Investigations on the Host-Parasite Interface of a Mycoparasite System

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Abstract

The cell wall composition of Choanephora cucurbitarum and the host-parasite interface, after infection with Piptocephalis virginiana, were examined in detail. The cell walls of C. cucurbitarum were determined to be composed of chitin (17%), chitosan (28.4%), neutral sugars (7.2%), uronic acid (2.4%), proteins (8.2%) and lipids (13.8%). The structure of hyphal walls investigated by electron microscopy of shadowed replicas before and after alkali-acid hydrolysis, showed two distinct regions: microfibrillar and amorphous. The microfibrils which were composed of mainly chitin, were organized into two distinct layers: an outer, thicker layer of randomly orientated microfibrils and an inner, thin layer of parallel microfibrils.

Electronmicrographs of the host-parasite interface of C. cucurbitarum and the mycoparasite, P. virginiana, 30 h following inoculation, showed that the sheath zone has a similar electron density to that of the host cell wall. The sheath was not present around the young (18 h old) haustorium. High-resolution autoradiographs of infected host hyphae showed that radioactive N-acetyl-D-glucosamine, a precursor of chitin, was incorporated preferentially in the host cell wall and sheath zone. Cell fractionation of label fed hyphae showed that 84% of the label was present in the cell wall and specifically in the chitin portion of the wall. The antifungal antibiotic, Polyoxin D, a specific inhibitor of the enzyme, chitin synthetase, suppressed the incorporation of the label in the cell wall and sheath zone and resulted in a decrease in electron density of the developing sheath. The significance of these results is discussed in the light of host resistance.

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Dedication

I wish to dedicate this manuscript to my uncle Larry, who passed away before its completion, for his constant encouragement and to my wife, Jane, who has been understanding and tolerant beyond all possible expectation. Finally, recognition must be given to my parents for their many many years of dedication to my education.

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Introduction

Parasitic fungi are found in all major fungal groups. Some of these fungi can obtain their required nourishment only from living hosts and are classified as obligate parasites. When such parasites attack certain higher vascular plants they cause mankind constant concern over great economic losses of food crops. Thus, the vast majority of the literature published on the topic of host-parasite relations has dealt with the activities of the rust and powdery-mildew fungi of economically important crops (Bushnell, 1972).

Despite the extensive investigations conducted on these obligate parasites, few questions have been answered concerning the two main interests of plant pathological research, the nutritional requirements of the parasite (ie. basis of parasitism), and the cellular and molecular basis of disease resistance. One of the reasons for the lack of significant findings is that the actual relationship of the host and parasite has been masked due to the complexity of the vascular host plant (Daly, 1967; Armentrout and Wilson, 1969). There are several factors that can complicate studies of host-parasite interactions in fungal-plant systems; this limits their usefulness as systems for studies aimed at discovering solutions to the basic problems posed in plant pathology (Daly, 1967). These factors include (i) the non-synchronous changes of the host and the parasite over the required lengthy growth period; (ii) in the case of plant diseases, where special conditions

are required for inoculation, changes in environmental conditions after inoculation may lead to spurious changes in metabolism; (iii) that the growth of plants during a comparative series of experiments under different, even though constant, environments provides these plants with different levels and amounts of substrates, and thus, the impact of disease is inconsistent in terms of its effect on the content of specific substrates.

The study of mycoparasitism (a fungus parasitic on another fungus) represents a relatively new approach to the elucidation of the basic principles underlying parasitism and resistance (Barnett and Binder, 1973). The use of a mycoparasitic system in studies aimed at the investigation of the principles of parasitism and host resistance has certain advantages over the more conventional plant-pathogen systems. These include a considerable saving of time and space, strict control over the cultural environment, complete regimentation of the host nutrition and the relative simplicity of the host cells compared to the plant cells typically used in the study of obligate parasitism (Barnett and Binder, 1973).

The mycoparasites are divided into two major groups based on the mode of parasitism; "necrotrophic" mycoparasites which excrete toxin, kill the host cells and derive nutrients from the dead host cells and "biotrophic" mycoparasites which secure nutrients from the living host cells. The biotrophic mycoparasites are assigned to three distinct categories according to their morphology and physiology (Barnett and Binder,

1973); (i) the internal mycoparasites which develop within cells of other fungi are represented by the chytrids; (ii) the contact mycoparasites that do not produce any haustorial or internal hyphae are found among the fungi imperfecti; (iii) the haustorial mycoparasites, whose members are of the order Mucorales, produce distinct haustoria within the host hyphae.

To date, there are only three well documented investigations of the host-parasite relationship in mycoparasites at the electron microscopic level (Armentrout and Wilson, 1969; Manocha and Lee, 1971 and 1972.) Armentrout and Wilson (1969) examined the fine structure of the interface of the mycoparasite Piptocephalis virginiana growing on a mucoraceous host Mycotypha microspora. Manocha and Lee (1971) investigated the developmental details of the host-parasite interface at the ultrastructural level of the same obligate mycoparasite but on another mucoraceous host, Choanephora cucurbitarum, itself a parasite of squash blossoms (Alexopoulos, 1962). The majority of electron microscopic investigations of fungal-plant systems have not dealt with the host-parasite interface as a structural entity in itself, but rather with the ultrastructural details of the haustorium and the host cell. The significance of the host-parasite interface in pathological studies should be emphasized since it is the region of direct interaction between the host and parasite and therefore forms the zone through which materials, that can elicit responses in either partner, are exchanged (Bracker and Littlefield, 1973).

Manocha and Lee (1971) showed that during the late stages of haustorial development, the sheath around the

haustorium is well developed and composed of materials with the same electron density as that of the host cell wall. In a later work, Manocha and Lee (1972) fed tritiated N-acetyl-D-glucosamine, a chitin precursor, to the host fungus, C. cucurbitarum, and observed using high-resolution autoradiography that the label was preferentially distributed in the host's cell wall area. When the infected host cells were exposed to the label at different time intervals following inoculation, the radio-activity was shown to be incorporated into the cell wall of the host and into the interface area after the sheath development had begun. These results suggested that the sheath is basically composed of the same material as the host cell wall.

However, these experiments did not rule out the possibilities of; (i) non-specific binding of the label; (ii) metabolism of tritiated N-acetyl-D-glucosamine and subsequent incorporation of a by-product. The presence of chitin although shown to exist in the cell walls of other members of the Mucorales (Bartnicki-Garcia, 1968b) has not been determined in the cell walls of the host fungus, C. cucurbitarum. In order to lend validity to any further research on this host-parasite interface, the determination of the presence of chitin in the host cell wall is imperative. Therefore investigations were carried out to determine the chemical composition of the cell wall of C. cucurbitarum and the origin and composition of the sheath which develops in the haustorium-host interface of the mycoparasite, P. virginiana and the host, C. cucurbitarum. Polyoxin D, a selective inhibitor of chitin synthetase, was

used as an experimental tool to manipulate chitin synthesis.

Literature Review

Due to the complex nature of the host-parasite interface, the review of literature is divided into three different sections to facilitate the appreciation and understanding of the problem. The first section of the review deals with the work done on the physical structure and chemical composition of fungal cell walls. The second section describes attempts to illustrate by light and electron microscopy the structures of haustorial parasites in both plant and fungal hosts. It also includes a review of studies on the host-parasite interface and on sheath development. The final section deals with the mode of action of an antibiotic, Polyoxin D, on the enzyme chitin synthetase and on wall development.

Physical Structure and Chemical Composition of Fungal Cell Walls

The importance of fungal cell wall architecture in the determination of the gross morphology of fungi (Aronson, 1965), including that of various fungal reproductive structures and of vegetative hyphae (Alexopoulos, 1962), should not be underestimated. A great deal of literature has been published, elaborating on the structural entities and the chemical composition of fungal cell walls. The majority of structural research stems from the development of sophisticated investigative techniques such as: polarization optics, heavy metal

shadow casting, biochemical analysis and specific enzyme hydrolysis. These techniques have made it possible for researchers to gain some added insight into the structural and physical organization of fungal cell walls at a level not previously revealed by light microscopy (Bartnicki-Garcia, 1968b).

Morphologically, the fungal cell wall has a characteristic two-phase system in which microfibrils of chitin or cellulose are embedded in an amorphous matrix (Aronson, 1965). Such microfibrils form the skeletal components of the cell walls of the vast majority of fungi. Bartnicki-Garcia and Nickerson (1962) showed that fungal morphology can be traced back to their differences in cell wall structure. They examined the dimorphic forms of the cell walls of Mucor rouxii and found that the yeast-like form is made of two layers. The outer layer appeared conspicuously fibrillar with random orientation whereas the inner layer was devoid of distinct fibrils. The filamentous form of M. rouxii showed none of the layering of the yeast-like forms, and appeared as a solidly packed meshwork of microfibrils, the main axis of which lay parallel to the surface of the cell. In Neurospora crassa, the well marked inner and outer layers as observed in the cell wall of the yeast-like form of M. rouxii were not observed by Manocha and Colvin (1967). The cell wall of N. crassa was shown to be a two-phase system composed of chitin microfibrils arranged randomly within an amorphous phase composed mostly of glucan and protein.

Bartnicki-Garcia and Reyes (1968a) found that the sporangiophore walls of M. rouxii were two layers thick, a granular inner layer and an outer layer of microfibrillar texture. This microfibrillar texture of the sporangiophore wall is thought to be related to the higher chitin content of the sporangiophore walls as compared to the filamentous hyphae. Marchant (1966) described the cell walls of Fusarium sulphureum as composed of three layers, the outermost microfibrillar and composed of chitin, the middle layer, non-fibrillar, and the inner one, electron transparent. The conidia of F. sulphureum were shown shown to have in addition to the three layers reported by Marchant (1966), a fourth outer layer. This layer consists of a mucilagenous material, xylan (Barran et al, 1975).

Whether randomly oriented microfibrils in fungal cell walls are representative of the walls of fungi in general is unclear. Manocha and Colvin (1968), employing the techniques of acid and alkali hydrolysis and snailgut enzyme digestion on the cell walls of Pythium debaryanum showed the existence of another microfibrillar network. The thick outer layer of the mature hyphal cell wall was observed to be composed of randomly orientated microfibrils whereas the thin inner layer displayed microfibrils arranged in parallel fashion and orientated transverse to the cell axis. Because the hyphal tips did not show the presence of this inner layer, the authors suggested that at least two different processes are responsible for the deposition of the cell wall material.

The subcellular site of cell wall microfibril formation is presently unresolved. It is considered unlikely that microfibrils are elaborated internally and then secreted in prefabricated form (Colvin, 1972). It is generally believed that microfibrils are assembled at the cell surface (Colvin, 1972) with the plasmalemma being actively engaged in their synthesis (Robinson and Preston, 1971). Thus a number of investigators have examined the hyphal apices of different fungi in an attempt to elucidate the cytoplasmic mode of microfibril organization, origin and deposition. Marchant, Peat and Banbury (1967) ascribed wall formation to a vesicular system in hyphal tips of several fungi. Large numbers of Golgi bodies and many lomasomes have been identified in the tips of extension hyphae of P. debaryanum (Manocha and Colvin, 1968). McClure et al (1968), in studying the apical organization of Asperigillus niger, Fusarium oxysporum and F. solani, observed the presence of "Spitzenkorpers" at the hyphal apices of all of these septate fungi. However, none of the Phycomycetes examined, including two species of Pythium, Mucor hiemalis and Zygorhynchus moelleri, showed these structures. These "Spitzenkorper" structures showed no rapid movement, a vague boundary and were visible only during the active extension growth of the hyphal tips, when viewed under light microscopy. When the hyphal growth was was temporarily inhibited the "Spitzenkorper" would fade out of sight to reappear only prior to renewed growth (McClure et al, 1968). Bartnicki-Garcia and Lippman (1969) proposed the existence of discrete minute apical organelles which

are responsible for the active region of synthesis of cell wall materials. They suggested two candidates for the role of cell wall depositor; a "Spitzenkorper", even though its presence in a Mucor species had not been observed (McClure et at, 1968); or, apical corpuscles, which were reported in the germ tubes of M. rouxii (Bartnicki-Garcia, 1968a).

Doubtless, the organization and growth of the fungal wall is under protoplasmic control; wall materials or at least wall precursors must traverse the plasmamembrane by some form of secretion (Bracker, 1968). Ledbetter and Porter (1963) suggested that microtubules beneath the surface of the plant cell protoplast somehow influence the orientation of microfibrils of the adjacent cell wall. However, Bracker (1967) stated that there is no evidence for the involvement of microtubules in the orientation or deposition of fungal cell materials.

It has been demonstrated that the enzyme chitin synthetase, which is responsible for the synthesis of chitin, a major cell wall constituent of mucoraceous fungi, from uridine diphosphate-N-acetyl-D-glucosamine, is present in "bound" form in the cell walls of the fungus M. rouxii (McMurrough et al, 1971). Bartnicki-Garcia and Lippman (1969) and McMurrough et al (1971) have shown, by autoradiography, that tritium labelled N-acetyl-D-glucosamine (chitin precursor) is preferentially localized in the apical dome of the hyphae of M. rouxii. Gooday et al (1971) confirmed these findings and added that the apical region is characterized by an accumulation

of cytoplasmic vesicles, which these authors implicated in cell wall formation. A periodic slowing in the growth rate of leading hyphae showed a concomitant variation in the intensity of incorporation of labelled chitin precursor, thus indicating a relationship between the rate of hyphal growth and label incorporation.

Subsequent investigations have shown the presence of a "soluble" form of chitin synthetase. It was separated from a membrane-rich fraction of the cell wall of M. rouxii, by exposure to the enzyme substrate (uridine diphosphate N-acetyl-D-glucosamine) and activated by N-acetyl-D-glucosamine. This solubilized enzyme catalyzed the in vitro synthesis of chitin microfibrils (Ruiz-Herrera and Bartnicki-Garcia, 1974).

Elucidating the nature of the cell wall structure is of course only part of the problem. The chemical composition of the cell wall constituents represents the other major faction of fungal cell wall research.

Until recently, the few investigations purporting to deal with cell wall constituents other than chitin and cellulose suffered from serious technical difficulties. The major problem has been in the preparation of cell walls which are free of both cytoplasmic and membranous contamination but still possess all of their constituents. With the development of mechanical methods of isolating cell walls, thereby minimizing component loss due to chemical isolation

techniques, there has been increased success in studies of fungal cell wall chemistry (Bartnicki-Garcia and Nickerson, 1962; Bartnicki-Garcia and Reyes, 1964; 1968a and 1968b; Manocha and Colvin, 1967 and 1968; Sangar and Duggan, 1973 and Barran *et al*, 1975). Through the use of these newer techniques, a marked degree of complexity and diversity in fungal wall chemical composition has become apparent.

On the basis of chemical composition, Bartnicki-Garcia (1968b) has divided the fungi into 8 different groups, with the criterion for categorization being the two most prominent polysaccharides which include, in addition to chitin and cellulose, chitosan, mannose, a glycogen-like polymer glucan, galactose and galactosamine. Bartnicki-Garcia (1968b) has shown that there exists a close correlation between the chemical composition of the cell walls and major taxonomic groupings elaborated on morphological criteria.

The classification is as follows:

Group I. Cellulose and glycogen are the major cell wall polysaccharides of the fungi of the first group. The combination of these polysaccharides has been reported in sporophores of *Dictyostelium*, *Acytostelium* and the microcyst walls of *Polysphondylium* (Acrasiales).

Group II. Glucan and cellulose are the predominant sugars of the hyphal walls of Oomycetes of the order Saproligniales and Peronosporales. The absence of chitin in any

of the fungi of this group was confirmed by Bartnicki-Garcia (1966) and only a small amount of hexosamine (1%) has been detected in the cell walls of oomycetous fungi. However, its polymeric state remains unknown.

Group III. Cellulose and chitin are present in the cell walls of the Hyphochytridiomycetes. The grouping is based only on the composition of the one fungus genus, Rhizidiomyces sp.

Group IV. Chitosan and chitin are the predominant aminosugarss of the wall of the Zygomycetous fungi.

Group V. Chitin and glucan are the dominant cell wall polysaccharides of the fungi of this category. This group contains all of the mycelial forms of the Ascomycetes, Basidiomycetes and Deuteromycetes and of some of the lower chytrids. The major classes of fungi included in this category differ only with respect to the presence of minor sugars.

Group VI. Mannan and glucan are the main sugars in the cell walls of the yeast forms of Ascomycetes and Deuteromycetes and this marked deviation in composition from the

mycelial forms, allows them to be grouped separately.

Group VII. Mannan and chitin are the marker polysaccharides of this group and are present in the cell walls of the genera Rhodotorula and Sporobolomyces.

Group VIII. Galactose and galactosamine polymers are present in the cell walls of the only Trichomycete so far examined in this group, Amoebidium parasiticum.

The fungi investigated in the present research, Choanephora cucurbitarum and Piptocephalis virginiana, are both members of the Zygomycetes and therefore the chemical composition of the cell walls of the zygomycetous fungi, which are categorized into Group IV of Bartnicki-Garcia's (1968b) classification scheme, is emphasised in this section of the literature review.

The cell wall compositions of a number zygomycetous fungi have been investigated over the past twenty-five years. Studies of Mucor rouxii (Bartnicki-Garcia and Nickerson, 1962; Bartnicki-Garcia and Reyes, 1964; 1968a and 1968b), Zygorhynchus veulliminii (Crook and Johnston, 1962), Rhizopus sp. (Frey, 1950) and Phycomyces blakesleanus (Kreger, 1954) all mucoraceous fungi, have shown the presence of chitin and chitosan as the major components of their walls. X-ray diffractometry has also been used to detect the existence of chitin in three species of the Entomophthorales; Basidiobolus sp, Entomophthorales sp, (Frey, 1950) and Paracoccidioides

brasiliensis (Moreno et al, 1969).

To date, the most thoroughly studied fungal cell walls have been those of the fungus M. rouxii. Bartnicki-Garcia and Nickerson (1962) originated a series of studies on the chemical composition of the cell walls of this dimorphic fungus. The chemical composition of the fungal walls was found, by acid and alkali extraction followed by chromatography of the soluble products, to be polysaccharides (glucosamine, galactose, mannose and fucose), phosphate, proteins, lipids, purines and pyrimidines, Mg^{2+} and Ca^{2+}. Chitosan was the most abundant component of the cell wall of both the yeast-like and filamentous forms of the fungus. There was no qualitative variation between the cell wall types investigated, but rather a quantitative difference mainly with regard to the mannose content, which was more abundant in the yeast-like form of M. rouxii. An absence of glucans (poly-glucose) was noted. This absence is a characteristic trait of the vegetative cell walls of this class of fungi (Bartnicki-Garcia, 1968b).

