do not move apart to form two groups of tubules until a point prior to metaphase. There is evidence in Heath's study to suggest that kinetochores are permanently attached to chromatids throughout the nuclear cycle. This being so, it might be postulated that partitioning of chromatids is occurring at prophase rather than at some later stage in the mitotic cycle. The kinetochore microtubules elongate during metaphase and anaphase, and no discrete metaphase plate is evident (Heath, 1980a).

As the intranuclear microtubules materialize and early migration of the centriole pairs (Fig. 22) takes place, extranuclear microtubules are found in the cytoplasm

radiating from the centriolar region (Fig. 22). As the centrioles move apart, some of the microtubules are in a position oriented parallel to the nuclear envelope (Fig. 22). Centriole-associated microtubules that extend outward from the poles of the nucleus (Fig. 22) appear to figure into the development of projections -- the so-called horns of the nuclear envelope -- beyond the centriole pairs (Fig. 23). Thus the mitotic "poles" of the nuclei do not lie at the opposite extremes of the elongate nucleus because the nuclear horns (associated with microtubules) project beyond the centriole pairs (Heath, 1974c, 1976). Mitochondria are of course associated with the extranuclear tubules (Heath, 1974a; see also next chapter, Slifkin's description of mitosis in encysted spores).

As nuclear division progresses toward telophase, the nucleus and nucleolus show evidence of equatorial constriction (Fig. 24). Kinetochore-like structures are visible within the dividing nucleus near each of the centriole pairs (Fig. 24). It is assumed that the kinetochores are locational points identifying accumulations of chromatin material (Heath, 1976; see also Heath, 1974a, 1980b).

The final stage in replication of somatic nuclei in representatives of the Saprolegniaceae that have been studied is partitioning of the genome. Precisely how this occurs is not known (Heath, 1974a, 1976). Heath and Greenwood (1970c) saw no identifiable chromosomes in dividing nuclei in the hyphae of *Saprolegnia ferax*, but earlier workers such as Höhnk (1935b) and Hartog (1895) reported actual numbers of chromosomes in the dividing nuclei of their specimens. It may be recalled that Bakerspiegel (1960) and F. E. V. Smith (1923), among others, doubted that chromosomes existed in this (and other) species of water molds, and certainly the divergent counts reported by various mycologists (see Chapter 24) suggest that the gross physical construction of chromosomes in the water molds is as yet unknown. In any case, during division two chromatin aggregates appear in late telophase. Subsequently, as separation of these two masses proceeds, the equatorial region constricts (Heath and Greenwood, 1970c), and, it is assumed (Heath, 1974a), new membranes are synthesized and deposited to complete the separation of the organelle into a pair of daughter nuclei.

Two structures associated with the mitotic process in the water molds warrant special attention, namely, the centrioles and the microtubules (Kubai, 1975). The possible role of the latter in centriole migration and chromatin separation also must be considered.

Centriole Structure and Replication: -- The onset of activity in the centriolar region is one very noticeable primary event in somatic nuclear replication in the water molds (Heath, 1974a, 1976; Heath and Greenwood, 1970c). A centriole -- equated at one time with microtubule organizing body, but generally no longer so considered -- consists basically of three parts, as seen in transverse section (Fig. 25; Heath and Greenwood 1970b). There is a central hub, and radiating from it nine spoke-like strands, the so-called "cartwheel". At the distal end of each "spoke" of the cartwheel there is a triplet of tubules (A, B, C, proximal to distal) embedded in a matrix. If viewed laterally, the triple tubules (there are nine such triplets in each centriole -- Fig. 25) are seen to be staggered in relation to one another such that one "end" of the stacked group

of three is chamfered (Heath and Greenwood, 1970c). It is postulated that chamfering of the distal end (but probably not the proximal) results from unequal elongation of the tubules as they are formed.

As mitosis proceeds (Heath, 1974a) in the very early stages, the centrioles (Fig. 26) duplicate. Precisely how this occurs is not entirely clear, but Heath and Greenwood (1970c) have postulated a possible sequential pattern to this development. The extant short-cylindrical centrioles are positioned end-to-end, and the "daughter" centrioles are formed between the "parent" ones. First, the hub of the newly forming centrioles appear, the cartwheel spokes are elaborated, and then the A, B, C tubules are added sequentially. The hubs of parent and daughter centrioles are evidently contiguous. The result of the duplication process (Fig. 26) is a linear series of centrioles, with one "parent" organelle at each end of the row of four. Subsequently, the pairs separate, and centriolar migration continues.

