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## Chemical and Ultrastructural Characterization of Trichophyton Mentagrophytes Arthrospore Walls and Septa

Jordan H. Pollack  
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CHEMICAL AND ULTRASTRUCTURAL CHARACTERIZATION OF  
TRICHOPHYTON MENTAGROPHYTES ARTHROSPORE WALLS AND SEPTA

by

Jordan H. Pollack

A Thesis Submitted to the Faculty of the Graduate School  
of Loyola University of Chicago in Partial Fulfillment  
of the Requirements for the Degree of  
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## LIFE

Jordan Harry Pollack was born to Israel and Sara Pollack in Chicago, Illinois, on October, 27, 1949.

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### Papers:

Hashimoto, T., J. H. Pollack, and H. J. Blumenthal. 1978. Carotenogenesis associated with arthrosporulation of Trichophyton mentagrophytes. J. Bacteriol. 136: 1120-1126.

Pollack, J. H., C. F. Lange, and T. Hashimoto. 1982. Non-fibrillar chitin associated with the walls and septa of Trichophyton mentagrophytes arthrospores. Submitted to J. Bacteriol.

### Abstracts:

Hashimoto, T., J. H. Pollack, and H. J. Blumenthal. Carotenogenesis associated with arthrosporulation of Tricho-

phyton mentagrophytes. Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, J21, p. 80.

Hashimoto, T., J. H. Pollack, and H. J. Blumenthal. Ultrastructure and chemical composition of Trichophyton mentagrophytes arthrospore walls and septa. Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, J23, p. 92.

Pollack, J. H., C. F. Lange, and T. Hashimoto. Characterization of fibrillar and non-fibrillar chitin associated with arthrospore wall of the dermatophyte Trichophyton mentagrophytes. 1982, J19, p. 94.

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## LIST OF ABBREVIATIONS

|        |   |     |  |
|--------|---|-----|--|
| A      | ampere  | mM  | millimolar                                       |
| Å      | angstrom  | min | minute   |
| °C     | degree Celsius                                      | M   | molar  |
| C.F.   | correction factor                                   | MW  | molecular weight                                 |
| cm     | centimeter  | nm  | nanometer  |
| DTT    | dithiothreitol                                      | N   | normal   |
| EDTA   | ethylenediamine<br>tetraacetic acid                 | %   | percent  |
| Fig.   | figure  | psi | pound per square inch                            |
| g      | gram  | rpm | revolution per minute                            |
| x g    | times force of gravity                              | sec | second   |
| galN   | galactosamine                                       | SDA | Sabouraud dextrose agar                          |
| glcN   | glucosamine   | SDB | Sabouraud dextrose broth                         |
| glcNAc | N-acetylglucosamine                                 | TCA | trichloroacetic acid                             |
| h      | hour  | UMS | urea, mercaptoethanol,<br>sodium dodecyl sulfate |
| IR     | infrared  | UV  | ultraviolet                                      |
| kV     | killivolt   | v/v | percent by volume                                |
| MPa    | megapascal<br>(1 Pa = 6.895 x 10 <sup>-3</sup> psi) | w/v | weight-volume percent                            |
| µg     | microgram   | YPD | yeast extract, neopeptone,<br>dextrose broth     |
| µl     | microliter  |     |  |
| µm     | micrometer  |     |  |
| mA     | milliampere   |     |  |
| mg     | milligrams  |     |  |
| ml     | milliliter  |     |  |
| mm     | millimeter  |     |  |

## INTRODUCTION

Arthrospores have been defined as asexual spores (conidia) which are formed by the septation (cross wall formation) and fragmentation of "fertile" hyphae which have ceased to elongate (Cole and Kendrick, 1969; Cole, 1975; Cole and Samson, 1979). Arthrospores are produced by a number of dermatophytic fungi including Trichophyton mentagrophytes and Microsporum gypseum (Rippon and Scherr, 1959). Unlike the microconidia and macroconidia which are produced by these organisms under saprophytic conditions, arthrospores in nature appear to be found almost exclusively in infected lesions (Rippon and Scherr, 1959; Miyazi et al., 1971b; Hutton et al., 1978). Despite this unique relationship between dermatophytic infections and arthrospores, the study of these organisms has been limited mainly to their hyphal forms. Although there have been a number of attempts to determine the factors affecting arthrospore formation (Rippon and Scherr, 1959; Miyazi et al., 1971a; King et al., 1976; Emyanitoff and Hashimoto, 1979; Weigl and Hejtmanek, 1979, 1980), it is only with the recent development of methods for producing large quantities of arthrospores to T. mentagrophytes in vitro (Bibel et al., 1977; Hashimoto and Blumenthal, 1977, 1978), that they have been characterized more fully (R. Emyanitoff, Ph.D. Dissertation, Loyola University of Chicago, 1978).