The composition of the spore cell walls of M. rouxii is markedly distinct from that of its vegetative walls (Bartnicki-Garcia and Reyes, 1964). These walls contain glucan and melanin as the main sugar components and totally lack fucose and galactose. Moreno et al (1969) reported on the presence of glucans in the cell walls of the yeast form of Paracoccidioides brasiliensis. Its sporangiophore walls were subsequently investigated and found to be composed of chitin, chitosan, D-glucuronic acid, fucose, mannose and galactose (Bartnicki-Garcia

and Reyes, 1968a). D-glucuronic acid is a major building block of both the vegetative and sporangiophore cell walls of M. rouxii (Bartnicki-Garcia and Reyes, 1968b). An identical complement of sugars (D-glucuronic acid, L-glucose, D-mannose and L-galactose) has been detected in the hydrolysates of extracted polysaccharides of different species of Mucor and Rhizopus (Martin and Adams, 1956). These sugars may form part of a heteropolyuronide such as the one isolated from M. rouxii (Bartnicki-Garcia, 1968b).

Proteins play an integral part in the structure of fungal cell walls (Bartnicki-Garcia and Nickerson, 1962). Proteins and various polysaccharides probably act as the cementing materials which bind together the cell wall components into macromolecular complexes (Bartnicki-Garcia, 1968b). Amino acid analysis has shown the common absence of hydroxyproline in fungi with chitinous walls (Crook and Johnston, 1962), although it is a characteristic amino acid of cellulosic walls of other fungi, algae (Punnett and Derrenbacker, 1966) and higher plants (Lamport and Northcote, 1960).

Dyke (1964), investigating Nadsonia elongata, demonstrated that the presence of lipid in the cell wall of this yeast was indeed a component of the cell wall itself and not a cytoplasmic contaminant, as had formerly been presumed. The presence of lipoidal material in isolated walls depends upon the fungus investigated (Aronson, 1965). Lipids may confer hydrophobic properties to certain cell structures such as ascospores and sporangiophores. Kreger (1954)

demonstrated that in the sporangiophore wall of Phycomyces, lipoidal material accounts for greater than 25% of the dry weight and that it was mostly of a cuticular nature. Some lipids are firmly bound to the cell wall and therefore they may have a role in the overall structural integrity of the cell (Nickerson, 1963).

Fine Structure of Host-Parasite Interface

In many instances the parasitism of a fungus on a susceptible host, when examined at an ultrastructural level, has been shown to follow a pattern of events. If environmental conditions are favourable, the germinating spore on a susceptible host produces a germ tube which comes into contact with the host cell surface. If infection or at least penetration is to occur, an appressorium must form prior to the actual penetration. These appressoria are adhesive discs securing a parasitic fungus to its host (Yarwood, 1956). The initiation, formation and action of the appressorium are integral parts of the infection process of many fungi (Emmett and Parberry, 1975). The actual mechanism of penetration of an infection peg into the host cell is still uncertain. There is evidence, in the literature, that penetration may be strictly a mechanical process, solely an enzymatic mechanism or a combination of the two processes. Nicholson et al (1972) detected, histochemically, the existence of transitory esterase activity in spores and germlings of Venturia inaequalis. Esterase aids in plasticizing the fungal spore wall and in softening the host cuticle. McKeen (1974) found

that the cuticle of Vicia fabia appeared to be enzymatically dissolved rather than mechanically broken by the fungus Botrytis cinerea. This conclusion was based on the fact that sharp, clean penetration pores, without signs of physical deformation, were made by the parasite through the host cuticle. Furthermore, esterase activity had also been detected in the tips of the germ tubes of Botrytis cinerea at seven hours following inoculation. This time coincides with the average time of host penetration (McKeen, 1974). Akai et al (1967) found that barley leaves infected with Erysiphe graminis showed chemical modification in the cellulose walls and around the penetration tubes. McKeen et al (1969) reported a chemical and morphological alteration of the epidermal walls of barley, clover, strawberry and sunflower occurring around the infection peg of Erysiphe species. When differentially stained, a "halo" region on the host cell wall around the infection peg of powdery mildew fungi appeared; whereas in the normal host cell wall this halo was not observed. They further demonstrated, cytochemically, that the "halo" region contained smaller amounts of reducing sugars, pentose and uronic acid. This indicates that there is at least a partial degradation of the host cell wall during the penetration process by these fungi.

Regardless of the degree of dissolution of cuticle and wall, some mechanical force is required for penetration by the infection peg (Bushnell, 1972). Goodman et al (1967) claimed that penetration through the host cell wall is a mechanical process. Akai et al (1968) noticed cracks in the

wall of barley leaves intersecting the infection peg of E. graminis, indicating that a large amount of mechanical pressure was being exerted. Manocha and Lee (1971) reported that the penetration of the mycoparasite Piptocephalis virginiana in Choanephora cucurbitarum involved mechanical means, although enzymatic penetration in this system was not ruled out.

Edwards and Allen (1970) support the concept that both modes of cell wall penetration are required. They revealed that during the infection of barley by E. graminis, penetration consists of both enzymatic digestion of the cellulose portion of the epidermis and the mechanical pushing of the infection peg through the papilla.

Once the penetration peg has gained entrance into the interior of the cell, the peg enlarges into a structure called the haustorium. The function of the haustorium has been regarded as an absorptive structure responsible for obtaining nutrients from the host cell (Bushnell, 1972). There is some experimental evidence that the transfer of nutrients occurs by way of the haustorium. Mount and Ellingboe (1969) observed that once primary haustoria had formed there was an increase in the movement of ^{32}P and ^{32}S from the host to the parasite. Manocha (1975) fed tritiated leucine to wheat leaves and demonstrated by high resolution autoradiography that labelled material was absorbed into the haustoria of wheat stem rust, Puccinia graminis tritici (race 56), during the early stages of infection and showing an absorptive

function for the haustorial structures.

In the infection process, although the host cell wall is breached, the host plasmalemma is only invaginated and remains intact during the early stages of infection (Peyton and Bowen, 1963; Berlin and Bowen, 1964; Shaw and Manocha, 1965; Ehrlich and Ehrlich, 1966; Manocha and Shaw, 1967; Bracker, 1968; Littlefield and Bracker, 1970 and 1972). This invaginated membrane, known as the extrahaustorial membrane possesses different characteristics from the non-invaginated host plasmamembrane. It differs in dimensions and stainability and it appears, when viewed following freeze etching, to be nongranular while the host plasmamembrane is granular in appearance (Bracker and Littlefield, 1973).

Shaw and Manocha (1965) showed that during very early stages of infection the extrahaustorial membrane is tightly appressed to the surface of the haustorium. As the infection proceeds into later stages, a separation develops between the membrane and the haustorial cell wall. This zone is composed of gels, semiliquids, solids or fibers (Chou, 1970; Zimmer, 1970; Hanchey and Wheller, 1971; Bushnell, 1972 and Manocha, 1975) and is termed the haustorial sheath (Bracker and Littlefield, 1973). The expression is equivalent to the "encapsulation" of Ehrlich and Ehrlich (1963), Berlin and Bowen (1964), and Shaw and Manocha (1965); and to the "zone of apposition" of Peyton and Bowen (1963). All these terms refer to the area between the haustorial cell wall and the extrahaustorial membrane. Virtually every haustorium is

surrounded by a sheath of one sort or another (Bracker and Littlefield, 1973).

The origin and the biochemical nature of haustorial sheaths are still speculative. Ehrlich and Ehrlich (1963) believed that the haustorial sheath, in wheat rust, was of haustorial protoplasmic origin. Peyton and Bowen (1963), Berlin and Bowen (1964) and Shaw and Manocha (1965) supported the contention that the haustorial sheath is of host origin. Shaw and Manocha (1965) reported the development of smooth endoplasmic reticulum in the host cell close to the sheath and noted that it could be associated with sheath formation, since it was not present in abundance in the non-infected cells. It has been suggested that smooth endoplasmic reticulum could be responsible for the secretion of host cell wall-like material into the sheath zone (Manocha, 1966). McKeen et al (1966) and Zimmer (1970) believed the sheath to be composed of by-products from the host-parasite interaction. Zimmer (1970) noted that apparently physiologically active haustoria were not surrounded by any sheath formations in cytoplasmic free host cells. He also observed lomasomes in both haustoria and hyphal cells of P. carthami which may be involved in cell wall deposition and suggested that if the sheath is not a product of the host cytoplasm then it must be a product of the host-parasite interaction. Chow (1970) and Hardwick et al (1971) on the other hand, regarded the sheath as being part of the fungal wall.

The haustorial sheath often changes in thickness with age (Shaw and Manocha, 1965; Bushnell, 1972). Chou (1970)

found that the sheath in cabbage cotyledons infected with Peronospora parasitica appeared to have three layers. Similarly, Zimmer (1970) reported three prominent regions of the sheath encircling P. carthami, an outer zone believed to be primarily liquid in composition, a middle area permeated by electron-opaque particles and an inner layer which may be deposited by-products of the physiological processes of the host-parasite interaction. Zimmer (1970) also noticed that the fibrillar portions of the sheath resembled loosely organized cell walls.

The extrahaustorial sheath may play a role in host-parasite relations. In a resistant reaction to Puccinia graminis (Shaw and Manocha, 1965; Manocha, 1966 and 1975) the sheath developed more rapidly than in the susceptible reaction of respective, khapli and little club, wheat varieties. Zimmer (1970) showed that the sheaths around haustoria in incompatible hosts were more amorphous than in a compatible host. Shaw and Manocha (1965), Stavely et al (1969) and Zimmer (1970) observed that the breakdown of the plasmamembrane and of other cytoplasmic structures in infected cells was more rapid in resistant than in susceptible hosts. Heath (1972) found that in immune hosts of Uromyces phaseoli, the extrahaustorial membrane lost stability and broke down. This was followed by the degradation of the haustorial sheath, thus starving the haustorium of its required nutrients.

Bushnell (1972) stated in his review that it was not known if the extrahaustorial sheath served as a barrier

to the movement of substances, facilitated such movement or was unrelated to the interchange of substances between host and parasite. Attempts to ascertain the role of the sheath in host-parasite relationships have recently been made utilizing the technique of autoradiography. Ehrlich and Ehrlich (1970) studied the transfer of ^{14}C from labelled germinating uredospores into wheat host cells. Their autoradiographs showed the presence of developed silver grains throughout the host tissues in the infection center, indicating the passage of materials from haustorium to host through the sheath. Manocha (1975), in an examination of haustoria and host parasite interfaces in wheat carrying the temperature sensitive Sr6 allele for resistance to race 56 of Puccinia graminis tritici, found that in the early stages of infection labelled leucine was incorporated into the haustoria of P. graminis. However, as the disease progressed in both susceptible and resistant hosts, there was a decrease in the amount of label incorporated. The uptake of ^{3}H-leucine into the haustoria ceased when the label was fed after 12 days in the susceptible host and after 4 days in the resistant host. These times coincide with the times of sheath development, and indicate that the sheath zone functioned as a defensive barricade against the parasite. Manocha (1975) and Ehrlich and Ehrlich (1970) showed that there was no accumulation of labelled nutrients in the sheath, thereby indicating that the sheath did not act as a nutritional sink for the parasite.

Recently, mycoparasites have been used in studies of host-parasite relations, owing to their ease in culturing, rigid control of the host nutrition, relative simplicity of the host cell and the apparent interface similarity with those of plant-fungal diseases (Manocha and Lee, 1971).

Much of the earlier research on mycoparasites concentrated on taxonomy, host ranges, nutritional requirements, cultural conditions and on the various modes of infection. Such investigations have been reviewed by Boosalis, 1956; Barnett, 1964; Madelin, 1968 and Barnett and Binder, 1973.

The filamentous haustorial mycoparasites belong to the morphological group, merosporangiferous Mucorales (Benjamin, 1959 and 1961). The principal genera are Syncephalis and Piptocephalis in the family Piptocephalidaceae and Dispira, Dimargaris and Tieghemiomyces in the family Dimargaritaceae (Barnett and Binder, 1973). The most extensively studied mycoparasite at the electron microscopic level is Piptocephalis virginiana. Armentrout and Wilson (1969) examined the fine structure of the haustorial formation of this mycoparasite infecting the host Mycotypha microspora. It was shown that upon infection the host cytoplasm became rich with tubular endoplasmic reticulum and mitochondria, but there were fewer spherosomes than in the non-infected host cells. The mature haustoria of P. virginiana were bounded by an electron dense sheath with a convoluted surrounding membrane. Armentrout and Wilson (1969) hypothesized a two way exchange through the sheath matrix which according to them was constructed of materials originating from both

the host and parasite. Acid phosphatase activity determined by the use of a lead sulphide precipitation procedure was shown to encompass the entire surface of the haustorium. From these results it was concluded that acid phosphatase and possibly other enzymes, present in the sheath matrix could be released into the host cytoplasm via "blebs" which were observed in the sheath matrix. It was also suggested that the return transport from the host could be accomplished by the endoplasmic reticulum which appeared close to the haustorial sheath at the time of sheath development. Rosenthal (1970) raised doubts concerning the interpretation of any acid phosphatase cytochemical data due to the non-specificity of the lead sulphide precipitation procedure. Thus rendering the interpretations of Armentrout and Wilson (1969) questionable.

Manocha and Lee (1971) studied the host-parasite relations of the mycoparasite, P. virginiana on the host Choanephora cucurbitarum. The young haustorium was seen to be filled with ribosomes, mitochondria, cisternae of endoplasmic reticulum and a few small vesicles. The host cell wall was breached by an infection peg which elongated and became the haustorial neck. The authors described the haustorial neck as being surrounded by a collar, which is an extension of the host cell wall. Studies conducted at different time intervals after inoculation, on the stages of development of the haustorium and sheath, indicated that the haustorial cell wall was closely appressed to the host

cell wall in the collar region. However, a thin electron-transparent zone became visible around the haustorium at 20-24 hours after inoculation. This zone was bounded by the plasmalemma of the host cell which was never penetrated by the haustorium. Near the host plasmalemma was an extensive network of smooth surface endoplasmic reticulum which sometimes completely encircled the haustorium. On the other hand, healthy host cells had only a few cisternae of endoplasmic reticulum (ER) and therefore the development of ER in the infected cell was a noticeable change. Vesicular activity of the host cytoplasm also increased in the vicinity of the haustorium and some dense vesicles were observed at the haustorial surface.

The sheath of older haustoria, 36 hours after inoculation, was composed of material with the same electron density as that of the cell wall of the host. The endoplasmic reticulum system, which had developed during 24-30 hours after inoculation, had completely disappeared by 36 hours and the haustoria began to show signs of vacuolation. Their electromicrographs did not show any "blebs" as had been found by Armentrout and Wilson (1969). Manocha and Lee (1972) used the technique of high resolution autoradiography to show that tritiated N-acetyl-D-glucosamine, a precursor of chitin, when fed to the infected cells at different time intervals after inoculation, was incorporated preferentially in the haustorial sheath and the host cell wall. These results suggested that the sheath is basically composed of the same material as the host cell wall.

Polyoxin D Studies

Polyoxin D, one of the main components of the polyoxin class of nucleotide antibiotics, is produced by Streptomyces cacaoi var. asoensis and effects the growth of some filamentous fungi, but has no effect on bacteria or yeasts (Isono et al, 1967; Isono and Suzuki, 1968). It has been found to be active against such plant pathogens as Cochliobolus miyabeanus, Alternaria kikuchiana and Piricularia oryzae (Sasaki et al, 1968; Ohta et al, 1970; Endo et al, 1970).

Sasaki et al (1968), using growing cells of C. miyabeanus, found that Polyoxin D inhibited the incorporation of ^{14}C-glucosamine into the cell wall fraction which was extractable by hot acid hydrolysis, whereas it showed no inhibitory effect on the uptake of the label by proteins and nucleic acids. Ohta et al (1970) showed that in the fungus Neurospora crassa, Polyoxin D at a concentration of 100 µg/ml, which is a level compatible with its antifungal activity, nearly halved the uptake of ^{14}C-glucosamine into the chitin fraction and caused an accumulation of the nucleotide, uridine diphosphate N-acetylglucosamine (UDP-GlcNAc). Since glucosamine must be converted to UDP-GlcNAc prior to its incorporation into chitin, the results indicated that the inhibition of chitin synthesis occurred via the enzyme, chitin synthetase (Isono and Suzuki, 1968). The chemical structure of Polyoxin D and UDP-GlcNAc are very similar (Isono and Suzuki, 1968), suggesting a structural basis for the competitive activity of Polyoxin D.

Kinetic investigations have shown that polyoxins are sensitive to varying pH values (Hori et al, 1974a) and it has been demonstrated that the nucleoside moieties of polyoxins and of UDP-GlcNAc act at the binding site of the enzyme, chitin synthetase. This specific binding site of the enzyme corresponds to the uridine moiety of UDP-GlcNAc and to the pyrimidine nucleoside moiety of polyoxins (Hori et al, 1974b). Accordingly, it was inferred that the inhibition of chitin synthetase by polyoxins results from the competition between nucleoside moieties of polyoxins and of UDG-GlcNAc on the binding site of the enzyme (Hori et al, 1974b).

Endo, Kakiki and Misato (1970) provided corroborating evidence for the earlier work of Endo and Misato (1969) by culturing C. miyabeanus in Polyoxin D which caused the fungus to produce osmotically sensitive protoplast-like structures, indicating that the primary site of action of the antibiotic, in vivo, is in the cell walls. Similar effects were noted by Bartnicki-Garcia and Lippman (1972) with Mucor rouxii when it was exposed to the effects of Polyoxin D. They observed that the bursting of the osmotically sensitive protoplast-like structures of M. rouxii occurred at the hyphal apices. These hyphal apices are the regions of high chitin synthetase activity and of chitin synthesis in the fungus. The localization of chitin synthesis was demonstrated, by means of autoradiographic studies, in the apices of M. rouxii by McMurrough et al (1971) and in C. cucurbitarum by Manocha and Lee (1972). Owing to the unique properties

of Polyoxin D, such as its specific competitive inhibition of chitin synthetase, it has been used to study the dimorphic properties of the fungus, M. rouxii, by altering the chitin content of the cell walls (Bartnicki-Garcia and Lippman, 1972).

Polyoxin D, therefore, has potential as an experimental tool to manipulate chitin synthesis and fungal morphology. In this investigation the action of this antibiotic on the cell walls of Choanephora cucurbitarum and on the host-parasite interface of C. cucurbitarum and the mycoparasite Piptocephalis virginiana was studied.

Materials and Methods

Cultures of Choanephora cucurbitarum (Berk. and Rav.) Thaxter and Piptocephalis virginiana Leadbeater and Mercer, were routinely grown at 23°C ± 1°C on a solid medium consisting of malt extract, 20 g; yeast extract, 2 g; agar, 20 g in 1 litre of distilled water; or on a liquid medium of the same composition as above minus agar.

I. CELL WALL STUDIES

Isolation of the Cell Wall

Cultures of C. cucurbitarum, grown for 48 h (unless otherwise stated) on a reciprocating shaker (40 strokes/min) in liquid medium at pH 6.8, were harvested by filtration on a coarse-sintered glass filter and washed with distilled water until free of the nutrient medium. A 10 g sample (wet weight) of the harvested mycelium was suspended in 25 ml of distilled water and homogenized in a Waring Blender, at maximum speed for 5 minutes. The slurry was centrifuged at 2000 x g for 10 min; the resultant pellet was resuspended in distilled water and immersed in an ice bath. The suspension was then sonicated with a Bronwill III sonicator, using the 10 x 1.9 cm probe (green) at maximum setting for a total sonication time of 1 h (10 min on followed by 10 min cooling). The blended, sonicated cell wall fragments were then re-isolated by centrifugation as described earlier. To ensure that the cell wall fragments were free of cytoplasmic and

membrane contamination, the centrifugation and washing procedures were repeated five times. The entire isolation procedure was carried out in a cold room at 4-5°C.