The question to be considered at this point is: what is the motive force behind centriolar movement? Unfortunately, a definitive answer is not at hand. Heath (1976) and Heath and Greenwood (1970c) postulated that the centrioles separate in response to the accompanying polymerization of intranuclear spindle tubules, and since the centrioles appear to be in close proximity to microtubules even very early in the division process, this is a plausible view. It is of course an hypothesis in need of confirmation. Although the deposition of new nuclear membrane substance most assuredly takes place as mitosis proceeds, Heath (1974a) did not believe that mere addition of such material could account for centriolar displacement.

Microtubules and Mitosis: -- Gleason reported in 1973(a) the discovery of intranuclear tubules, smaller than those of the spindle apparatus, in interphase and actively dividing somatic nuclei in the hyphal tip of a nonsexual *Saprolegnia*. Their chemistry and function remain undetermined, and it is not known if these structures were in fact what are now recognized as "microtubules".

The results of a detailed ultrastructural analysis of nuclei in *Thraustotheca clavata* led Heath (1974a) to conclude that there are four positional types of spindle microtubules in this species although all are of the same structure, and all have one free end adjacent to the nuclear envelope pockets (Fig. 27). In *T. clavata* some microtubules are continuous, that is, they extend between the two nuclear pockets (seen most prominently at anaphase/telophase). A second type extends for most of the length of the nucleus, but with one free end distal to the adjacent centriole having no differentiated terminal structure. These were interdigitating microtubules. Polar microtubules radiate (intranuclear) for rather short distances away from the centriolar pockets, and also bear no detectable terminal organelle. The fourth type is one for which Heath proposed the name chromosomal microtubules; these terminated in identifiable kinetochores. A fifth kind of spindle microtubule was defined by Heath (1978) as a free microtubule. Such organelles lie free in the spindle without any evident connection to kinetochores or to spindle pole regions. In the species of water molds that have been studied at the subcellular level, about 8-10 kinetochores (Heath, 1974c) ultimately

appear near the centriolar “poles” at anaphase. Perhaps this division into types may prove to be too cumbersome since there may in fact be partial fixation-associated microtubule depolymerization. In any case, serial sectioning (Heath, 1974a, 1978a) clearly shows various placements of these organelles, some of which might most properly be described as nonkinetochore spindle microtubules.

Although much is known of microtubule self-assembly *in vitro*, there is a scarcity of information pertaining specifically to the water molds. There is some suggestion (Heath and Greenwood, 1971) that in *Saprolegnia ferax* the origin of spindle tubules might be traced either to endoplasmic reticulum or to the Golgi apparatus. As Heath and Greenwood (1970c) remarked, microtubules could arise by the synthesis of subunits of tubular material followed by polymerization of these units into linear configurations.

Mitosis in the water molds taking place as it does within the intact nuclear envelope raises a pertinent question: what is the mechanism that divides the genome equationally? It is not our purpose to review the various concepts of subcellular structure, but a brief digression into ultrastructural theory -- as it applies to mitosis in the Saprolegniaceae -- is justified. At the outset it may be postulated that two events in the division process require a force accompanied by some mechanism of synthesis, namely, the continuous elongation of the spindle and separation of the chromatin.

In 1968, Heath and Greenwood demonstrated conclusively that there is an intranuclear spindle present during some phases of somatic nuclear replication in *Saprolegnia ferax* and, except for metaphase, in *S. furcata*. Nearly simultaneously Slifkin (1968) showed that colchicine could penetrate the hyphae of *S. delicata*[†] but did not block mitosis (Slifkin, 1967a, b). Her observation was taken as partial evidence that equational division takes place in this species, at least, in the absence of an intranuclear spindle.

Slifkin's study was essentially repeated on *Saprolegnia ferax* by Heath (1975a, b) seeking to uncover any antimitotic action of colchicine, colcemid, camphor, vinblastine, and griseofulvin. The first two of these compounds presumably combine with microtubule subunits and prevent polymerization. Heath (1975a) found that even at lethal levels, none of the antimitotic agents prevented the synthesis and assembly of microtubules. He proposed that resistance of *S. ferax* to disruptors of mitosis might be due to variations in nontubulin components of the microtubules, or to the “cytoplasmic environment” of the matrix surrounding these organelles. Heath explored these two hypotheses by analyzing chromatographically hyphal extracts to determine if colchicine or colcemid binding occurred. The results of his experiments indicate that there are two binding fractions in *S. ferax*, a stable and a nonstable one. He was unable to assemble positive proof that either (or both) of these fractions were tubules, but circumstantial evidence (from molecular weight determinations, for example) pointed to such a composition. Precisely what conveys resistance in the microtubules in *S. ferax* and *S. delicata*[†] is yet to be discovered.