No studies have yet been done on the chemical changes that occur in the cell wall during arthrospore formation in T. mentagrophytes or

other dermatophyte species. Indeed, only cursory chemical studies have been done on the arthrospore cell walls of any fungal species (Wheat et al., 1977; Ebina et al., 1978; Kier et al., 1980). This is despite the importance of these studies to the understanding of the immunological manifestations of dermatophyte infections (Grappel et al., 1974) and to the understanding of arthrospore formation in general, since the cell wall is ultimately directly responsible for the shape of the organism (Bartnicki-Garcia, 1968; Brody, 1973; Mishra, 1977). One of the purposes of this study was to determine the chemical composition of the arthrospore walls of T. mentagrophytes and to relate its chemical structure with that of the hyphal walls from which they originate.

This introduction will review some of the earlier work on arthrospore formation in T. mentagrophytes and the chemical and ultrastructural studies that have been done on the hyphal forms of dermatophytes, and on arthrospores and related forms of other species.

The most obvious structural change during arthrosporulation occurs in the cell wall. The earliest change appears to be the appearance of an inner wall layer, initially 0.1  $\mu\text{m}$  thick, along the length of the hyphae. This wall layer can be distinguished by its being less electron dense than the rest of the wall, which is approximately 0.2  $\mu\text{m}$  thick. The two zones are separated by a thin dark layer. Whether this new layer is the result of deposition of new material or the result of rearrangement of existing wall material is not clear (R. Emyanitoff, Ph.D. dissertation, 1978).

The next step is the formation of sporulation septa. Like the

vegetative septum, the arthrospore septum appears to be formed by the invagination of the inner, but not the outer, wall layer (Pock-Steen and Kobayasi, 1970; Bibel et al., 1977). The single septal pore which forms is initially also flanked by osmiophilic granules or Woronin bodies. However, further development of the septum is different from the vegetative septum. The septa, which are rarely closer than 20  $\mu\text{m}$  in the vegetative state, occur at intervals between 3 and 5  $\mu\text{m}$  in the arthrosporulating hyphae. The septal walls, in cross section, have a bilayered appearance, the two layers separated by a septal "furrow". Finally, the edges of the wall which form the pore eventually fuse so that the septal furrow is continuous throughout each septum (R. Emyanitoff, Ph.D. dissertation, 1978).

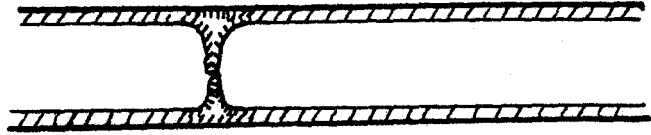
The next step consists of thickening of the inner wall with a slight decrease in the thickness of the outer wall layer. At maturity the outer wall layer is only 0.1  $\mu\text{m}$  thick and tends to detach from the inner wall layer, which may be as thick as 0.45  $\mu\text{m}$ . Finally the cells become more spherical and eventually separate into single cell units or spores (Bibel et al., 1977; R. Emyanitoff, Ph.D. dissertation, 1978). These steps are diagrammed in Fig. 1.