For the ultrastructural investigation the isolated cell walls were immediately fixed for thin sectioning, or dried on clean glass microscope slides for heavy metal shadow coatings. Isolated cell walls, used for analytical studies were lyophilized and stored at -4°C until required.

Light and Electron Microscopy

The whole hyphae and isolated cell wall fragments were examined and photographed under a phase-constrast (Leitz-Wetzlar, Orthoplan) microscope. The complete breakage of the hyphae and the purity of the isolated cell walls were tested by a differential staining technique based on the reaction of I^{3-} with chitosan (Bartnicki-Garcia, 1962). Intact cells stained reddish brown when a drop of dilute Lugol's iodine solution was added, whereas the isolated cell walls appeared pink to violet in color indicating the absence of membranous or cytoplasmic contamination.

The replicas of the isolated cell walls for electron microscopy were prepared according to the method described by Manocha and Colvin (1967). A part of the isolated cell walls was acid hydrolyzed in 1 N HCl at 100°C for 1 hour. Suspensions of non-hydrolyzed and acid hydrolyzed isolated cell walls were smeared on to clean glass slides and allowed to dry. The slides were dipped in 0.4% Formvar

(E.F. Fullam, Inc.) in ethylene dichloride. The dried film carrying the residue was stripped from the slide to the surface of a 10% solution of ammonium hydroxide. The solution was replaced with distilled water until the pH was between 6 and 7. The replicas were mounted on copper (200 mesh) grids by depositing the grids on the film and then removing the film and grids together from the solution. The dried replicas were shadowed with Pd-Au alloy at an angle of 15°.

Thin sections of the isolated cell walls were prepared as described under the section for preparation of thin sections for electron microscopy and autoradiography.

Analytical Procedures

Chitin Analysis

The presence of chitin in the isolated cell walls of C. cucurbitarum was determined by a modified method of Sangar and Duggan (1973). The isolated cell walls were suspended in 2 N NaOH for 12 h at 23°C ± 2°C. The alkali-treated cell wall fragments were recovered by centrifugation at 2000 x g for 10 minutes. The pellet was washed with distilled water and was reisolated by centrifugation as previously described. This isolated pellet was then treated with 1 N H_2SO_4 for 16 h at 60°C. Following this treatment the alkali-acid extracted cell wall fragments were centrifuged and the pellet was again treated with 2 N NaOH for 30 min at 23°C ± 2°C. The insoluble wall

material was recovered by centrifugation and washed with distilled water and then reisolated and lyophilized. This insoluble cell wall fraction, designated as the alkali-acid insoluble cell wall fraction, was used for both infrared spectroscopy and X-ray diffractometry.

The percentage of chitin in the isolated cell wall material was obtained gravimetrically by determining the percentage of alkali-acid insoluble material present in the original weight of the lyophilized cell walls.

Reducing Sugars

Isolated cell walls were incubated in 22.5 N H_2SO_4 at 30°C for 3 hours. The acid was then diluted to 0.8 N and reheated to 97°C for 4 hours. The hydrolysates were neutralized with $BaCO_3$ and deionized with a double bed of Dowex-I (Acetate form) + Dowex-50 (H^+ form) as described by Wang and Bartnicki-Garcia (1970). The hydrolysates were evaporated to dryness at 40°C, in a rotary evaporator. The resultant residue was redissolved in 1 ml of dimethsulfoxide, silylated with 0.2 ml of hexamethyldisilazane in 0.1 ml of trimethylchlorosilane, and extracted with 1 ml cyclohexane (Ellis, 1969). The silylated monosaccharides were injected into Hewlett Packard 5700 A gas-liquid chromatograph fitted with a copper column, 1/8 inch x 7 foot, packed with 4% SE-52 on 60-80 mesh Chromosorb W (Sweeley et al, 1963). N_2 flow rate 20 ml/min, air 240 ml/min, H_2 60 ml/min. The programmed column temperature was 110-300°C at a rate of 4°C/min, with the flame-ionization detector temperature at

300°C, and the injection port temperature at 200°C. Qualitative determination of sugars was made by co-chromatography with known standards. Quantitative estimations were made using an internal standard.

Amino Sugars

Isolated cell walls were hydrolyzed with 6 N HCl in sealed ampules under N_2 at 105°C for 12 hours. The HCl was evaporated to dryness under vacuum over NaOH pellets at 45°C. The residue was silylated and the determinations were made as described for the H_2SO_4 hydrolysates.

Protein Determination and Amino Acid Analysis

Twenty-milligram samples of lyophilized, isolated cell walls were homogenized in 10 ml of cold (4-5°C) 5% trichloroacetic acid (TCA). The proteins were resolubilized with 1 N NaOH and the insoluble cell wall material was removed by centrifugation at 2000 x g for 10 minutes. The protein determinations on the solubilized proteins were made according to the procedure of Lowry et al (1951). The amino acid analysis of the isolated proteins was achieved according to the method described by Manocha and Colvin (1967). The samples of protein were analyzed at the National Research Council through the courtesy of Dr. J. R. Colvin.

Lipid Extraction and Fatty Acid Analysis

The lipids from lyophilized isolated cell walls were extracted 3 times with 20 volumes chloroform-methanol (fraction I) as described by Deven and Manocha (1975). The

same cell walls were further extracted with 10% methanolic potassium hydroxide (fraction II) as described by Safe (1974). The fatty acid composition of lipid fractions I and II were determined by gas-liquid chromatography (glc) as described by Deven and Manocha (1975). A Hewlett Packard 5700 A gas-liquid chromatograph was used for the analysis of the fatty acid methyl esters. The 180 cm x 3 mm glass column was packed with 10% Silar 10C on Chromosorb Q AW-DMCS 100-120 mesh size. The temperature was programmed for 135-200°C at a rate of 2°/min.

Ash Content

The percentage of ash was determined by heating a known amount of lyophilized isolated cell wall material in a muffle furnace to 700°C for 6 h and then weighing the residue.

II. PREPARATION OF THIN SECTIONS FOR ELECTRON MICROSCOPY AND AUTORADIOGRAPHY

Electron Microscopy

Thoroughly washed mycelia of both host and parasite, cultured on solid medium (previously described) were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer at pH 6.8 for 2 h and post-fixed in 2% OsO_4, prepared in the same buffer, for 2-3 h, *in vacuo*. The mycelia were washed in distilled water and stained with 0.5% uranyl acetate overnight. The isolated cell walls were fixed by the same procedure as described above, omitting the uranyl acetate staining. The

samples were dehydrated in an ethanol and propylene oxide series and were embedded in a Spurr mixture (Spurr, 1969). Thin sections were cut on a Reichert Ultramicrotome UM3 using a Dupont diamond knife and were examined in a Philips EM 300 at 60 Kv after staining with lead citrate (Reynolds, 1963).

Autoradiography

To determine the incorporation level of chitin precursors in the cell wall, spores of the host and parsite fungus were inoculated on a liquid medium and allowed to grow for 14 h, then transferred to a medium labelled with 25 µCi/ml of tritiated N-acetyl-D-glucosamine (specific activity 4 Ci/mM) and allowed to feed for 2 h. The labelled hyphae were then transferred to an identical medium containing non-radioactive N-acetyl-D-glucosamine for 1 hour. To investigate the incorporation of label in the host-parasite interface (sheath zone) the infected host cells 16, 20, 24, 28, 32 and 36 h after inoculation, were fed on labelled medium for 2 h. In controls, tritiated N-acetyl-D-glucosamine was replaced by the non-radioactive compound. All samples prior to fixation for electron microscopy were thoroughly washed in non-radioactive N-acetyl-D-glucosamine and distilled water.

The method of fixation and embedding was the same as described under electron microscopy. Thin sections were mounted on carbon coated grids. A gelled mono-granular layer of Ilford L-4 emulsion was applied on the sections following the method of Caro and VanTubergen (1962). The

grids were attached to clean glass microscope slides with cellophane tape (sticky both sides), and exposed for 2-3 weeks at 4°C in sealed light proof boxes containing silica gel. The autoradiographs were developed in Microdol X at 20°C for 5 min and fixed in acid fixer for 5 minutes. The sections were stained with lead at high pH (Karnovsky, 1961) for 10-15 minutes. Numerous micrographs were examined for each treatment and typical representatives of each were selected.

III. WHOLE CELL FRACTIONATION

Fourteen hour old cultures of C. cucurbitarum were fed on labelled medium (as described in autoradiography) for 2 h followed by a chase period of 1 h in identical medium containing non-radioactive compound. The mycelia, harvested by filtration, were washed thoroughly with distilled water and were homogenized in a Waring blender. The homogenized cells were sonicated for 1 h (10 min on 10 min cooling) with a Bronwill Biosonik III using a 10 x 1.9 cm probe. The sonicated mixture was centrifuged at 2000 x g for 10 min in a Sorval B20 centrifuge. The pellet was resuspended in distilled water and sonicated as above, and this process was again repeated. The pellet from the last centrifugation was checked for purity of cell wall fraction as described under cell wall studies, and was lyophilized for further investigation. The combined supernatants were centrifuged at 10,000 x g for 10 min in an ultracentrifuge IEC/B-60. The pellet represented the mitochondrial fraction and the

supernatant was further centrifuged at 100,000 x g for 20 minutes. The pellet contained the microsomal fraction, the supernatant being the cytoplasmic fraction (for purposes of simplification see flow chart fig. 1).

Aliquots (0.5 ml) from each separation in the fractionation procedure were mixed with 9.5 ml of Aquasol Universal LSC Cocktail (New England Nuclear Corp.) and counts were taken on a Packard, model 3310, Tri-Carb Liquid Scintillation Spectrometer. The quenching curve was determined using an external standard.

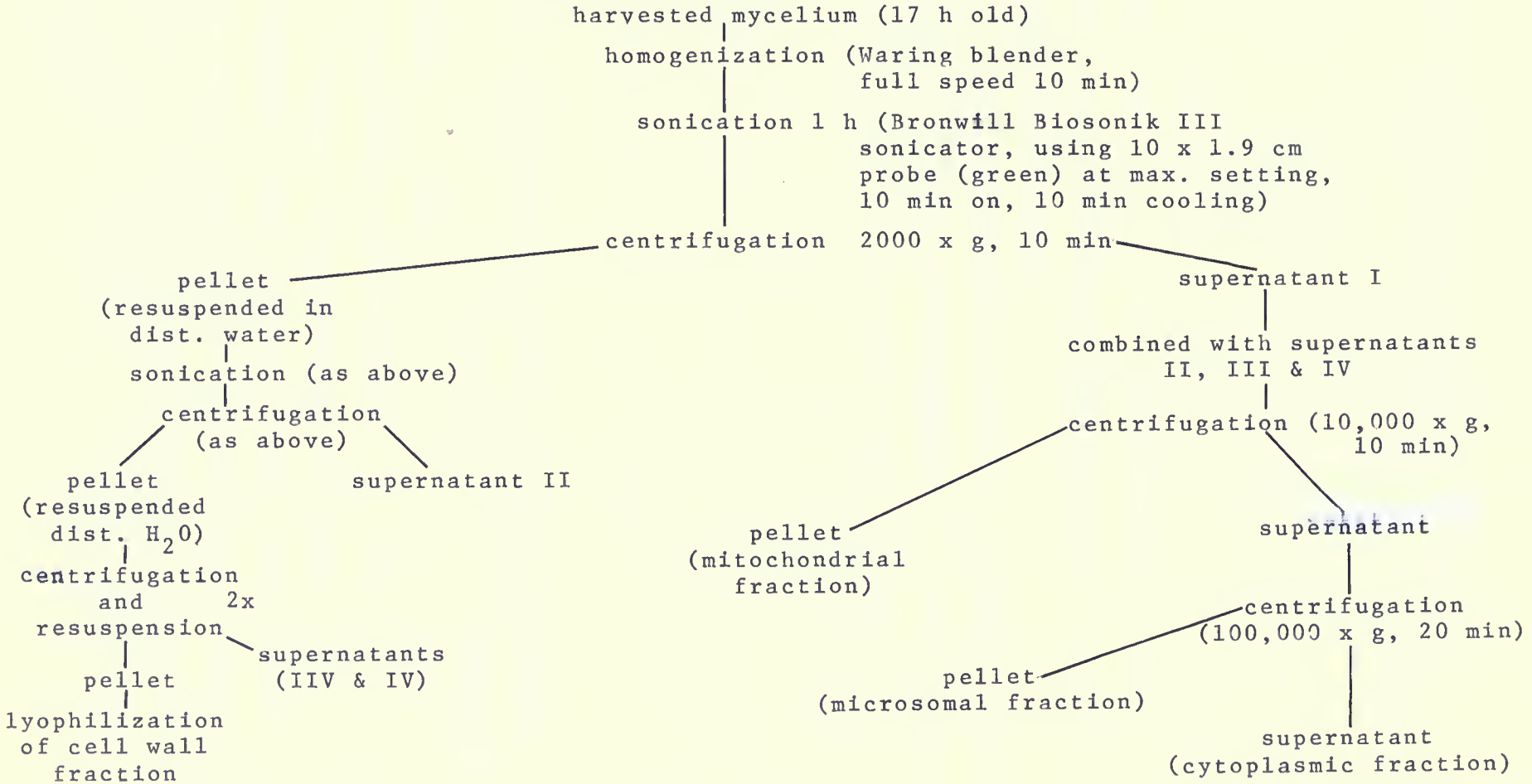
For the subfractionation experiment, isolated radioactive cell walls of C. cucurbitarum were prepared as described above. The isolated walls from the fractionation experiment were subfractioned and the insoluble material (chitin) was isolated as described under analytical procedures. The radioactivity counts were made as previously described.

IV. POLYOXIN D STUDIES

For the growth study, equivalent spore suspensions of C. cucurbitarum were incoulated in 250 ml Erlenmeyer flasks containing 100 ml of liquid medium supplemented with either 19, 95, 190, 380 and 760 µM Polyoxin D. Specimens were sampled after 24, 48 and 72 hours. Mycelia were collected on 0.45 µ millipore filters, washed with distilled water, dried in an oven at 100°C for 30 min, and finally dessicated under vacuum to a constant weight. The control flasks contained no Polyoxin D.

Fig. 1

Whole Cell Fractionation of C. cucurbitarum



Note: The high speed centrifugations (10,000 and 100,000 x g) were carried out using an IEC/B-60 Ultracentrifuge at $4^{o}C \pm 1^{o}C$. The remainder of the fractionation procedure was accomplished in a cold room at $4^{o}C \pm 1^{o}C$.

Effect of Polyoxin D on the Label Incorporation

Spores of C. cucurbitarum and P. virginiana were grown on liquid medium for 14 h then transferred to a medium containing 25 Ci/ml of ^{3}H N-acetyl-D-glucosamine and 380 µM Polyoxin D for 2 h, followed by another transfer to a medium supplemented with non-radioactive N-acetyl-D-glucosamine (chase period) for 1 hour. Controls were treated as described for the experimental condition except Polyoxin D was omitted from the medium.

To determine the effect of Polyoxin D on ^{3}H N-acetyl-D-glucosamine incorporation into the sheath zone, host and parasite spores were allowed to grow for 25 and 27 h and then transferred to the labelled medium with and without Polyoxin D for 2 h, then chased 1 h on non-radioactive liquid medium containing N-acetyl-D-glucosamine.

In both studies, specimens were prepared for autoradiography as previously described.

Effect of Polyoxin D on the Host-Parasite Interface

Spores of C. cucurbitarum and P. virginiana were inoculated on liquid medium for 20 h and transferred to a medium containing 380 µM Polyoxin D. Samples were taken at 8, 12, and 16 h following the transfer (or 28, 32 and 36 h following inoculation). Control cultures were transferred to fresh medium at 20 h and sampled at the same time intervals as described above. Thin sections of the cells were obtained as detailed in the section under electron microscopy.

Results

Section I; Structure and Composition of the Cell Wall of *Choanephora cucurbitarum*

A: Structure of the Cell Wall

The elongated, branched, coenocytic hyphae of *C. cucurbitarum* (Fig. 2) when subjected to mechanical isolation procedures and viewed under phase contrast microscope yielded a clean, cytoplasmic-free preparation of cell walls (Fig. 3). The purity of the preparation was verified by a histochemical differential staining technique and by electron microscopy. When the isolated cell walls were exposed to a dilute Lugol's solution, the I^{3-} reacts with chitosan to give a pink to violet colour. However, in the presence of cytoplasmic or membranous contamination the colour becomes reddish brown (Bartnicki-Garcia and Nickerson, 1962). Furthermore, thin sections of the mechanically isolated cell walls, when examined in an electron microscope, showed the walls to be free of cytoplasmic contamination and attached membranes (Fig. 4).

Electron micrographs of the intact hyphae of *C. cucurbitarum*, fixed in glutaraldehyde and postfixed in OsO_4, show the cell walls to be composed of two distinct layers differing in electron density (Fig. 5). Paladium-gold replicas of isolated, sonicated, purified walls show a uniform outer surface with amphorous texture (Fig. 6).

Fig. 2 Light microscope picture of 48 h old hyphae of C. cucurbitarum. x 2,200.

Fig. 3 Light microscope photograph of 48 h old isolate cell walls of C. cucurbitarum. x 2,200.

Fig. 4 Electronmicrograph of isolated cell wall. x 34,000.

Fig. 5 Thin section of 48 h old intact hypha of C. cucurbitarum. x 34,000.