Certain theories of chromosome movement have been linked to lengthening and shortening of microtubules. For example Inoué and Sato (1967) hypothesized that polymerization and depolymerization of subunits induced changes in the length of

microtubules, while Dietz (1972) held to the notion of an interaction between the tubules and a spindle matrix against which individual elements could push as the nucleus elongated. As Heath (1975c) has pointed out, the known nature and distribution of microtubules (in *Saprolegnia ferax* and *Thraustotheca clavata*) accommodates Dietz's views. The cross-bridge and sliding motion hypothesis (McIntosh *et al.*, 1969), Bajer's (1973) "zipper" theory, and Forer's (1974) explanation do not appear to us to be supported by the known ultrastructural events in somatic mitosis in the water molds. Heath (1975c) also has suggested that Margulis' (1974) concept of chromosomal movement under the pushing force of interchromosomal microtubules is not appropriate to explain movement in dividing vegetative nuclei of *T. clavata*. In the mitotic figures of the *Thraustotheca*, to be sure, complete, interchromosomal tubules have not been detected.

Heath (1975c, d) reviewed some of the general theories to explain chromosome movement, and suggested that a surface shear force operates in microtubules such that they move in the spindle matrix. He proposed that an interaction between tubule-linked actin (which he was unable to demonstrate; Heath, 1975c) and matrix-linked myosin was very possibly the motive force. This view certainly is not at variance with theory, but there is as yet no evidence for either actin or myosin in the Oomycete nucleus.

According to Heath and Greenwood (1970c) the mitotic spindle probably only generates sufficient force to separate the chromatin. A second force, they suggested, must function in the actual division of the nucleus. That a physical space relationship exists between the nuclear envelope (in, for example, *Saprolegnia ferax*) during mitosis and cytoplasmic microtubules is clear. This close proximity of tubules to membrane, Heath and Greenwood (1970c:145) suggested, was evidence for "... a mechanism whereby the nuclear envelope is able to actively 'crawl' along the apparently rigid tubules, thus pulling the nucleoplasm with it and aiding the elongation and eventual separation of the daughter nuclei." The precise mechanism remains undiscovered (Heath, 1974a, 1976), even though the hypothesis (Heath, 1974c) is supported by some morphological evidence.

MITOSIS IN THE PARASITIC SPECIES

In 1972(a) Hoch and Mitchell characterized the nature of the subcellular structure of *Aphanomyces euteiches* during sporogenesis. A part of their account, however, dealt with the somatic nuclei. They were unable to find centrioles associated with these nuclei, but did detect small, fibrous bodies -- in a position at which one might expect to find centrioles -- which they called procentrioles. The illustrations provided of these structures (Hoch and Mitchell, 1972a) suggest that improved preparative techniques are probably needed before the true structure of procentrioles can be determined. It is clear that characteristic "cartwheel" centrioles are associated with mitotic divisions accompanying primary spore (encysted) germination in *A. euteiches*.

Heath's study (1974b) of the subcellular events in mitosis in hyphae of *Aphanomyces astaci* demonstrated that this species was typically saprolegniaceous in nuclear behavior. Centriole structure, position, replication, and migration, simple kinetochores, and spindles composed of microtubules were features of nuclei in *A. astaci* comparable to, for example, those of *Thraustotheca clavata* and *Saprolegnia ferax*. As in the other species of Saprolegniaceae where nuclear division has been studied ultrastructurally, there was no metaphase plate formed during mitosis. This evidence certainly brings into question the rather precisely illustrated typical mitotic figures shown by some of the earlier mycologists who employed classical cytological staining methods to bring out details of the division process!

ULTRASTRUCTURAL CYTOCHEMISTRY

The very noticeable progress made toward an understanding of the fundamental aspects of organelles and their interrelatedness cannot be entirely credited to the mere discovery of structural characteristics. Some investigators have assembled data and observations which provide the hypothesis that the organelles in the mycelium of water molds are very probably connected functionally. Nowhere has this approach toward functional integration been more extensively explored (in the water molds) than in the search for a pathway leading from the endomembrane system to the elaboration of hyphal wall material. (See also, in Chapter 6, Hill and Mullins, 1980b.)