The most basic type of chemical analysis is the determination of the monomers present in the cell wall. A number of studies have been done on the hyphal walls of different dermatophytes (Eveleigh and Knight, *Bacteriol. Proc.* 11:27, G-82, 1965; Shah and Knight, 1968; Noguchi et al., 1971, 1975; Kitazima et al., 1972; Nozawa et al., 1973). The results show that, as with all fungal walls (See reviews by Aron-

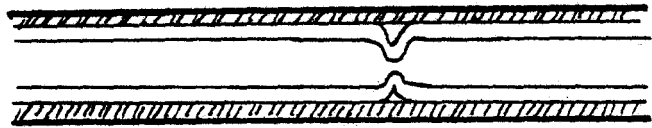


Fig. 1. Diagrammatic representation of the sequential cell wall changes which occur during development of T. mentagrophytes arthrospores. For detailed explanation see text. Hyphal wall structure based on Pock-Steen and Kobayasi (1970). Remaining steps based on R. Emyanitoff (Ph.D. dissertation, 1978). A. Hyphal wall and septum. B. Early arthrosporulation-deposition of new arthrospore wall layer. C. Early septation. D. Late septation and wall thickening. E. Arthrospore maturation - rounding of spores, disarticulation, sloughing off of outer layer.

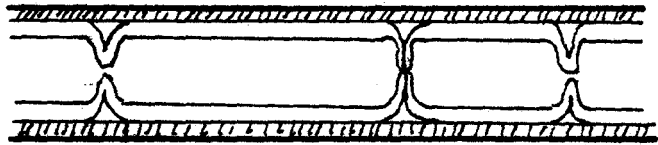
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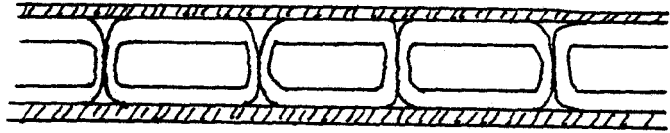
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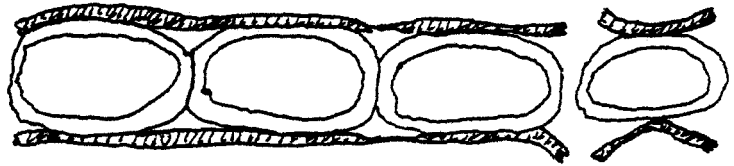
C



D



E



son, 1965; Bartniki-Garcia, 1968, 1973b; Gander, 1974; Rosenberger, 1976), neutral sugars are the main components ranging from 30-55% of the total cell wall dry weight of the thirteen dermatophyte species studied (including Microsporium audounii, M. canis, M. gypseum, T. mentagrophytes, T. rubrum, T. tonsurans and Epidermophyton floccosum). The only monosaccharides consistently recovered from the different dermatophyte species studied were glucose and mannose. The ratio of glucose to mannose was considered to be characteristic of each dermatophyte genus if culture conditions were controlled (Noguchi et al., 1975). Galactose was found in trace amounts in some studies of T. mentagrophytes (Noguchi et al., 1971, Kitazima et al., 1972; and Wu-Yuan and Hashimoto, 1977) and in E. floccosum (Nozawa et al., 1973). Between 22 and 30% of the hyphal wall, (16% of the microconidial wall of T. mentagrophytes, Wu-Yuan and Hashimoto, 1977) consists of N-acetylglucosamine. Shah and Knight (1968) were the only group to report the presence of galactosamine in the walls of any dermatophyte species. The remainder of the wall consists of protein (7-10%), lipid (3-6%) and ash (2-8%).

The next stage in the chemical analysis of the cell wall is the determination of the linkages of the sugar monomers. While not necessary if the proper lytic enzymes are available, in many instances it is first necessary to fractionate the polysaccharides from each other. The most widely used procedure is that of Mahadevan and Tatum (1965), originally used for determining the structure of Neurospora crassa, which separates these polymers based on their solubility in acid and

alkaline solutions. Once the polysaccharides are fractionated the linkages between the individual monomers can be determined by specific lytic enzymes, Smith degradation (Hay et al., 1965) or methylation analysis (Hakomori, 1964). The procedure of Mahadevan and Tatum (1965) is extremely useful for it enables one to separate (1 $\rightarrow$ 3)- $\beta$ -glucan, which is alkali soluble following acid treatment (Fraction III), and chitin, which is acid and alkali insoluble (Fraction IV), the two most important structural fungal polymers (Rosenberger, 1976) from the alkali and acid soluble polysaccharides (Fractions I and II). This procedure was used to detect the presence of the alkali soluble polysaccharides, (1 $\rightarrow$ 6) and (1 $\rightarrow$ 2)- $\alpha$ -galactomannan, and the alkali resistant polysaccharides, (1 $\rightarrow$ 3)- $\beta$ -glucan and chitin in the cell wall of T. mentagrophytes hyphae (Kitazima et al., 1972).