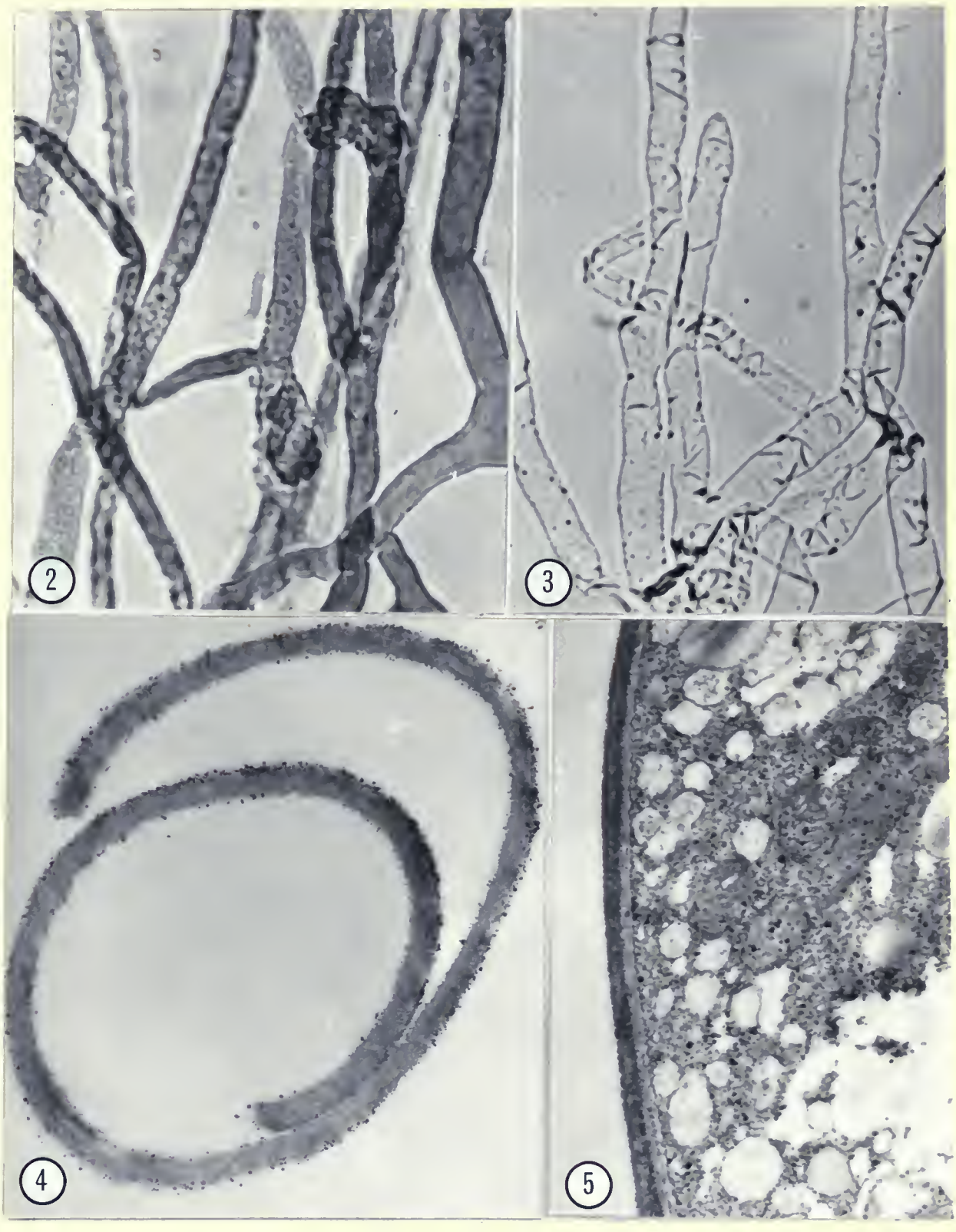

2
3
4
5

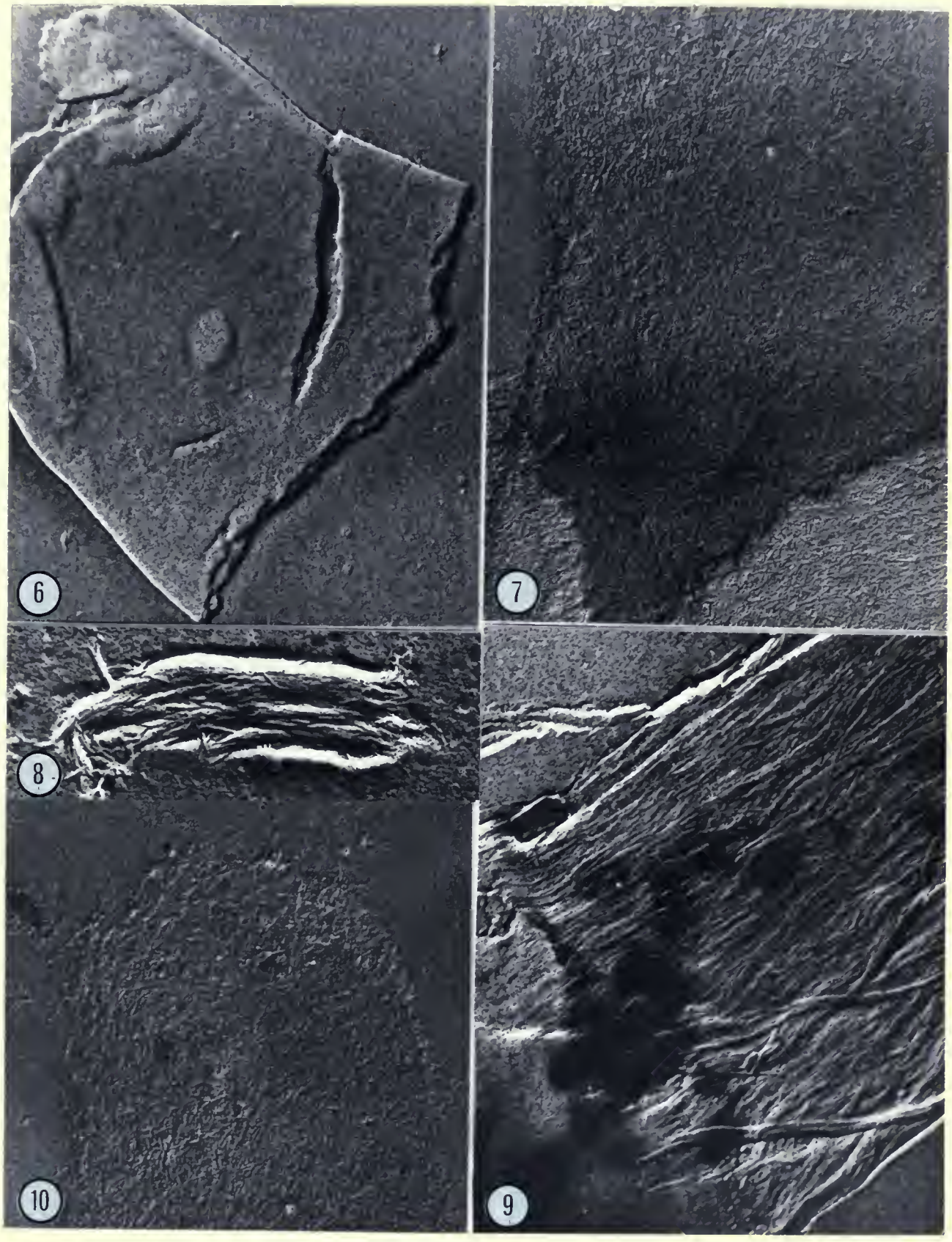
Fig. 6 Replica of the outer surface of an isolated, sonicated hyphal wall. x 12,500.

Fig. 7 Replica of the same material as figure 6 after acid hydrolysis. The chitin microfibrils are closely interwoven and arranged at random. x 12,500.

Fig. 8 Replica of a bundle of parallel arranged microfibrils of 48 h old, isolated and hydrolyzed cell wall. x 21,000.

Fig. 9 Replica of 5 day old wall showing the regular parallel orientation of the microfibrils in the inner layer of the wall. x 21,000.

Fig. 10 Replica of the surface of a hyphal tip after acid hydrolysis. Note the loose network at the apex and increasing rigidity further back. x 12,500.


6
7
8
10
9

In contrast, the replicas of the cell walls hydrolyzed with 1N HCl at 100°C for one hour displayed a network of randomly oriented microfibrils of a diameter between 100 and 200 Å (Fig. 7). Occasionally small bundles of parallel arranged microfibrils were also observed (Fig. 8). In the 5-day-old hyphae, subjected to the same chemical treatment as the 48-hour hyphae, it was found that the microfibrils on the inner side of the walls were oriented parallel to each other (Fig. 9). It was not possible to determine the angle of the microfibril orientation relative to the longitudinal axis of the cell because of the diminutive size and irregularity of the cell wall fragments. Figure 10 is a replica of a hyphal tip with random oriented microfibrils similar to those of a mature hyphal wall. However, the network is loosely arranged at the extreme tip as compared to the arrangement in the mature part of the hypha.

B: Chemical Composition of the Cell Wall

The total chemical composition of the isolated cell walls is presented in Table 1. Conclusive evidence for the presence of chitin in the cell walls of C. cucurbitarum was obtained by infrared spectrometry and X-ray diffraction analysis. The spectra of the acid-alkali resistant residues of the isolated cell walls were compared to those of authentic chitin. The infrared spectrum (Fig. 11), with absorption maxima at 895, 955, 1015, 1072, 1115, 1160, 1228, 1300, 1375, 1420, 1550, 1624, and 1655 cm^{-1}, is identical to the control. Figure 12 compares the X-ray diffraction patterns of the

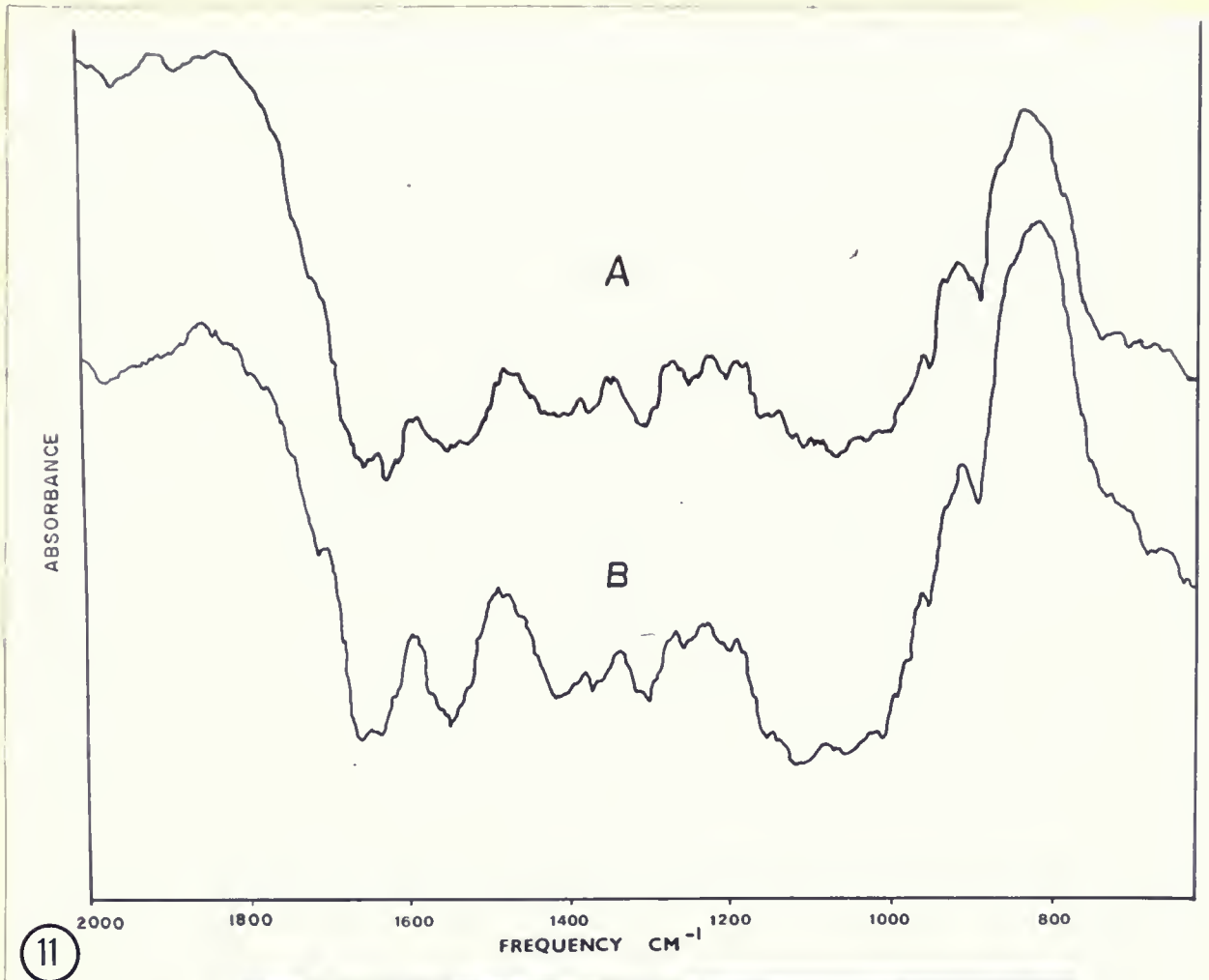
TABLE I

Composition of cell walls of Choanephora cucurbitarum

Component	% cell wall dry weight
Protein	8.2
Lipid	13.8
Acid soluble carbohydrate	35.6
Insoluble carbohydrate (chitin)	17.0
Ash	15.3

Fig. 11 Infrared spectra of authentic chitin (A) and of chitin extracted from the cell walls of C. cucurbitarum (B).

Fig. 12 X-ray diffraction pattern of authentic chitin (A). Pattern of chitin extracted from cell walls of C. cucurbitarum (B).


A
B
ABSORBANCE
2000
1800
1600
1400
1200
1000
800
FREQUENCY CM⁻¹

11


A
B

12

acid-alkali hydrolyzed cell wall with authentic chitin and confirms, by identical interplanar spacings, the existence of chitin. Quantitatively, chitin comprises a total of 17.0% of the total cell wall dry weight (Table I).

The neutral (reducing) sugars were separated by gas-liquid chromatography (glc) following conversion to their corresponding silyl derivatives. The mobilities of the silyl products were determined by co-chromatography with the known standards, and the results were summarized in Table II. The relative proportions of the neutral sugars were : L-fucose: D-mannose: D-galactose = 2.7: 1: 10.7. The H_2SO_4 hydrolysis procedure also extracted a hexuronic acid which was identified by co-chromatography to be D-glucuronic acid and it accounted for 2.4% of the total cell wall dry weight. Quantitative estimates of the reducing sugars were obtained using an internal standard. The peak areas were calculated using a recorder fitted with an integrator. The results of the calculations are listed in Table II. A doublet peak which had a shorter retention time than fucose could not be identified. The total percent of the cell wall dry weight composed of H_2SO_4 soluble sugars was 7.2%.

The amino sugars were identified by co-gas-liquid chromatography of HCl hydrolysates following silylation. The products of this extraction procedure were two amino-polysaccharides of which N-acetyl-D-glucosamine accounted for only a minor fraction. The prominent amino sugar, D-glucosamine, accounted for 28.4% of the total cell wall dry weight (Table II) and was calculated against an internal

TABLE II

Acid soluble carbohydrates of Choanephora cucurbitarum cell wall, as determined by gas-liquid chromatography

Carbohydrate	% cell wall dry weight
Fucose	0.8
Mannose	0.3
Galactose	3.2
Glucuronic acid	2.4
Unknown	0.5
Sum of H_2SO_4 soluble carbohydrates	7.2
*Glucosamine (chitosan)	28.4
Total acid soluble carbohydrates	35.6

* all determinations were made on H_2SO_4 hydrolysates except for glucosamine which was extracted by HCl hydrolysis

standard. Since chitin (poly N-acetyl-D-glucosamine) is known to be both alkali and acid insoluble, and chitosan (poly-D-glucosamine) is acid soluble (Bartnicki-Garcia and Nickerson, 1962), the glucosamine fraction was interpreted as originating from chitosan. The identity of the glucosamine was further confirmed by amino acid analysis (Table III). It comprised 67.7% of the total amino acid composition of the walls.

The protein content of the hydrolyzed, isolated cell walls was solubilized with trichloroacetic acid (TCA). The extracted protein concentration, determined by the Lowry (1951) method against hydrolyzed bovine serum albumine as a standard, was estimated as 8.2% of the total cell wall dry weight. The amino acid composition of the cell wall proteins (TableIII) showed a high proportion of aspartic acid, glutamic acid, lysine, threonine and alanine. The notable absence of cysteine and tryptophane can be explained as artifacts of the amino acid determination procedure. Upon hydrolysis, cysteine is degraded to form cystic acid, whereas tryptophane, being unstable, can not be detected using our methods (Lehninger, 1960). The amino acid profile is also devoid of hydroxyproline, however, this is well documented for fungi with chitinous walls (Crook and Johnston, 1962). Hydroxyproline exists only in fungi, algae (Punnett and Derrenbacker, 1966) and higher plants possessing cellulosic walls (Lamport and Northcote, 1960).

The extraction of the isolated hydrolyzed cell

TABLE III

Amino acid composition of cell wall proteins of *Choanephora cucurbitarum*

Amino acid	% composition of total amino acids
Aspartic acid	4.0
Threonine	2.3
Serine	2.1
Glutamic acid	3.2
Proline	1.3
Cysteine	---
Glycine	1.5
Alanine	2.2
Valine	1.4
Methionine	0.2
Isoleucine	0.9
Leucine	1.7
Tyrosine	0.6
Phenylalanine	2.1
Histidine	0.8
Lysine	2.6
Arginine	1.4
Tryptophane	---
Cysteic acid	0.1
Glucosamine	67.7
Ethanolamine	3.9

walls with chloroform-methanol yielded a lipid fraction (I) which comprised 12.8% of the cell wall dry weight. A second extraction with methanolic potassium hydroxide resulted in an additional lipid yield (fraction II) of 1% by weight. Ten different fatty acids were identified from the cell wall lipids (TableIV). In both the isolated lipid fractions (TableIV), the major fatty acids were palmitic (16:0), stearic (18:0), oleic (18:1) and linoleic (18:2) with lesser amounts of myristic (14:0), palmitoleic (16:1), pentadecanoic (15:0) and γ-linolenic (18:3) acids also being recorded. Only trace elements of lauric (12:0) and heptadecanoic (17:0) acids were detected. Although the second extraction allowed recovery of the identical acids as in fraction I, there were significant decreases in the percent composition within each fraction for oleic (31.2 to 6.8%) and palmitic (30.6 to 10.1%) acids. In addition, fraction II showed a large increase in its proportion of linoleic acid (10.7 to 71.3%). γ-linoleic acid constitutes 6.6% of the total fatty acids in fraction I; on the other hand, in fraction II, only trace amounts of this acid were present.

The incineration of the isolated hydrolyzed cell walls yielded a substantial amount of ash (15.3%), the composition of which was not determined in the investigation.

TABLE IV

Fatty acid composition of total lipid fraction of the cell wall of Choanephora cucurbitarum

Fatty acid esters	Percent composition of fatty acids	
	Fraction I	Fraction II
$C_{12:0}$ (Lauric acid)	T *	T
$C_{14:0}$ (Myristic acid)	3.1	2.0
$C_{15:0}$ (Pentadecanoic acid)	1.2	T
$C_{16:0}$ (Palmitic acid)	30.6	10.1
$C_{16:1}$ (Palmitoleic acid)	6.5	3.5
$C_{17:0}$ (Heptadecanoic acid)	T	T
$C_{18:0}$ (Stearic acid)	10.2	6.4
$C_{18:1}$ (Oleic acid)	31.2	6.8
$C_{18:2}$ (Linoleic acid)	10.7	71.3
$C_{18:3}$ (γ-Linolenic acid)	6.6	T

* T = trace

Section II: Fine Structure and Composition of the Host-Parasite Interface

A. Studies on the Fine Structure of Host-Parasite Interface

As previously reported for other haustorial parasite-host systems (Shaw and Manocha, 1965; Manocha and Lee, 1971), the young haustorium, enclosed in its own cell wall, was surrounded by the host plasmamembrane which was highly invaginated and was called the extrahaustorial membrane. This extrahaustorial membrane did not rupture and was appressed to the haustoria during the early stages of infection, i.e. 16-20 h after inoculation (Fig. 13). As the host-parasite relationship developed (22 h) the extrahaustorial membrane began separating from the haustorium (Fig. 14). The extrahaustorial membrane was completely encircled by the endoplasmic reticulum. The host cytoplasm also showed a concomitant increase in vesicular activity near the haustorium. By 22-24 h, the membrane completely separated from the haustorium and a distinct electron transparent zone became apparent (Fig. 15). The enlarged interface zone began to show an accumulation of electron dense material by 28-32 h following inoculation. This material was termed the haustorial sheath (Fig. 16). By 36 h, the sheath was fully mature and showed distinct layers which had similar electron densities to those of the host cell wall (Fig. 17). Figures 18 and 19 show the point of penetration and structure of the host-parasite interface as examined at 22 and 36 h after inoculation. There was no sign of sheath at the early stage of haustorium development.