In 1967, Thiéry demonstrated by cytochemical means that polysaccharides could be detected in preparations made for ultrastructural analysis. Later, P. J. Harris and Northcote (1971) showed that these compounds were associated with Golgi bodies, and were similar to ones deposited in cell walls. While these observations were not derived from study of any of the Saprolegniaceae, they did establish a basic principle.

In 1965, Palczewska reported that dictyosomes in the hyphae of a nonsexual *Achlya* contained thiamin pyrophosphate, while other organelles -- which she called spherosomes (lysosomes of some authors) -- contained acid and alkaline phosphatases. Later (1972), Palczewska and Jagodzka published an additional report on enzymes associated with these granular (0.2-1.0 μm diameter) inclusions. By various cytochemical tests, they found alkaline and acid phosphatase and an esterase at the sites of spherosomes in the hyphae, spores, sporangia, undifferentiated oogonia, oospheres, oospores, and antheridial branches of *Achlya flagellata*[†]. Acid phosphatase activity was particularly high in differentiating sporangia, in antheridial cells, and in disintegrating oospheres. The enzyme β -galactosidase was present in all the stages of development of the isolate of this species except the spores. The precise nature of "spherosomes" is yet to be determined; perhaps ultrastructural analysis would yield something of their morphology and origin. Spherosomes are not mentioned by others who have probed into the subcellular structure of the water molds. The periodic acid-silver hexamine (PASH) reaction in vesicle-containing hyphae of *Saprolegnia ferax*, for example, demonstrates that these organelles contain polysaccharides (Heath *et al.*, 1971).

Two major classes of vesicles (some of both classes appearing to derive from the Golgi apparatus) have been found (Hill and Mullins, 1980a) in the growing hyphal tips of *Achlya ambisexualis* (E87). The large vesicles (about 150 nm in diameter) are periodic acid-silver methenamine positive, and uniformly phosphotungstic acid-chromic acid negative. Acid phosphatase and IDPase also occur in these large vesicles. Smaller vesicles -- about 80 nm in diameter -- are PASM-negative, and mostly PTA-CrO₃-positive.

Several details of subcellular localization of particular chemicals have come from studies by Dargent (1975, 1977), and Dargent and Denisse (1976) on the hyphae of *Achlya bisexualis*, and by Fèvre (1976, 1977) and Fèvre and Dumas (1977) working with *Saprolegnia monoica*[†]. Dargent reported that polysaccharides in *A. bisexualis* are localized on particular surfaces of the Golgi apparatus, on the surface and interior of apical vesicles, in the plasmalemma, and in association with the hyphal wall, but none apparently is evident on the vesicles blebbing from the sides of Golgi bodies or on the endoplasmic reticulum. Alkaline phosphatase activity, Dargent found (1975), was associated with the dictyosomes and larger vesicles in the hyphal apex. Thus, there appears to be simultaneous occurrence of polysaccharide deposition (synthesis) and alkaline phosphatase activity in the dictyosomal region and in the apical vesicles that are presumably Golgi-derived (Grove and Bracker, 1970; Grove, *et al.*, 1970; Dargent, 1977; Roland, 1973). Dargent and Denisse (1976) applied the Gomoroi technique (1952) for the detection of acid phosphatase by iron precipitation, and found activity of this enzyme complex to be very intense at the site of the evaginations ("sacculles") on the dictyosome surface, and in the vesicles at the hyphal apex. There were no precipitated granules (indicative of acid phosphatase) on the plasmalemma or on the surfaces of ER.

Dargent (1977) placed substantial emphasis on chemical polarity (or gradation) within the hyphal tip, postulating that the Golgi apparatus synthesized polysaccharides and phosphatases which were then incorporated into sacculles, and subsequently transported within vesicles to the plasmalemma. The vesicles then fused with this membrane, and in doing so contributed precursors toward the synthesis (hydrolysis of phosphate esters to activate monomers of polysaccharides) of the hyphal wall. Thus, the presumed sequential stages in the derivation of wall vesicles from Golgi bodies (Roland, 1973: fig. 14, and Grove *et al.*, 1970) serve also to transport essential enzyme systems coordinately.

Various other enzymes also are sequestered within organelles in the hyphae of water molds. Nolan and Bal (1974) used an EM histochemical technique based on deposition of copper to localize cellulase activity -- in dictyosomes, vesicles, and (in some instances) between the plasmalemma and hyphal wall in *Achlya ambisexualis*. They considered that the synthesis of cellulase (probably both an endo- and exo-cellulase) took place within the dictyosomes, and the enzyme complex was then transported to the plasmalemma and released to the region between the membrane and the cell wall. Since the technique used by Nolan and Bal may not be specific for cellulase, further exploration is necessary.