Knowing the different polymers present is insufficient to understand the makeup of the fungal cell wall, for the cell wall components are localized in different areas of the wall. This is best illustrated by the fact that fungal walls appear stratified in ultrathin sections (Aronson, 1965; Hunsley and Burnett, 1970; Rosenberger, 1976; Farkas, 1979). The most widely used method for localizing cell wall polymers has been electron microscopic examination of carbon shadowed specimens before and after chemical or enzymatic treatment. By this method one can distinguish fibrillar and non-fibrillar components. The treatment, particularly if enzymatic, (such as [1 $\rightarrow$ 3]- $\beta$ -glucanase), which reveals the microfibrils is used to identify the matrix polymers, while the treatment which disrupts the microfibrils (such as chitinase), is used to identify their chemical composition (Rosenberger, 1976).

The localization of the cell wall components of T. mentagrophytes hyphae was reported by Kitazima et al. (1972). They identified the outer, rough, crusty, material seen in shadowed preparations with an alkali soluble galactomannan with 1→2 and 1→6 glycosidic linkages. They assumed that the galactomannan must be associated with the pigment found in the outer wall layer since Noguchi et al. (1971) had reported not seeing an electron dense layer in thin sections of non-pigmented hyphae. Although the alkali treatment removed some of the amorphous material from the inner wall surface (which might have been either galactomannan or  $\beta$ -glucan) revealing fibrils, no fibrils were visible on the outer surface. Acid removed material containing glucose and mannose in a ratio of 1.4:1 and much peptide. More important, the long fibrils evident on the inner wall surface following the alkali treatment were no longer visible. Instead, both inner and outer wall surfaces showed short thick fibrils. They then reasoned that the middle wall layer must have consisted of the long fibrils which must have been composed of a glucomannan-peptide complex. In all probability what they probably observed was the partial degradation of the long chitin microfibrils, along with the transformation of amorphous (1→3)- $\beta$ -glucan into short microfibrils (Jelsma and Kreger, 1975; Wu-Yuan and Hashimoto, 1977). The innermost layer consisted of  $\beta$ -glucan which, after the previous treatments became alkali soluble, and the "short" fibrils which they then identified as chitin.

Wu-Yuan and Hashimoto (1977) determined that the microconidial wall of T. mentagrophytes consisted of three layers. The outermost layer

consisted of a glycoprotein-lipid complex which was extracted with urea, mercaptoethanol, and sodium dodecyl sulfate (UMS). The middle layer consisted of proteinaceous material associated with a melanin-like pigment. Freeze etched preparations of this layer, prepared by treating the extracted wall residue with glusulase, showed the presence of a pattern of rodlet patches. This middle layer, which was extremely resistant to wall lytic enzymes, including proteases, could only be dissolved with 1 N NaOH (Hashimoto et al., 1976). The inner layer, which remained after extraction with UMS and NaOH, consisted of amorphous glucans, which were partially susceptible to (1 $\rightarrow$ 3)- $\beta$ -glucanase, and microfibrillar chitin susceptible to chitinase.

Kitajima and Nozawa (1975) were able to separate an "exolayer" at the surface of Epidermophyton floccosum, by lysing the inner layer with glusulase. The exolayer was found to contain a large amount of protein (63% of the fraction). The only neutral sugar found in more than trace amounts was mannose (10%), while the rest of the fraction consisted of glucosamine (17%). Wu-Yuan and Hashimoto (1977) have shown that the "exolayer" is not analagous to the rodlet layer found in T. mentagrophytes microconidia (Hashimoto et al., 1976) for it is soluble in cold alkali and in sodium dodecyl sulfate in the presence of dithiothreitol, while the rodlet layer is not. However, it might be analagous to the outermost layer found in T. mentagrophytes microconidia, the thin pellicle overlaying the rodlet layer, which also contains a high concentration of protein (42.5%) (Wu-Yuan and Hashimoto, 1977).