Abbreviations: Ex = extrahaustorial membrane; H = haustorium; S = sheath; W = host cell wall

Fig. 13 Thin section of host-parasite interface 20 h following inoculation. x 27,500.

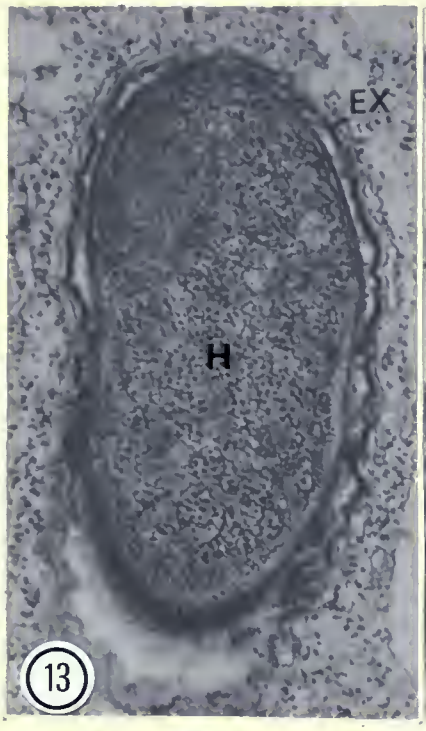
Fig. 14 Thin section of host-parasite interface 22 h following inoculation. x 27,500.

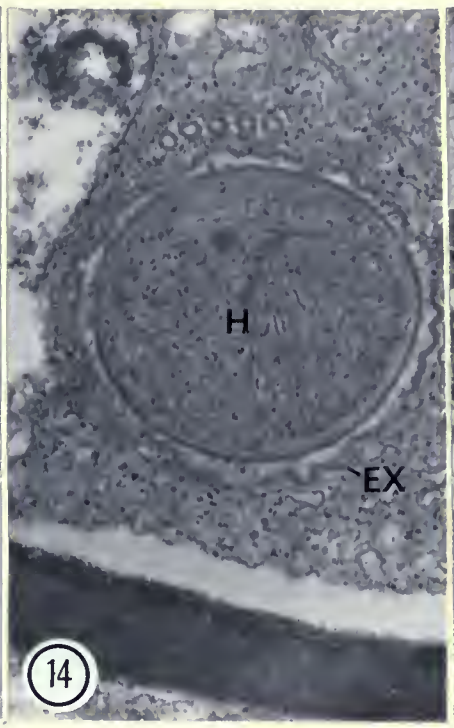
Fig. 15 Thin section of the host-parasite interface 24 h after inoculation. x 27,500

Fig. 16 Thin section of host-parasite interface 28 h following inoculation. x 27,500.

Fig. 17 Thin section of host-parasite interface 36 h following inoculation. x 27,500.

Note the absence of sheath development in figures 13, 14 and 15, and the presence of a distinct sheath around the haurtoria in figures 16 and 17.


EX
H
13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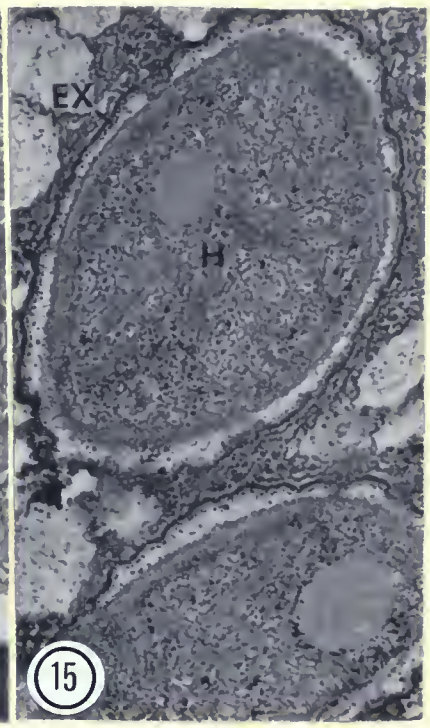
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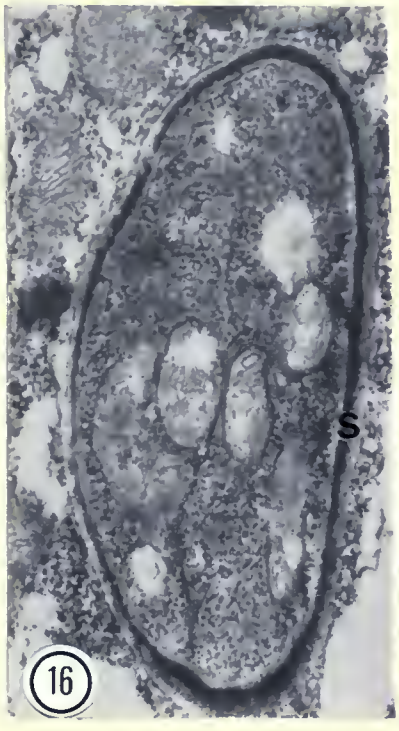
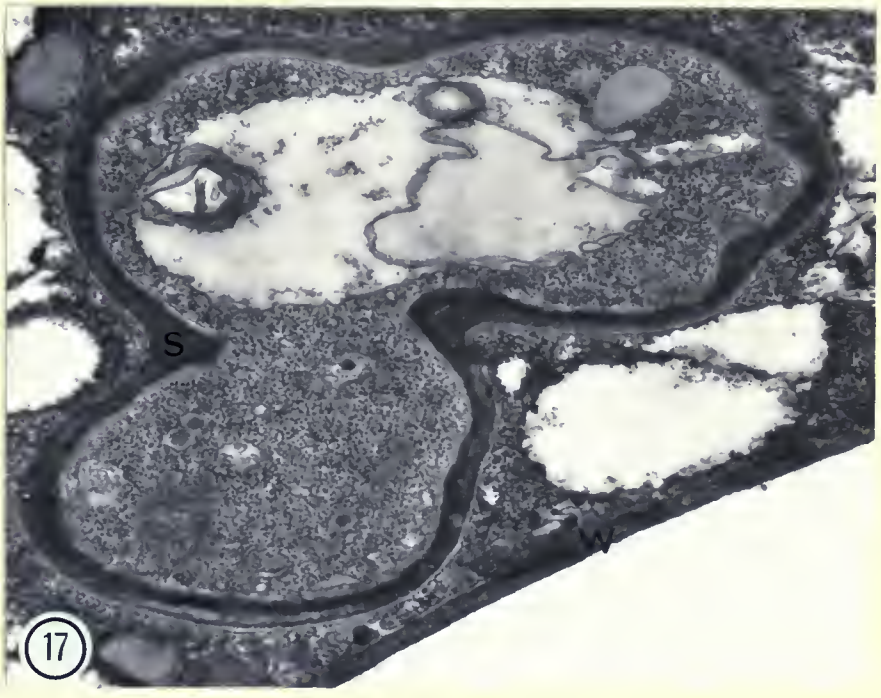
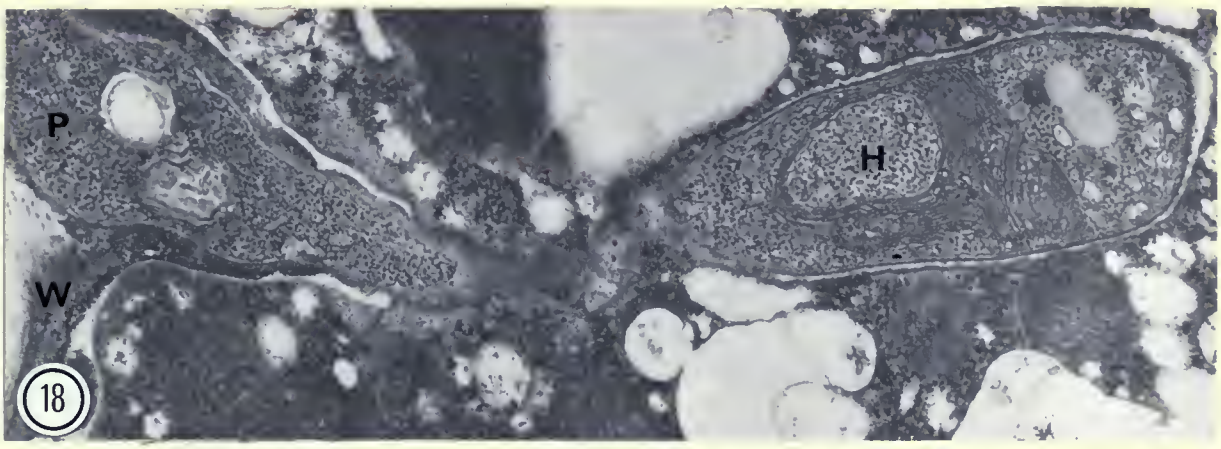

S
16

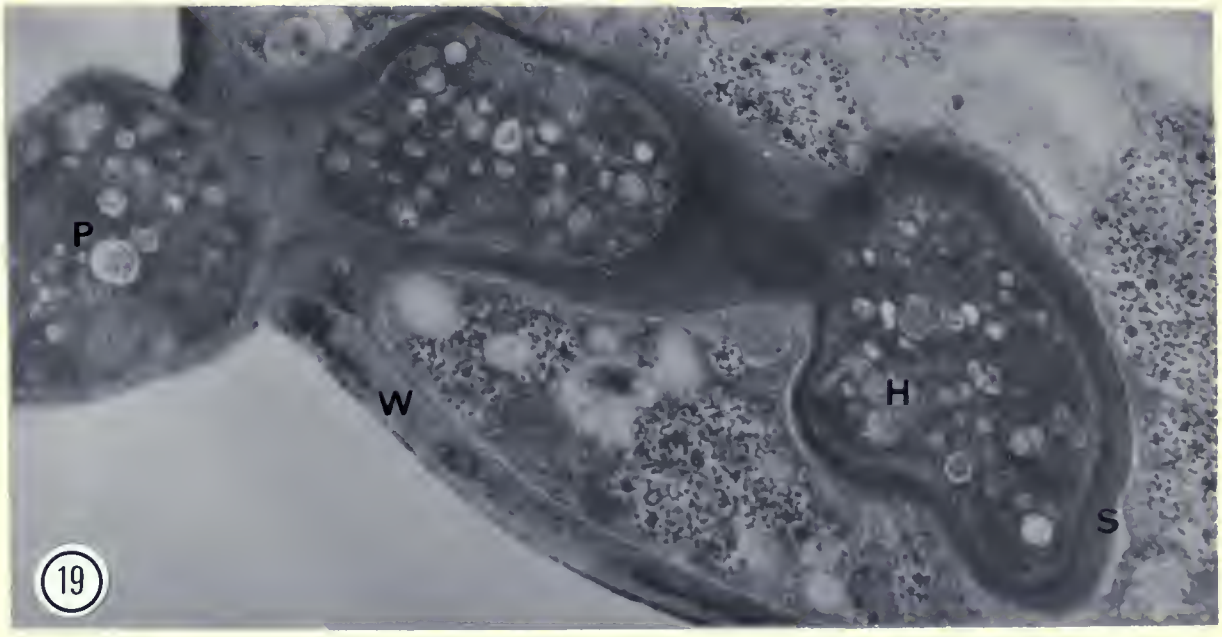

S
W
17

Abbreviations: P = parasite; H = haustorium; W = host cell wall; S = sheath

Fig. 18 Longitudinal section of 20 h old infected host cell. x 25,000.

Fig. 19 Longitudinal section of 36 h old infected host cell. Note the presence of a distinct sheath around the haustorium. x 25,000.


P
H
W
18


P
W
H
S
19

B. Composition of the Sheath

(i) High Resolution Autoradiography

(a) Incorporation of ^{3}H N-acetyl-D-glucosamine in the Host Cell Wall

Due to the similarity in the electron density and the layered appearance of the sheath with that of the host cell wall, the possibility that the sheath was composed of host cell wall material was investigated using the technique of autoradiography. When the spores of the host and parasite, C. cucurbitarum and P. virginiana respectively, were grown on a medium containing tritiated N-acetyl-D-glucosamine, the hyphal walls of C. cucurbitarum (17 h old) became heavily labelled with the radioactivity as indicated by the number of silver grains exposed in the autoradiographs (Figs. 20 and 21). However, no grains were observed on the parasite (Fig. 22) or in the control condition of the host fungus (Fig. 23).

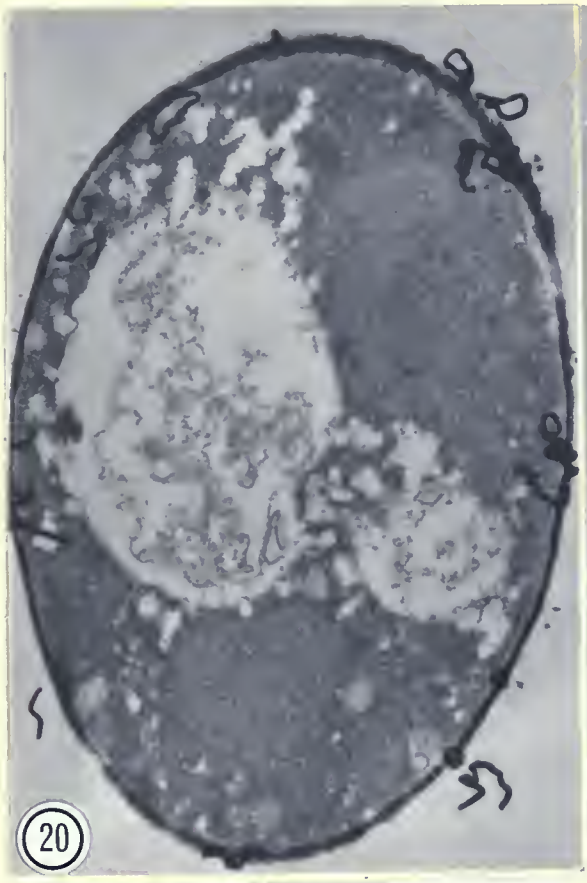
(b) Incorporation of ^{3}H N-acetyl-D-glucosamine in the Sheath Zone

An examination of autoradiographs of young infected host (16-20 h after inoculation) fed with ^{3}H N-acetyl-D-glucosamine, showed that the silver grains developed only along the host cell wall and they were not observed in or around the host-parasite interface. When the label was fed for 2 h to 28 h old infected hyphae, the silver grains were also observed in or around the vicinity of the host-parasite interface (Fig. 24). The autoradiographs prepared from older infected cells (32 h and more) showed sheath development around the haustorium and also the

Figs. 20 and 21 Autoradiographs of ^{3}H N-acetyl-D-glucosamine fed host hyphae (17 h old) showing numerous silver grains along the cell walls. x 12,500.

Fig. 22 Autoradiograph of tritiated N-acetyl-D-glucosamine fed parasite spore (17 h old). Note the absence of silver grain development. x 30,000.

Fig. 23 Autoradiograph of C. cucurbitarum, control host hypha. Note the absence of silver grain development. x 12,500.



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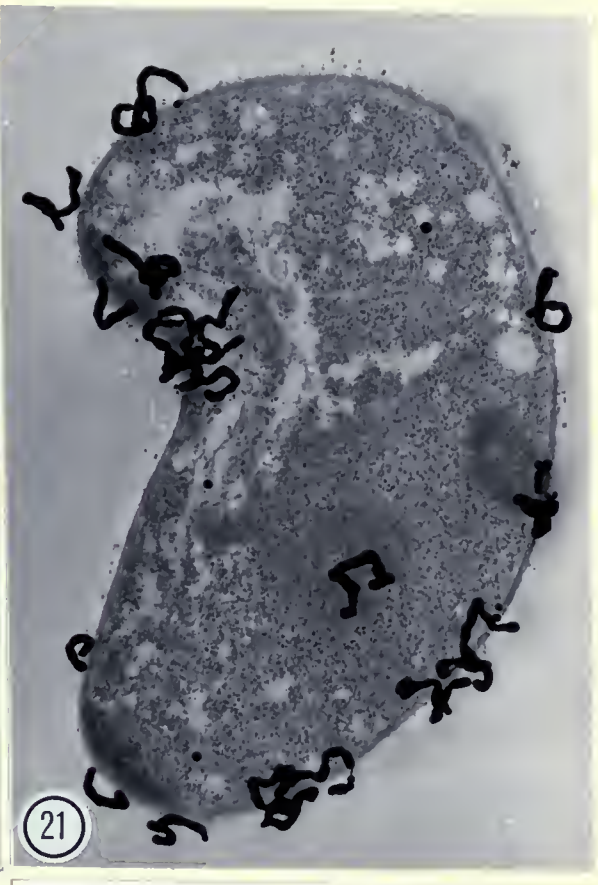

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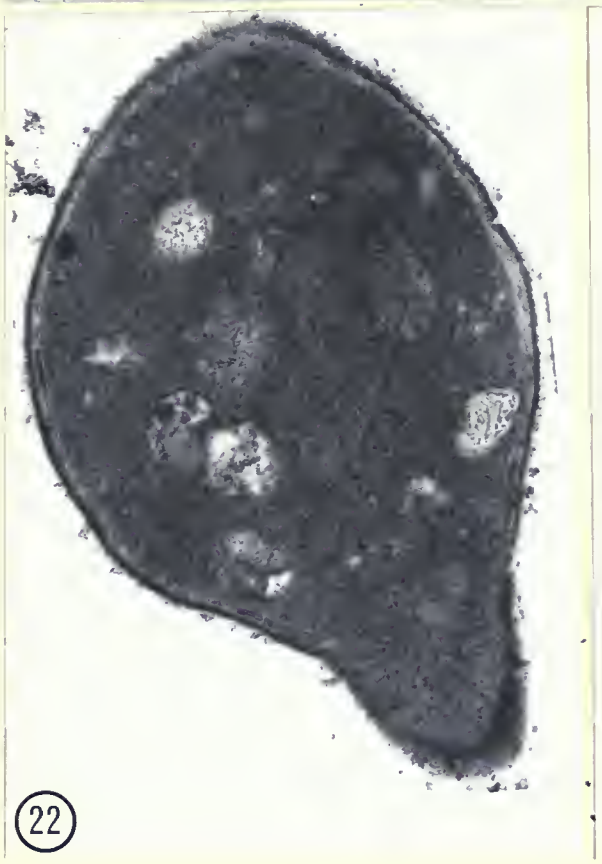