Assuming Dargent's (1977) scheme of vesicle functioning in the transport of chemicals to the site of wall synthesis to be correct, the transfer of cellulases from origin to place of action could be accomplished by wall vesicles as well. Cellulase in the hyphal system is, of course, involved in the growth of lateral branches in the antheridiol-activated development of the antheridial apparatus (Chapter 21). Heath (1976) stated that it is possible for precursors of glucan (a major wall fraction) to be present in wall vesicles, but there is no evidence of wall fibril synthesis within the membrane-bound enzyme packets.

Fèvre's (1976, 1977) work -- and that of Fèvre and Dumas (1977) -- employed gel electrophoresis, labeled polysaccharide analysis, protein synthesis inhibitors, differential centrifugation, and electron microscopy, to study the chemistry of ultrastructural moieties. Density bands of glucanase and cellulase from particulate fractions corresponded to a location in endoplasmic reticulum, Golgi bodies, and plasmalemma. Glucanase activity was traced also to the sites of vesicles. Fèvre and Dumas (1977) found that wall fractions and particulates from the cytoplasm harbored most of the β -glucan synthetase (1-4 linked) activity of the hyphae of *Saprolegnia monoica*[†], and the magnitude of this enzyme's activity was related to the morphological state of the mycelium: greater activity in branched than in unbranched filaments. Within the cytoplasm of this *Saprolegnia* most β -glucan synthetase activity is said to be associated with dictyosomes and vesicles, and dictyosomes also contain (1-4)-linked polysaccharides (possibly glucan oligomers, the precursors to cellulose). Chromatographic analyses performed by Fèvre and Dumas (1977) suggested that laminaribiose and laminaritriose occurred in the mycelial fractions of their isolate of *S. monoica*[†] indicating the possible presence of the (1-3)-linked β -glucan synthetase as well. Their conclusions regarding the mechanism of enzyme transport to the hyphal wall coincides exactly with Dargent's (1977) hypothesis.

From the foregoing account, it is evident that cytochemical analyses support the view that there is a functional and physiological continuity of organelles involved in genesis of the plasmalemma and the hyphal wall in the water molds. Final proof is of course lacking with respect to this particular group of fungi but the assemblage of circumstantial evidence is difficult to avoid.

Although microbodies have been found in the hyphal apices of *Aphanomyces euteiches* by Maxwell *et al.* (1975), nothing could be determined of the enzymes associated with them (Maxwell, communication). Extracts from the mycelium of *Achlya ambisexualis* containing microbodies (but not entirely free of mitochondria) were analyzed by Coinski and Mullins (1977) for enzymatic activity (they treated preparations with 3-3'-diaminobenzidine tetrahydrochloride to identify catalase, for example). Certain enzymes were associated with the extracts: catalase, glycolate oxidase, isocitrate lyase, malate dehydrogenase, citrate synthetase, fumarase, and glutamate:oxaloacetate transaminase. Their data showed only very low activity levels of malate synthetase and uricase. These analyses suggest that microbodies are of the glyoxysomal type (but this can be little more than supposition because of the mitochondrial contaminants in the preparations). Typical microbodies are present in

the hyphae of *Saprolegnia terrestris* (Howard and Moore, 1970), and such organelles characteristically contain catalase. However, this has not been demonstrated for *S. terrestris*. Cytochemical analysis coupled with EM methodology was used by Murrin and Nolan (1977) in some analyses of the mycelium of *S. megasperma*. The circular or shallow U-shaped mitochondria of this fungus harbored membrane-bound succinate dehydrogenase (a nonheme iron protein functioning to oxidize succinate to fumarate).

Beakes (1976) explored the effect of streptomycin on the ultrastructure of some species of *Saprolegnia*. In the vegetative phase of this particular watermold, the activity of mitochondria seemed to be altered by the antibiotic. The structure of dense-body vesicles and vacuoles in sporulating filaments were altered by streptomycin as was the cytology of the nuclei. During the sporulating phase of the various isolates Beakes studied, there was an increased sensitivity to streptomycin. He suggested that the antibiotic competes successfully for divalent cation binding sites on the plasmalemma and perhaps also on membranes of other organelles; the number of such sites might be increased, he proposed, during the reproductive phases of the isolates (*see* also Fletcher, 1979a).