The structure of the hyphal septum of T. mentagrophytes has been studied by Kitajima et al. (1976). They observed that papain could

remove randomly arranged microfibrils from the septal surface, leaving microfibrils with a spiral orientation around the septal pore similar to the observation of Hunsley and Gooday (1974) for the septa of N. crassa. They concluded that the septum consists of a central chitin-disc overlaid with protein. Preliminary results of the structural study of the arthrospore septum (Hashimoto et al., Abstr. Annu. Meet. Am. Soc. Microbiol., 1979, J23, p. 92) have shown that the concentric rings could be seen without any pretreatment, which might indicate either a lack of or thinner protein layer.

Although the chemical compositions of dermatophyte arthrospores have not been analyzed, the compositions of a few other fungal arthrospores have been studied. Jones et al. (1968) found that the arthrospore walls of Mucor ramannianus were chemically similar to the vegetative walls with respect to their susceptibility to lysis by Streptomyces culture filtrates, even though the arthrospore walls were much thicker.

Wheat et al. (1977) studied the differences in susceptibility of arthrospore and mycelial walls of Coccidioides immitis to various extraction procedures, and also analyzed the relative composition of the extracts. They found that the mycelial wall residues following extraction with detergent and digestion with pronase, contained 1.6 times more glucosamine than did the same arthrospore wall fraction. On the other hand, a fairly large amount (the exact percent is unclear) of glucosamine was extracted by pronase from the mycelial wall but not from the arthrospore wall. They attributed this to glucosamine being present in a form other

than chitin or, alternatively, to chitin being crosslinked with peptide. In addition, the arthrospore walls contained 1.5 times more protein than the mycelial walls. After pronase digestion the arthrospore walls contained no sulfur amino acids, no tyrosine, and almost no proline, whereas the mycelial walls contained cystine, no methionine and little serine. The arthrospore walls also contained five times the galactose and only 60% of the glucose of the mycelial walls. This study did not determine the chemical composition of the intact walls as the authors were more concerned with their relative composition.

The only previous chemical analyses of intact arthrospore walls of any species were done by Ebina et al. (1978) and Kier et al. (1980) on the arthrospore walls of Geotrichum candidum. Both groups found no significant differences between the arthrospore and mycelial cell walls. Both walls contained approximately 60% neutral sugars, 9% amino sugars, 10% protein and smaller amounts of lipid, phosphorus and ash. Ebina et al. (1978) reported no differences in the types or quantities of individual neutral sugars, fatty acids or amino acids.

The chemical composition of the cell walls of a few arthrospore like cells have also been reported. These are cases of organisms which normally show filamentous growth but can be induced to grow in ways which resemble arthrospore formation.

One such case is that of Aspergillus niger which, when grown on manganese deficient medium (conditions which favor citric acid production), form yeast-like structures which resemble arthrospores (Detroy and Ciegler, 1971; Garrison and Boyd, 1974). The cell walls of these



cells were analyzed by Kissler et al. (1980). They found that the cell walls of A. niger grown in manganese containing medium consisted of 80.8% neutral sugars and 7.8% amino sugars while those grown on manganese deficient medium contained only 65.0% neutral carbohydrates and 18.0% amino sugars. The cell walls were also analyzed according to the fractionation procedure of Mahadevan and Tatum (1965). The yeast-like cells contained more of Fraction II (acid soluble glucan) while the filamentous cells contained more of Fraction I (alkali soluble glucan, galactosaminogalactan, galactoglucomannan) and Fraction III [(1→3)-β-glucan]. No differences were found in the amino acid composition.

The effect of the antibiotic ramihyphin A on a number of fungi was studied by Barathova et al. (1975). In sublethal doses it causes the hyphae of some species to become undulated with swollen yeast-like structures. In other species it causes much hyphal branching. The chemical composition of Neurospora sitophila treated with ramihyphin A has been reported by Betina et al. (1976). The cells grown in the presence of the antibiotic, which are highly branched with "vesicular" structures, had almost double the amount of glucosamine found in the cells grown in the absence of the antibiotic (5.3% compared to 2.9% of the cell wall). There was also a doubling of the amount of β-glucan in the antibiotic-grown cells. In addition, they thought that there was a change in the crystalline structure of the chitin of the antibiotic-grown cells since the X-ray diffraction pattern was diffuse while that of cells grown without the antibiotic still showed some diffraction rings.