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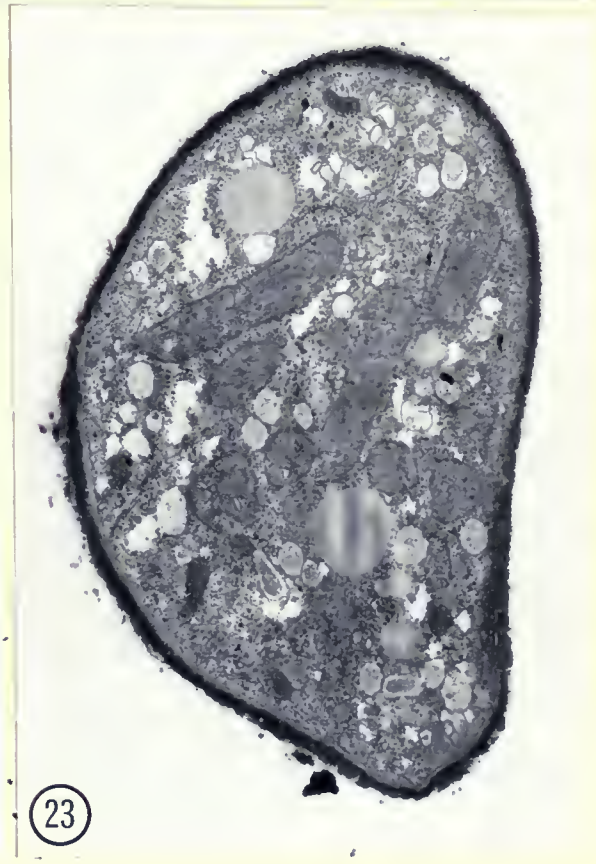
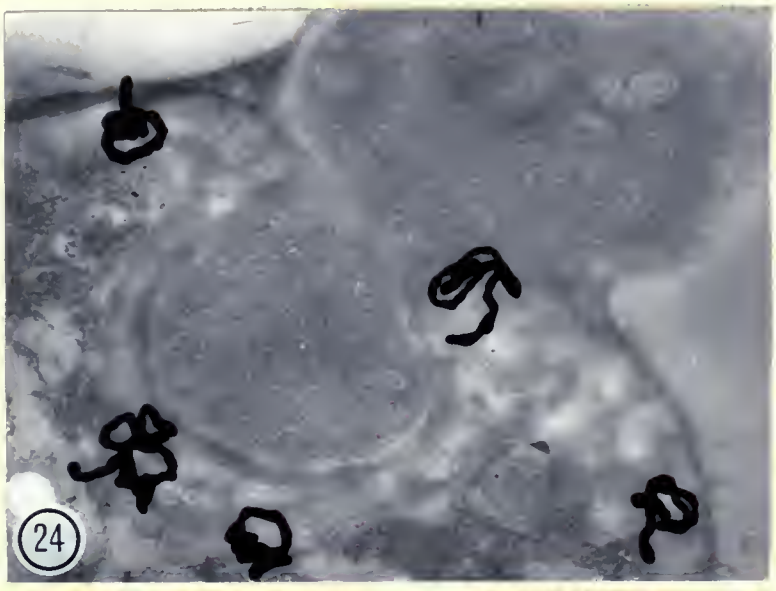


23

Fig. 24 Autoradiograph of 30 h old infected hypha fed ^{3}H N-acetyl-D-glucosamine. x 27,500. Note the presence of silver grains along the host cell wall and around the haustorium.

Fig. 25 Autoradiograph of 32 h old infected hypha fed ^{3}H N-acetyl-D-glucosamine. x 27,500.



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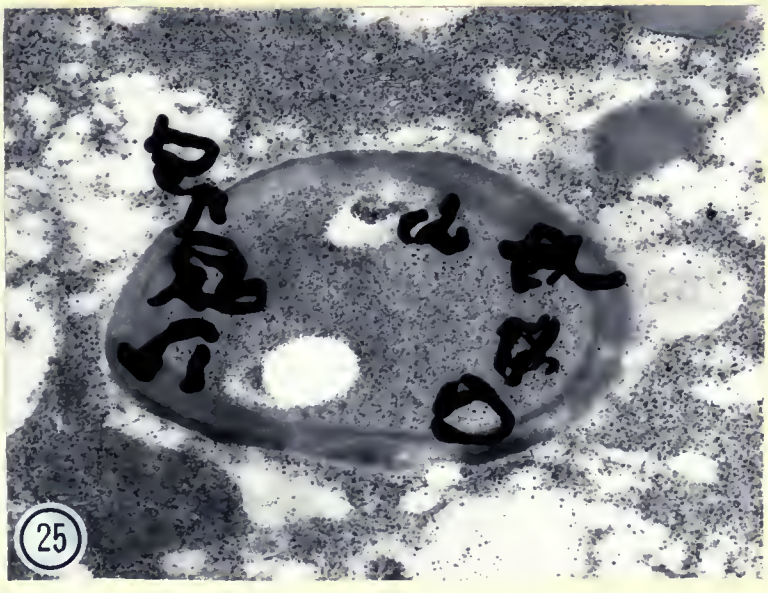

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presence of silver grains over the sheath area (Fig. 25).

(ii) Incorporation of ^{3}H N-acetyl-D-glucosamine in Cellular Fractions

The preferential distribution of silver grains in cell walls and sheath region does not rule out the possibility of non-specific binding of ^{3}H N-acetyl-D-glucosamine to the cellular material. Therefore whole mycelia of 17 h old C. cucurbitarum (fed 2 h with tritiated N-acetyl-D-glucosamine) were fractionated into mitochondrial, microsomal, cytoplasmic and cell wall fractions and the radioactivity in each was determined. The distribution of the label into the various fractions is presented (Table V) in both disintegrations per minute (dpm) and microcuries (μC) and also in percent (%) distribution for each fraction. Analysis showed a preferential distribution of the labelled material to the cell wall fraction. The first experiment indicated that 72.42% of the radioactivity was incorporated into the cell wall, and 82.51% of the total counts were recovered in this same fraction during the second experiment. Total percent of the counts recovered during the first and second experiments improved from 85.62% to 96.65%, respectively, indicating an improvement in the separation and isolation techniques. In both experiments between 13.20 and 14.14% of the total counts were present in the combined mitochondrial, microsomal and cytoplasmic fractions.

The fractionation experiment ruled out non-specific binding of the label; however, the possibility

TABLE V

Distribution of tritium labelled N-acetyl-D-glucosamine in cell fractions of Choanephora cucurbitarum

first experiment			second experiment		
Cell fraction	Radioactivity incorporated (μC)	Total activity (%)	Cell fraction	Radioactivity incorporated (μC)	Total activity (%)
whole cells	0.417 (924,958) *	100.00 (100.00) **	whole cells	2.355 (5,229,302) *	100.00 (100.00) **
mitochondria	0.016 (35,535)	3.84 (3.84)	mitochondria	0.064 (140,698)	2.72 (2.72)
microsomes	0.022 (49,815)	5.28 (5.39)	microsomes	0.029 (63,907)	1.23 (1.22)
cytoplasm	0.017 (38,468)	4.08 (4.16)	cytoplasm	0.240 (532,404)	10.19 (10.18)
cell wall	0.302 (669,121)	72.42 (72.34)	cell wall	1.943 (4,313,331)	82.51 (82.48)
total of fractions	0.357 (792,939)	85.62 (85.73)	total of fractions	2.275 (5,050,340)	96.65 (96.58)

* figure in parenthesis show radioactivity in dpm

** figure in parenthesis show percent of radioactivity distributed based on dpm totals

remained that the labelled chitin precursor was being metabolized and subsequently, the cell walls and sheath areas were incorporating some radioactive by-product of the metabolic process into cell wall and sheath constituents other than chitin. Therefore labelled isolated cell walls of C. cucurbitarum were subjected to alkali and acid hydrolysis and the distribution of the tritium cell wall counts was determined (Table VI). The residue after hydrolysis (previously determined to be chitin) contained between 59.6 to 66.2% of the recovered radioactivity. The H_2SO_4 soluble material possessed 19.5 to 16.6% of the label; however, these counts might have originated from the partial degradation of the newly synthesized chitin or from short chains of N-acetyl-D-glucosamine in the process of being polymerized into the acid insoluble chitin (McMurrough and Bartnicki-Garcia, 1971b). Therefore it is possible that as much as 79.2 to 82.8% of the tritiated N-acetyl-D-glucosamine is incorporated into chitin. These findings clearly indicate that the host fungus incorporated the free chitin precursor from the medium and utilized it in the synthesis of new cell wall material, namely, in the form of chitin (poly-N-acetyl-D-glucosamine).

C: Polyoxin D Studies

(i) Effect of Polyoxin D on the Growth of C. cucurbitarum

The dry weight yield of mycelia of C. cucurbitarum decreased when cultured on liquid medium supplemented with the antibiotic, Polyoxin D. The results indicated that at

TABLE VI

Distribution of tritium label in the isolated cell wall subfractions of Choanephora cucurbitarum

first experiment			second experiment		
Fraction	Radioactivity incorporated (μC)	Total activity (%) @	Fraction	Radioactivity incorporated (μC)	Total activity (%) @
NaOH soluble	0.053 (117,832) *	17.55 (17.61) **	NaOH soluble	0.275 (611231) *	14.17 (14.17) **
H_2SO_4 soluble	0.059 (130,077)	19.54 (19.44)	H_2SO_4 soluble	0.323 (717,739)	16.62 (16.64)
Insoluble residue	0.180 (399,465)	59.60 (59.70)	Insoluble residue	1.286 (2,854,247)	66.19 (66.17)
Total of subfractions	0.292 (647,374)	96.69 (96.75)	Total of subfractions	1.884 (4,183,217)	96.96 (96.98)
Cell wall	0.302 (669,121)	100.00 (100.00)	Cell wall	1.943 (4,313,331)	100.00 (100.00)

@ percentage based on the total cell wall μC
* figure in parenthesis show radioactivity in dpm
** figure in parenthesis show percent of radioactivity distributed based on cell wall dpm total

a concentration of 19 µM, Polyoxin D had little or no inhibitory effect on the dry weight yield. However, concentrations of 190 and 380 µM Polyoxin D caused approximately 20 to 60% inhibition of growth. It was also discovered that at a concentration of 760 µM Polyoxin D totally inhibited mycelial development (Appendix 1). Inhibition curves showed that the greatest effect occurs between 24 and 48 h after inoculation. By 72 h following inoculation the rate of inhibition of the various concentrations decreases; however, they all continued to inhibit mycelial growth (Fig. 26).

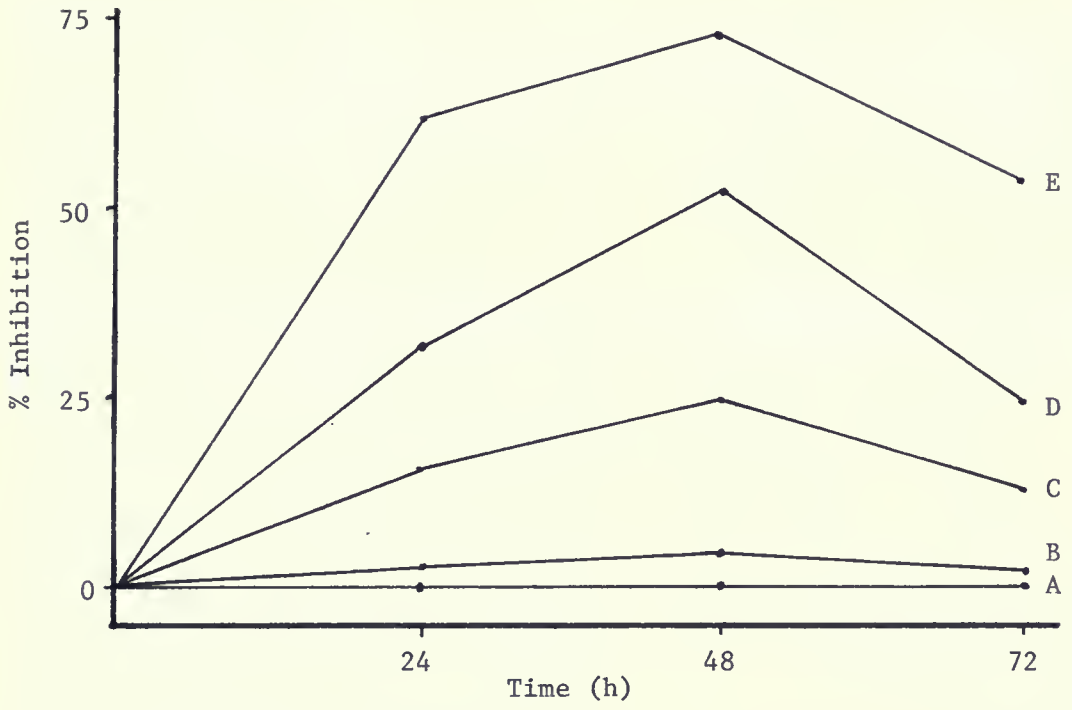
(ii) Effect of Polyoxin D on the Incorporation of Tritiated N-acetyl-D-glucosamine in the Host Cell Wall

Polyoxin D, a competitive inhibitor of chitin synthetase, was used to demonstrate that the preferential distribution of silver grains in the cell wall of C. cucurbitarum and in the sheath around the haustorium of P. virginiana is in fact a result of the activity of the enzyme chitin synthetase. When 14 h old hyphae of C. cucurbitarum grown with P. virginiana were transferred onto a medium containing ^{3}H N-acetyl-D-glucosamine and 380 µM of Polyoxin D, autoradiographs showed a remarkable decrease in the number of silver grains and hence label incorporation in the cell wall (Fig. 27) as compared to the control without Polyoxin D (Fig. 28).

The distributional data of the silver grains in the autoradiographs of control (label only) and Polyoxin D

Fig. 26

Polyoxin D inhibitory effect on the growth of Choanephora cucurbitarum



Polyoxin D concentrations

A = control

B = 19 µM

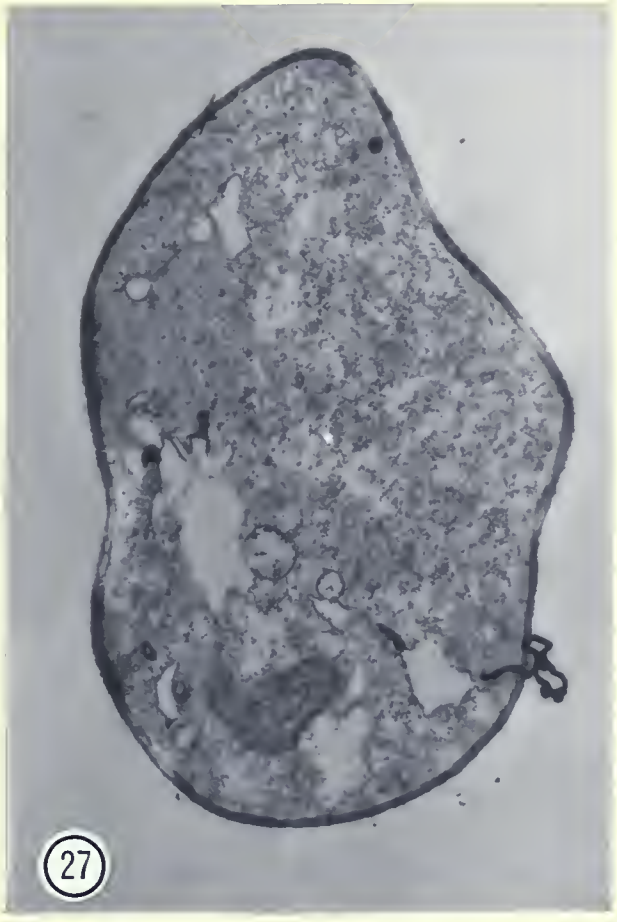
C = 95 µM

D = 190 µM

E = 380 µM

Fig. 27 Thin section showing the effect of Polyoxin D on the incorporation of ^{3}H N-acetyl-D-glucosamine in the cell wall of 17 h old hypha of C. cucurbitarum grown in the presence of both label and antibiotic. x 27,500.

Fig. 28 Thin section of 17 h old C. cucurbitarum cultured in medium containing labelled N-acetyl-D-glucosamine. x 27,500.



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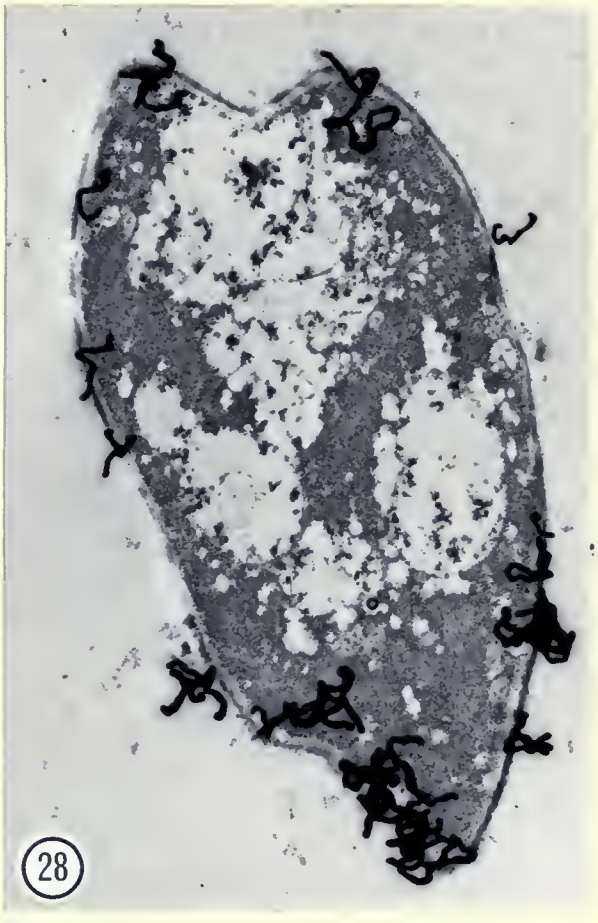

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(label and Polyoxin D) treated cells is presented in Table VII. The significance of the silver grain distribution over the autoradiographs was statistically analysed by Chi square (χ^2) and student t-test methods. The expected grain distribution values (Appendix 2) for the χ^2 analysis of the control and the Polyoxin D treated cells were determined by calculating the average cell wall area to that of the overall cell using a 'compensating polar planimeter'. The average cell wall area was calculated as 7.3% of the total cell area. For the null hypothesis that the distribution pattern of the silver grains is proportional to the component areas throughout both control and Polyoxin D treated cells, χ^2 values of 2,876.4 ($P \leq 0.001$) and 9.26 ($P \leq 0.01$) were obtained respectively. Therefore the null hypothesis is rejected and the distribution patterns are interpreted as being highly disproportionate with the cell wall areas of both control and Polyoxin D treated cells having significantly greater number of silver grains than expected by either random distribution or proportional areas.

It should be noted that the control cell walls of the host autoradiographs contained 78.6% (Table VII of all the grains present and this value is between the values found by the liquid scintillation counting of the radioactivity distributed in the isolated cell wall fraction (Table V) of the host fungus, thereby offering corraborating evidence for the validity of the autoradiographic results.

To determine if the autoradiographs of Polyoxin D treated cells showed a statistically different number of

TABLE VII

Distribution of label from ^{3}H N-acetyl-D-glucosamine in autoradiographs from control and Polyoxin D treated Choanephora cucurbitarum (in 25 autoradiographs per treatment)

Treatment	Component									
	Host fungus cell wall area		Host fungus cytoplasm region		Parasitic fungus		Background		Totals	
	Grains counted	% total	Grains counted	% total	Grains counted	% total	Grains counted	% total	Total counted	Total %
Control	591	78.6	81	10.8	42	5.6	3 8	5.0	752	100
Polyoxin D	26	56.6	9	19.6	1	2.2	9	19.6	45	100

Statistical Analysis

Control cell wall versus remaining control components combined: χ^2 = 2,876.4 P =<0.001

Polyoxin D treated cell wall versus remaining Polyoxin D treated components combined: χ^2 = 9.26 P =<0.01

Total grains of control versus Polyoxin D treatment: t = 9.246 P =<0.001

Distributional patterns for control and Polyoxin D groups: Heterogeneity χ^2 = 10.52 P =<0.001

silver grains (i.e., label) incorporated from the control, a student t-test was conducted on the data summarized in Table VII. Due to the non-normal distribution pattern of the data (Appendix 3 and 4), the grain totals for each autoradiograph were transformed ($\sqrt{x}$ + .5, Steel and Torrie, 1960) in an attempt to normalize the pattern. The null hypothesis tested was that there is no significant difference in the intensity of the labelling between the control and the Polyoxin D treated cells. A t-value of 9.246 was calculated and the null hypothesis was rejected ($P=<0.001$). Clearly the control cells are more heavily labelled than the Polyoxin D treated mycelia and that the Polyoxin D has a statistically significant effect on decreasing the incorporation of the tritiated N-acetyl-D-glucosamine into the cells.

The heterogeneity of the distribution patterns of the silver grains of the control and the Polyoxin D experimental autoradiographs were analysed in a 2x2 Chi square (Appendix 5). The data from either the control or the Polyoxin D cell wall groups were paired against the respective sums of the data of the remaining three fractions. For the null hypothesis, that the distribution patterns of the control and experimental groups were identical, a homogeneity χ^2 value of 10.52 was obtained. Therefore the null hypothesis was rejected ($P < 0.001$) and the distribution of the silver grains is different between the control and the experimental groups. The Polyoxin D has caused a significant decrease in the percentage of label going to the cell wall

area of the experimental class as compared to the control situation. Concomitantly, the antibiotic caused an increase in the percentage of label (silver grains) found in the non-cell wall areas. This is explained on the basis that a lesser quantity of labelled N-acetyl-D-glucosamine is incorporated into the cell wall material of the experimental groups due to the mode of action of the antibiotic Polyoxin D, as a competitive inhibitor of chitin synthetase.

The silver grains totalled in the 'parasite component' of Table VII are randomly distributed over the parasite area and are believed to be background grains and not truly incorporated label. This belief is strengthened by the fact that the 17 hour host cells are not infected by the parasite and that the autoradiographs of 17 hour old axenically grown spores of P. virginiana do not show silver grains over the parasitic hyphae with any consistency greater than that of the background distribution.

(iii) The Effect of Polyoxin D on Label Incorporation into the Host-Parasite Interface and on Sheath Development

High resolution autoradiographs of 28 h old infected hyphae of C. cucurbitarum (fed ^{3}H N-acetyl-D-glucosamine for 2 h followed by a 1 h chase period) showed silver grain development over the developing interface zone (Fig. 29). In the experimental situation, where Polyoxin D is added to the culture medium, only occasional silver grains developed over the sheath areas (Fig. 30). It was calculated that in the control there was an average of 4.0 silver grains per

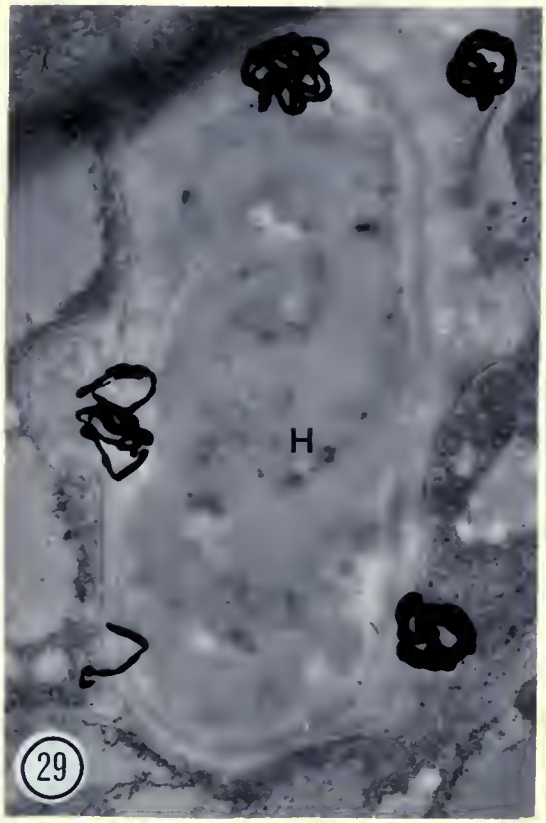
Fig. 29 Thin section of infected host hypha (28 h old) fed tritiated N-acetyl-D-glucosamine. x 30,000.

Fig. 30 Autoradiograph of infected host hypha (28 h old) showing the effect of Polyoxin D on the incorporation of label in the sheath zone. x 30,000.

Note: Compare the sheath development in figures 29 and 30.

Fig. 31 Longitudinal section of 32 h old infected hypha of C. cucurbitarum. x 22,500.

Fig. 32 Longitudinal section of 32 h old infected hypha grown on 380 µM Polyoxin D for 12 h prior to harvesting. x 22,500.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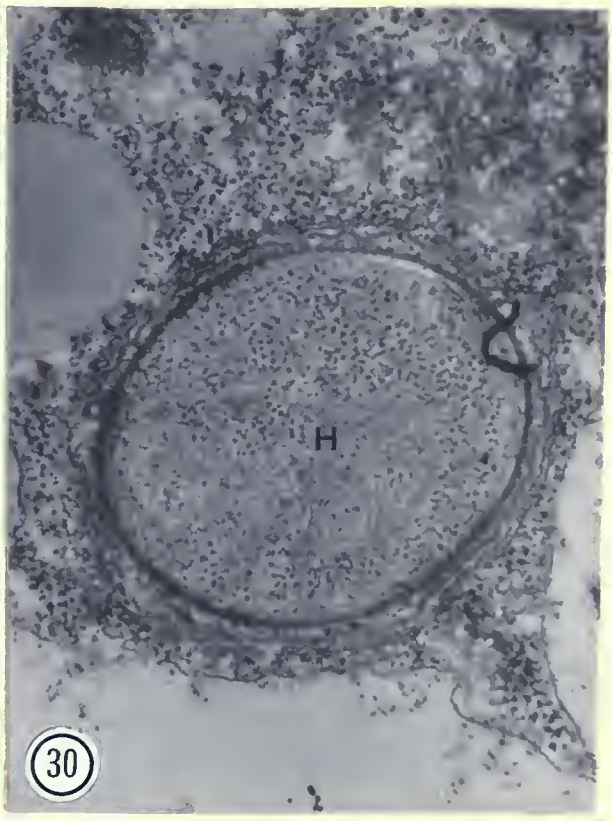
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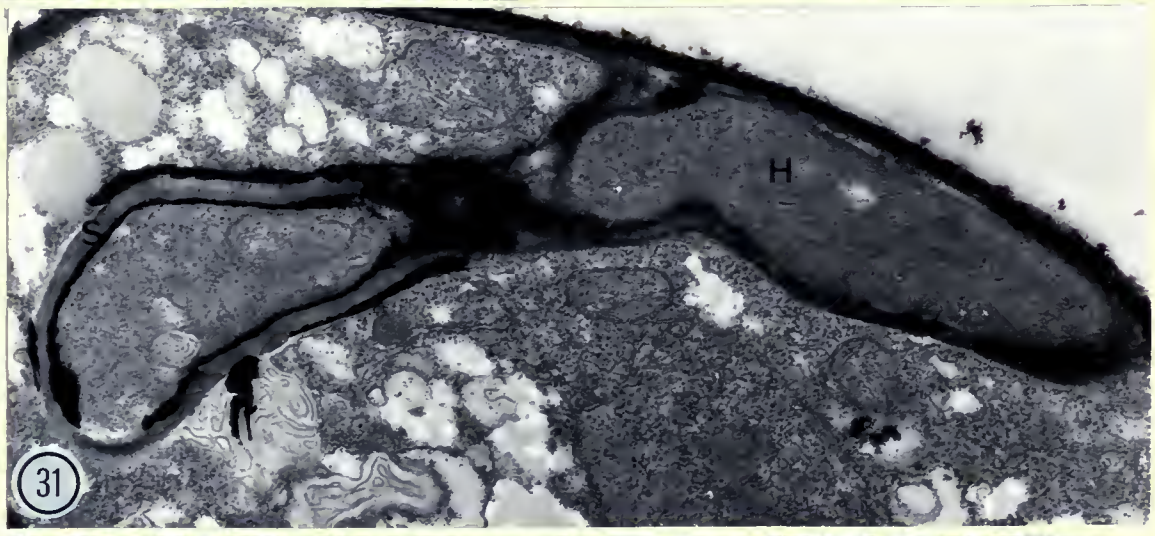
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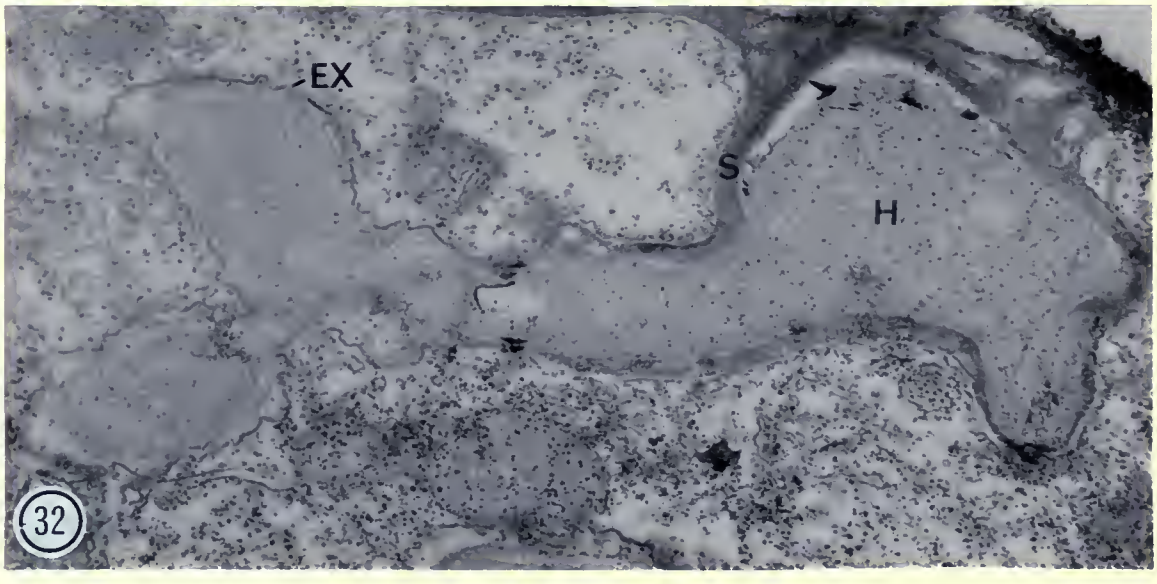
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sheath zone; however, an average of 0.2 silver grains were present in the Polyoxin D treated sheaths. In order to determine if Polyoxin D had a statistically significant effect on the number of silver grains developed, a student t-test was conducted on the data presented in Table VIII (see Appendix 6). The null hypothesis that the number of silver grains present in control and experimentally treated interfaces are proportionately equal, was rejected ($t=4.986$; $P=<0.001$). Therefore, the distribution of the silver grains into the developing sheath zone of the Polyoxin D treated cells is significantly less than that of the control and was due to the presence of the chitin synthetase inhibitor.

Longitudinal sections of 32 hour old infected hyphae of C. cucurbitarum (Fig. 31) showed a continuous sheath completely encompassing the haustorium of the parasite. At this age, the sheath had not yet reached full maturity and appears as a single layer of electron dense material. A similar longitudinal section of a host-parasite interface (32 h after inoculation) following 12 h on liquid medium supplemented with 380 µM Polyoxin D (Fig. 32) showed an incompletely developed electron dense sheath around the haustorium. The remainder of the interface had only slight electron density. The comparison of the interfaces from the Polyoxin D treated specimen with that of the control indicated that non-synchronous sheath development is not the habitual developmental pattern and therefore is attributed to the effect of the antibiotic, Polyoxin D.

TABLE VIII

Distribution of silver grains over the sheath zone of infected C. cucurbitarum. with and without Polyoxin D treatment

	Control	Polyoxin D
No. of haustorial sheath examined	15	14
No. of silver grains observed	56	3

Statistical Analysis

Total grains in control versus Polyoxin D treated sheaths: $t = 4.986$ $P = <0.001$

Discussion

The host-parasite interface is an important region for the investigation of diseased organisms, for it is the area through which materials are exchanged in order to elicit responses from either partner (Bracker and Littlefield, 1973). The interface which develops between Choanephora cucurbitarum and the mycoparasite Piptocephalis virginiana has been previously investigated by Manocha and Lee (1971 and 1972). Their findings indicated that the sheath zone which develops between the host and parasite has a similar electron density to that of the host cell wall. However, the chemical composition of the cell wall of C. cucurbitarum was not known. To further elucidate upon the chemical composition of the host-parasite interface, it was felt pertinent to analyze the cell walls of C. cucurbitarum in detail. For the sake of convenience, the discussion is dealt with in two sections.

A. Structure and Composition of the Cell Wall of Choanephora cucurbitarum

Mechanically isolated and highly purified cell walls of C. cucurbitarum upon chemical and physical analysis revealed the presence of chitin, chitosan, fucose, mannose, galactose, glucuronic acid, proteins, lipids and ash. Glucosamine was found to be the major cell wall carbohydrate and is present as N-acetylglucosamine in chitin and as D-glucosamine in chitosan. Chitin comprised 17% of

the total wall material and its presence was confirmed by infrared spectrophotometry and X-ray diffraction of alkali-acid hydrolyzed cell walls. Chitosan, the predominant component of C. cucurbitarum cell walls, accounting for 28.4% of the total dry weight, was identified by gas-liquid chromatography and by amino acid analysis. No evidence for the presence of cellulose was obtained in our study.Holligan and Drew (1971) determined that the method of gas-liquid chromatography for the quantitative and qualitative analysis of the carbohydrate mixture extracted from the cell wall, as used in the present work, to be more accurate and less tedious than paper chromatography and spectrophotometry.

The chemical composition of the cell walls of C. cucurbitarum resembles several other representatives of the Zygomycetes, whose cell wall compositions have been studied (Frey, 1950). The presence of chitin and chitosan, and the absence of glucose from the vegetative hyphae are the prominent features of the cell walls of zygomycetous fungi. Bartnicki-Garcia (1968b) suggested that the cell wall composition in fungi could be correlated with their taxonomic position. The presence of chitosan in the Zygomycetes probably fulfills the structural role played by glucan in other fungi and along with protein and lipid may fill the interstices between the microfibrils as reported in Neurospora crassa (Manocha and Colvin, 1967). Fucose, mannose, galactose, and D-glucuronic acid have also been reported in other zygomycetous fungi. The hyphal walls

of C. cucurbitarum, however, contained more galactose than either fucose or mannose. Bartnicki-Garcia and Reyes (1964) showed that the amount of these sugars in the walls of Mucor rouxii varied with the stage in the life cycle. Mannose was the most abundant in the yeast form and fucose in the hyphae and sporangiophores. This report provides further evidence for the presence of hexuronic sugar, glucuronic acid, in the cell wall of zygomycetous fungi. This sugar acid has been reported in only a few fungal cell walls (Bartnicki-Garcia and Reyes, 1968a).

The cell wall of C. cucurbitarum appears to be composed of two distinct regions: microfibrillar composed of chitin and an amorphous region composed of a mixture of chitosan, protein and lipids. The protein contained no hydroxyproline or cysteine but did contain relatively large amounts of aspartic and glutamic acids. The general distribution of the protein within the cell wall is unknown and probably forms an integral part of the cell wall of filamentous fungi (Manocha and Colvin, 1967). Wang and Bartnicki-Garcia (1970) found that protein was associated with the heteropolysaccharide and the alkali-insoluble network of the chitin-glucan microfibrils in Verticillium albo-atrum. The heteropolysaccharide and the protein were probably surface and (or) interfibrillar components, which on removal by alkali treatment exposed the randomly interwoven network of microfibrils.

The cell wall lipids contained at least two extractable fractions (Iand II) which differ in their concentration and fatty acid composition. Dyke (1964) was

the first to show that the lipid found in the isolated cell walls of Nadsonia elongata is a true constituent and not a cytoplasmic contaminant. He showed that the cell wall lipids of N. elongata were mainly composed of fatty acids and lacked palmitoleic acid. The cell wall lipids of C. cucurbitarum contain palmitoleic acid and they differ from the cellular lipids in their fatty acid composition (Deven and Manocha, 1975). The role of the cell wall lipids in the fungi remains unknown. It is likely that lipids along with some of the proteins may participate in the formation of macromolecular complexes in the cell walls or at their surfaces.

In this study only 89.9% of the cell wall material was identified. The composition of the remaining 10.1% is presently unknown. Incomplete breakdown in the hydrolysis procedures and (or) inadequacies in the techniques used may partly explain the discrepancy. Furthermore, the nature of the cell wall components, from which the ash was derived remains to be determined.

Manocha and Lee (1971) studied the fine structure of C. cucurbitarum and described the cell wall as distinctly two-layered . The present study confirms the previous findings. The heavy metal shadow castings revealed that the two layers have a different orientation of microfibrils in mature hyphae and in this respect resemble the cell wall of Pythium debaryanum (Manocha and Colvin, 1968). In contrast Neurospora crassa possesses a cell wall which contains only a random arrangement of chitin microfibrils (Manocha

and Colvin, 1967). The presence of the two layers of cell wall having different orientation of microfibrils in mature hyphae and the absence of an inner layer at the tips of the hyphae may indicate that at least two different processes are responsible for the deposition of wall in C. cucurbitarum. Though this study provides no direct information concerning the mechanism of apical extension growth, it would be interesting to assume, as in the case of most fungi, that the hyphal wall extension in C. cucurbitarum occurs solely through the apical deposition of newly synthesized wall material. This mechanism may be applicable to the formation of the thicker outer layer of randomly arranged microfibrils. After formation of this layer, the thin inner layer of parallel oriented microfibrils may be deposited. The thin inner layer was never observed in 48 hour cultures; instead, occasionally, small bundles of parallel arranged microfibrils were observed. It could be due to either the lack of cementing material required for the parallel arrangement of the microfibrils or the susceptibility of the thin layer of the young cell walls to drastic treatment.