The purpose of comparing the chemical composition of the mycelial

form with that of the arthrospore form is to discern the chemical changes which may account for the transformation. While a number of quantitative differences have been recorded in various wall components, no recurring change has been found which would implicate that change as responsible for or even associated exclusively with arthrosporulation. This latter distinction is very important. A number of comparative studies, mostly with dimorphic organisms (Bartnicki-Garcia and Nickerson, 1962a; Domer et al., 1967; Pine and Boone, 1968; Kanetsuna et al., 1969, 1972, 1974; Domer, 1971; Kanetsuna and Carbonell, 1970, 1971; San Blas and Carbonell, 1974; Azuma et al., 1974; Rippon, 1980) have found quantitative differences in outer wall components such as mannan and  $\alpha$ -glucan. These differences, which also seemed to correlate with structural changes seen in electron micrographs, seemed sufficient to hypothesize mechanisms by which alterations in the synthesis of these components, either directly or indirectly, influenced the dimorphic transformations of these organisms (Bartnicki-Garcia, 1963, 1968; Kanetsuna et al., 1972; Valentine and Bainbridge, 1978; Cole et al., 1980). More recent evidence using mutants and cultures which have lost their virulence (San Blas et al., 1976, 1978, 1981; San Blas and San Blas, 1977; Kanetsuna, 1981) has questioned the assumptions upon which some of these hypotheses are based, since dimorphic change can occur without the expected changes in these outer wall components. Thus chemical differences may be associated with some transformations but not be their cause.

To differentiate associated chemical changes from transformation causing changes may be difficult, if not impossible. It is clear that

one necessary, but not sufficient, criterion would be that the chemical change must correlate with an ultrastructural change associated with the transformation.

The prominent architectural changes during arthrospore formation in T. mentagrophytes are the deposition of septa at close intervals and the addition of a new wall layer which is contiguous with the septa (R. Emyanitoff, Ph.D. Dissertation, 1978). The prominent chemical change, therefore, expected during arthrospore formation should be an increase in the component, or components, which comprise the septa. In those instances where septal composition has been determined, the primary component of the septa appears to be chitin, or a chitin like substance.

The primary septum of Saccharomyces cerevisiae consists mainly of chitin, (Bacon et al., 1966; Cabib and Bowers, 1971; Beram et al., 1972; Seichertova et al., 1973). Only after the primary deposition are secondary materials such as mannans and  $\beta$ -glucans overlaid (Baur et al., 1972; Molano et al., 1980). Similarly, in the fission yeast Schizosaccharomyces pombe a primary septum, called the annular rudiment, is formed first and serves as the template for deposition of the secondary septum (Johnson et al., 1973). The primary septum was not analyzed chemically, but was shown to have a high affinity for primuline, a fluorescing dye, as does chitin (Seichertova et al., 1973). In addition the cell was shown to incorporate much more 2-deoxyglucose during septum formation than during cell extension.

The development of septa in N. crassa is characterized by a heavy incorporation of N-acetylglucosamine into the septal rings. The septal chitin was probably overlaid with some protein, but not with the other

lateral wall components (Hunsley and Gooday, 1974). Chitin also appears to be the main component of the crosswalls of Schizophyllum commune (but not of the dolipore swellings). As in *N. crassa*, the surface components (S-glucan) were not present on the septa (van der Valk et al., 1977).