However, in older cultures a definite layer of parallel microfibrils was observed. The deposition of this layer and the thickening in general in these older hyphae may explain their resistance to infection, i.e. penetration by Piptocephalis virginiana.

B. Structure and Composition of the Host-Parasite Interface

Electronmicrographs of the infected Choanephora

cucurbitarum (22-24 hours after inoculation) showed that the young haustoria, lacking electron density in the interface, were encircled by cisternae of endoplasmic reticulum and that there was a concentration of vesicular activity in the vicinity of the haustoria. As the haustoria aged, the interface became distinct and the electron density of the sheath was noticed. When full sheath maturity was attained, the sheath was composed of two layers of material possessing similar electron density to that of the host cell wall. Concomitant with the maturation of the sheath, the vesicular activity and the encircling cisternae of endoplasmic reticulum in the area of the haustoria dissipated. These observations are in complete accord with those previously reported by Manocha and Lee (1971).

For the present study, the technique of autoradiography was used to obtain photographs of localized radioactive substances from prelabelled specimens. The autoradiographs were obtained by laying photographic emulsion on a thin section of the tissue and developing the image produced by the radioactivity. A considerable number of researchers have utilized this technique in studies of host-parasite relationships (Ehrlich and Ehrlich, 1970; Manocha and Lee, 1972; Manocha, 1975; Mendgen and Heitefuss, 1975). This indirect method of determining the composition of the sheath must be utilized since technical limitations render the isolation of the sheaths from infected host hyphae a physical impossibility.

As implied by the definition of autoradiography, the exposure of the photographic emulsion to develop silver grains indicates the location of radioactivity in the biological specimen. Since chitin was shown to exist in C. cucurbitarum, autoradiographs of the infected host hyphae, prefed with labelled chitin precursor, N-acetyl-D-glucosamine, were expected to show silver grain concentrations over the areas containing newly synthesized host cell wall. The preliminary high resolution autoradiographic results corroborated the earlier work of Manocha and Lee (1972) and demonstrated that tritiated N-acetyl-D-glucosamine (chitin precursor) is preferentially incorporated into the sheath and host cell wall areas. This result provides supportive evidence for the assumption, based on similarities in electron density of Manocha and Lee (1971), that the sheath is composed of host cell wall material.

According to the present autoradiographic results and those of Manocha and Lee (1972), the parasite, P. virginiana does not incorporate the free chitin precursor even though it is a member of the Mucorales, as is the host fungus. Perhaps the parasite walls are of a different chemical composition than those of its related species or possibly the parasitic fungus is not capable of utilizing or absorbing the labelled material for the synthesis of its own cell walls. However, these suggestions are purely speculative since P. virginiana is an obligate parasite which as yet has not been axenically cultured, thus making it difficult to examine its cell wall composition.

The fractionation of the host mycelia indicated that between 72.4 and 85.5% of the ^{3}H N-acetyl-D-glucosamine was preferentially distributed into the cell wall fraction, thus ruling out the possibility of non-specific binding of the label to non-cell wall components. The radioactivity present in the cytoplasmic, mitochondrial, and microsomal fractions can be explained as contamination or as originating from the short chain chitin polymers synthesized by the enzyme, chitin synthetase, present in these fractions. McMurrough et al (1971) demonstrated that approximately 10% of the total chitin synthetase activity isolated from Mucor rouxii was present in the microsomal fraction and was interpreted as possibly representing nascent enzyme prior to its migration to the cell wall. Traces of the chitin synthetase activity were also found by McMurrough et al (1971) in both the mitochondrial and cytoplasmic fractions of M. rouxii. They showed that when the individual cell fractions were incubated with ^{14}C-UDP-N-acetyl-D-glucosamine, that N-acetyl-D-glucosamine comprised 80-90% of the radiactive product synthesized. This indicates that various cell fractions, when incubated with chitin precursors, produce chitin polymers. It should be noted that the possibility of ^{3}H N-acetyl-D-glucosamine being incorporated into the lipid intermediates, as shown in the biosynthesis of bacterial cell wall polysaccharides has not been ruled out (Sherr et al, 1968; Lennarz et al, 1972). However, various attempts to detect a lipid intermediate in the chitin synthetase system of fungi have been inconclusive (Endo

et al, 1970; McMurrough et al, 1971). McMurrough and Bartnicki-Garcia (1971) observed that there was a considerable stimulation of chitin synthetase when a crude lipid extract of M. rouxii was added to the incubation mixtures containing chitin synthetase from the microsomal cell fraction. Unfortunately, attempts to isolate and identify the lipid intermediate were unsuccessful.

The subfractionation of isolated labelled cell walls of C. cucurbitarum demonstrated that between 59.6 and 66.2% of the radioactivity is incorporated into the alkali-acid insoluble fraction which was previously determined to be chitin (a polymer of N-acetyl-D-glucosamine). McMurrough et al (1971) calculated that nearly 85% of the total chitin synthetase activity of M. rouxii was present in its cell walls. If C. cucurbitarum, which is clearly related to M. rouxii, has a similar chitin synthetase distribution, then there should be a greater percentage of radioactivity in the residual fraction. The acid soluble counts, comprising 19.5 to 16.6% of the total, might have originated from some acid soluble aminopolysaccharide. This appears to be a feasible explanation since the dominant cell wall constituent is an acid soluble aminosugar, chitosan (poly-glucosamine) which makes up 28.4% of the wall. However, the chitosan biosynthetic pathway is not presently known. Furthermore, McMurrough et al (1971) recovered nearly identical amounts of chitin when synthesized from UDP N-acetyl-D-glucosamine labelled in either the glucosamine

or acetyl moieties. These results indicated that the polymer synthesized was made of N-acetyl-D-glucosamine residues, i.e. chitin, and excluded the possibility that chitosan, the corresponding ß(1-4) polymer of D-glucosamine had been synthesized concurrently. These authors were able to isolate, after partial acid hydrolysis, radioactive oligomers of the labelled product and confirmed that they were N-acetyl-D-glucosamine residues. Therefore, the most plausible explanation of the radioactivity in the present acid and alkali soluble fractions is that it represents newly synthesized short chain polymers of N-acetyl-D-glucosamine (chitin) contaminating the fractions and does not originate from the related aminosugar.

The subfractionation results of labelled cell wall show that at least two thirds and possibly much more of the radioactivity present in ^{3}H N-acetyl-D-glucosamine is incorporated directly and specifically into the cell wall chitin without being metabolized and (or) distributed to other cell wall entities. However, it should be noted that the radioactive compounds solubilized in either the acid or the alkali solutions were not analysed, and therefore, the possibility of some synthetic pathway utilizing N-acetyl-D-glucosamine to form some non-chitin compounds has not been ruled out.

It has been reported (Endo et al, 1970) that Polyoxin D selectively inhibits the synthesis of cell wall chitin in some filamentous fungi and that the primary effect is the competitive inhibition of chitin synthetase. This

enzyme catalyzes the transfer of N-acetyl-D-glucosamine from uridine diphosphate N-acetyl-D-glucosamine to an endogenous acceptor. Hori et al (1974b) found that the degree of inhibition of ^{14}C glucosamine incorporation into chitin in Alternaria kikuchiana by Polyoxin B, a nucleotide antibiotic closely related to Polyoxin D, varied in direct proportion to the concentration of the inhibitor used. The growth study utilizing Polyoxin D was required to determine if C. cucurbitarum, the host fungus of the mycoparasitic system studied, was susceptible to the effects of this antifungal antibiotic and if so, what concentrations would be appropriate for the host-parasite investigations.

In a biological context, autoradiography is founded on the principle of assimilation of radioactive substances by living organisms. Therefore, it was logical to assume, if Polyoxin D, which inhibits chitin synthesis, was added to a medium containing labelled chitin precursor (^{3}H N-acetyl-D-glucosamine) and if autoradiographs were made of chitin containing organisms grown on such a medium, that they would show altered silver grain distribution patterns from those of the controls.

Autoradiographs of C. cucurbitarum, prepared in the above mentioned fashion, were statistically analysed against the controls. The Polyoxin D treated cells were found to have significantly fewer silver grains overall ($P=<0.001$) than the controls. Both the control (label only) and the experimental (label and Polyoxin D) autoradiographs showed statistically biased silver grain distribution

patterns for the cell wall areas ($P=<0.001$ and $P=<0.01$, respectively). These results are further supported by the fractionation experiments which showed that approximately 72 to 82% of the labelled chitin precursor was incorporated into the host fungal cell wall. The autoradiographs of the treated cells showed fewer cell wall silver grains than the controls. Although the label entered the cell, it could not be incorporated into the chitin due to the inhibiting action of the Polyoxin D at the site of chitin synthetase activity, i.e. the cell wall. The mode of action of Polyoxin D allowed greater distribution of the label in the non-cell wall areas of the treated cells. This was verified by the test for homogeneity ($P=<0.001$).

Autoradiographic studies of infected host cells show label incorporation in the cell wall and sheath areas. When specimens of the same age as the above mentioned controls were fed a 2 hour pulse of the label and Polyoxin D concurrently, there was statistically fewer silver grains developed over the interface ($P=<0.001$) as compared to the control autoradiographs.

It is therefore assumed that chitin synthetase activity is present in and around the interface zone. Extended culturing periods of the host and parasite on Polyoxin D supplemented medium showed drastic effects on the development of the sheath contents. In controls, the development of the interface followed a sequential pattern as previously described. However, in the presence of the

competitive inhibitor of chitin synthetase, Polyoxin D, the cisternae of the endoplasmic reticulum and the vesicular activity which normally dispersed 28 h after inoculation was still present. Furthermore, the electron density of the sheath was consistently less than that observed in the controls. These observations indicate that the sheaths of the Polyoxin D treated cells are not maturing at the normal rate of the controls.

Prior to the present study, it was assumed that chitin was present in the sheaths of C. cucurbitarum encircling the haustoria of the mycoparasite, P. virginiana (Manocha and Lee, 1971 and 1972). The cell fractionation and the cell wall subfractionation experiments of the present investigation confirmed that ^{3}H N-acetyl-D-glucosamine was indeed incorporated into the chitin. Also the autoradiographic results showed that Polyoxin D significantly inhibited the incorporation of ^{3}H N-acetyl-D-glucosamine into chitin, specifically in the sheath zone and the cell wall areas. Therefore, these results provide unequivocal evidence that the sheath, which develops in the host-parasite interface of C. cucurbitarum and the mycoparasite, P. virginiana, is composed, at least in part, of chitin. Any contribution to the sheath composition by the parasite could not be determined and the possibility is not ruled out.

The findings of Ehrlich and Ehrlich (1970) using inoculated prelabelled ($^{14}CO_2$) uredospores on wheat leaves demonstrated the passage of radioactivity from the pathogen

to the chloroplasts and the cell walls of the host. Mendgen and Heitefuss (1975) inoculated Phaseolus vulgaris with labelled uredospores of Uromyces phaseoli and made a time course study of the early stages of infection. Their autoradiographs showed that the fungal cell walls and the haustoria were heavily labelled. However, no label was detected in either the host cells or the sheath zones. These results are in contrast to those of Ehrlich and Ehrlich (1970) who showed the migration of label from pathogen to the host. Mendgen and Heitefuss (1975) interpret this as a result of nonspecific diffusion of $^{14}CO_2$ from the parasite to the host. Regardless, both studies confirm that the sheath zone does not act as a sink for the radioactive substances from the parasites.

Manocha (1975) studied the fine structure of host-parasite temperature-sensitive combinations of wheat rust. The author reported that the sheaths developed earlier around the incompatible host-parasite combinations than in the compatible (susceptible) combinations. These findings were supported by Coffey (1976) who studied the major gene resistance involving the flax K-gene and the rust fungus Melamspora lini using electron microscopy. His observations showed that in resistant cells, there was a progressive increase in fibrillar definition in the extrahaustorial matrix (sheath). However, in the susceptible host, the sheath did not develop. The intriguing possibility exists that the earlier development of the sheath zone is a major determinant in the resistance of the host

to its parasite (Manocha, 1975 and Coffey, 1976). Autoradiographic studies support this view and have shown that there is a greater incorporation of ^{3}H-leucine from the host to the parasite in susceptible combinations than in the resistant combinations (Manocha, 1975). The incorporation declined in the former reaction with the age of the infection and closely coincided with the development of the sheath. That the sheath plays a role in resistance rather than action as a nutritional sink has been suggested in other studies (Shaw and Manocha, 1965; Manocha, 1966; Manocha and Lee, 1972 and Coffey, 1976). It should be noted that the composition of sheath would not be the same in different host-parasite systems and it would depend primarily on the chemical composition of the host cell wall.

On the basis of our present evidence and the literature available it seems that the ability and the rapidity with which the host can deposit cell wall material at the area of penetration may well determine the host's susceptibility or resistance to infection. In order to further elucidate upon the functional properties of the sheath, a number of investigations are presently being considered. If the sheath is indeed a restrictive barrier to parasitism, then its development should cause a cessation in the movement of materials to and or from the haustorium. This consideration may be approached by further time course autoradiographic studies utilizing prelabelled parasitic spores. Another approach involves cell wall mutants. Since the sheath zone contains host cell wall material (chitin)

possible genetic mutations for thickness, composition or the host's ability to rapidly deposit its walls may play a critical role in resistance. Discovering the mechanism(s) which render the host immune to parasitism is undoubtedly a focal point to the understanding of host susceptibility and resistance and hence would be a major advance towards solving the problems of pathogenicity.

Conclusion

The results of the present study have shown the hyphal walls of Choanephora cucurbitarum to be composed of two distinct regions: microfibrillar and amorphous. The microfibrils, which were composed mainly of chitin, were organized into a thick randomly orientated, outer layer and a thin inner layer of parallel arranged microfibrils. Chemically the walls were found to be composed of lipids, proteins, uronic acid, neutral sugars, chitosan and chitin (poly N-acetyl-D-glucosamine).

Electron microscopy revealed that the young haustorium of the parasite, Piptocephalis virginiana had no distinct sheath development immediately after penetration of the host, C. cucurbitarum. In fact, the haustorial sheath development only became evident long after penetration. Both the sheath, which developed in the host-parasite interface and the host cell wall displayed similar electron densities. When radioactive N-acetyl-D-glucosamine was fed to the infected host hyphae it was incorporated preferentially in the host cell wall and sheath zone. Chitin isolated from the cell walls of ^{3}H N-acetyl-D-glucosamine fed C. cucurbitarum was found to contain most of the labelled precursor. Polyoxin D, a specific inhibitor of the enzyme chitin synthetase, inhibited the incorporation of tritiated N-acetyl-D-glucosamine in both the cell wall and the sheath zone. These results provided unequivocal evidence that the sheath zone around the mature haustorium is composed, at least in part, of chitin, originating from the host cell wall. The sheath's role is interpreted as being a resistant barrier synthesized to retard parasitic advancement.

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APPENDIX 1

Polyoxin D effect on growth of Choanephora cucurbitarum

	Growth (dry weight in mg)		
Treatment	24 h	48 h	72 h
Control	53.5	108.5	261.2
19 µM	51.9	103.1	254.7
95 µM	44.9	81.9	228.5
190 µM	36.9	52.1	197.2
380 µM	20.9	29.6	120.6
760 µM	----	----	-----

APPENDIX 2

Statistical analysis: Chi square analysis of data from Table VII

Null hypothesis: The silver grain distribution, of both control and experimental autoradiographs, is proportional to the cell wall area.

Note: The expected number of grains are calculated on the basis that 7.3% of all grains should be found over the cell wall area.

A. Control autoradiographs

Chi square analysis of control autoradiographs

	obs	exp	obs-exp	$(obs-exp)^2$	$\frac{obs-exp}{exp}^2$
Cell wall	591	97	494	243,048	2505.3
Total of remaining components	161	655	-494	243,048	371.1
Total	752	752			2876.4

$\chi^2 = 2876.4$ P=<0.001

B. Experimental autoradiographs (Polyoxin D treated specimen)

Chi square analysis of experimental autoradiographs

	obs	exp	obs-exp	$(obs-exp)^2$	$\frac{obs-exp}{exp}^2$
Cell wall	26	16.2	9.8	96.04	5.93
Total of remaining components	19	28.8	-9.8	96.04	3.33
Total	45	45			9.26

$\chi^2 = 9.26$ P =<0.01

APPENDIX 3

Distribution of silver grains over 25 control autoradiographs in Polyoxin D study

AREA				
Host cell wall	Host cytoplasm	Parasite	Background	Total
32	2	2	3	39
14	1	2	1	18
20	3	3	0	26
94	9	1	8	112
12	2	3	4	21
2	3	0	1	6
26	3	0	1	30
14	1	3	0	18
84	11	3	1	99
25	2	1	0	28
15	3	1	0	19
7	0	1	1	9
22	1	4	1	28
14	3	2	0	19
8	1	2	0	11
15	3	1	0	19
9	1	0	4	14
12	0	0	4	16
39	10	0	7	56
40	9	0	0	49
7	0	2	0	9
16	3	3	1	23
13	6	1	1	21
36	4	4	0	44
14	0	3	0	17
Total 591	81	42	38	752

Note: For student t-test the totals for each autoradiograph were transformed in order to normalize the distribution of the data. (Steel and Torrie, 1960)

APPENDIX 4

Distribution of silver grains over 25 autoradiographs of Polyoxin D treated specimen

AREA				
Host cell wall	Host cytoplasm	Parasite	Background	Total
2	0	0	5	7
11	0	1	0	12
0	0	0	0	0
0	1	0	0	1
0	0	0	0	0
1	1	0	0	2
2	7	0	0	9
0	0	0	0	0
0	0	0	0	0
5	0	0	0	0
0	0	0	4	4
0	0	0	0	0
2	0	0	0	2
2	0	0	0	2
1	0	0	0	1
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
Total 26	9	1	9	45

Note: For student t-test the totals for each autoradiograph were transformed in order to normalize the distribution of the data. (Steel and Torrie, 1960)

APPENDIX 5

Statistical analysis: Test of heterogeneity of data in Table VII

Null hypothesis: The silver grain distributional patterns are identical in both control and treated cells.

Heterogeneity χ^2 of silver grain distribution in control and experimental autoradiographs

	Cell wall	Total of remaining components	Total
Control	591	161	752
Polyoxin D treated	26	19	45
Total	617	180	797

Heterogeneity χ^2 = 10.52 P =<0.001

APPENDIX 6

Distribution of silver grains over the sheath zone of infected *C. cucurbitarum* , with and without Polyoxin D treatment

Control: Sheath	# of grains present
1	3
2	3
3	6
4	5
5	9
6	0
7	2
8	5
9	5
10	2
11	1
12	3
13	4
14	4
15	6
Total 15	56

Average number of silver grains per sheath = 4.0

Polyoxin D treated: Sheath	# of grains present
1	1
2	0
3	1
4	0
5	0
6	0
7	0
8	0
9	0
10	0
11	1
12	0
13	0
14	0
Total 14	3

Average number of silver grains per sheath = 0.2

Note: For student t-test the total grain number of each autoradiograph was transformed in order to normalize the distribution of the data. (Steel and Torrie, 1960)