One would probably not be wrong in presuming that the prominent event in arthrospore formation would also be the deposition of chitin-containing septa. It is also possible that the inner wall thickening might be associated with this increased chitin formation. Vannini (1979) showed that increased temperature, an inducer of dimorphic change (Romano, 1966; Anderson, 1978) and of arthrosporulation (Rippon and Scheer, 1959; Miyazi and Nishimura, 1971; Emyanitoff and Hashimoto, 1979, Weigl and Hejtmanek, 1979, 1980) stimulates "subapical deposition of internal sheets of chitin". Conversely, coumarin, a drug that affects hyphal polarity by disrupting the orderliness of chitin deposition, causes wall thickening (Dall'Olio and Vannini, 1979). It is also possible that continued deposition of chitin might cause spherical shape (Valentine and Bainbridge, 1978). Alternatively, spherical shape might be the result of internal pressure.

This argument would be considerably strengthened if some distinctive difference could be found in the newly deposited lateral wall layer which is also found in the septa. In fact, some workers have found that the septal rims of *N. crassa* and *S. commune*, although they contain high amounts of chitin, are resistant to lysis by chitinase (Mahadevan and Tatum, 1967; Hunsley and Gooday, 1974; van der Valk et al., 1977). These results seem to correspond to similar observations, namely that as

arthrospores mature they are more resistant to both antibiotics and enzymatic lysis (Duran et al., 1973; Hashimoto and Blumenthal, 1978).

This study will examine the chemical differences between the arthrospore and mycelial walls of T. mentagrophytes. It will show that the septa and inner wall layer of the arthrospores contain a chitinase-resistant chitin, which is otherwise located only in the septal rims of the hyphae. This chitinase-resistant layer will be further characterized ultrastructurally, chemically and biophysically. This newly deposited material may account for the change in arthrospore morphology and for their unusual resistance to enzymes and antibiotics.

## MATERIALS AND METHODS

### Organisms

The organism used in this study was Trichophyton mentagrophytes ATCC 26323. In some comparative experiments, Saccharomyces cerevisiae strain YB 1014 was used. Stock cultures of both fungi were maintained at room temperature on Sabouraud dextrose agar (SDA, Difco). Pleomorphic transformation of T. mentagrophytes from the granular colony phenotype to the cotton phenotype was prevented by monthly transfer of microconidia from granular type colonies showing no evidence of the cotton phenotype.

### Growth Conditions

A. Preparation of arthrospores: Microconidia were produced and harvested as described by Hashimoto et al. (1972). T. mentagrophytes was grown on SDA at room temperature (23-26°C) for 2 weeks. Microconidia were collected only from colonies which were free of the cotton phenotype and were transferred to a 50 ml polycarbonate centrifuge tube (Nalge) containing sterile distilled water. They were dispersed by immersing the tube in an ultrasonic cleaner (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) and filtered through 10 layers of sterile cheesecloth in a conical funnel to remove any hyphae. Arthrospores were produced and harvested as described by Hashimoto and Blumenthal (1978). Approximately 0.5 ml of this microconidial suspension ( $4-5 \times 10^6$  spores per ml of distilled water) was inoculated on a cellulose dialysis mem-

brane (Union Carbide Corp., New York, N.Y.) which had been placed on Sabouraud dextrose agar containing 0.2% sodium acetate. The inoculated plates were incubated at 37°C for 24 h before 2-3 ml of Sabouraud dextrose broth (Difco) was added aseptically over the dialysis membrane. The addition of the broth did not disturb the germinated microconidia, which adhered to the membrane. The plates were placed in a covered jar and further incubated for an additional 7 days at 37°C. By the end of the incubation period essentially all hyphae were transformed into arthrospores.

The arthrospores were collected by gently scraping the membrane surface with a spatula. They were then washed in distilled water by centrifugation (2,600 x g, 10 min at 4°C) four times and stored frozen at -20°C until use.

B. Preparation of hyphae: Approximately 10 ml of a microconidial suspension ( $4-5 \times 10^6$  spores per ml distilled water) were inoculated into 1 liter of Sabouraud dextrose broth (4% glucose, 1% neopeptone, Difco) and incubated on a rotary shaker (200 rpm) at 37°C for 26-28 h. The resultant hyphae were collected and washed by vacuum filtration on sintered glass using a Millipore filtration apparatus. The hyphae were stored frozen at -20°C until use.

C. Growth of *Saccharomyces cerevisiae*: *Saccharomyces cerevisiae* was inoculated into YPD broth (1% yeast extract, 2% neopeptone, 2% dextrose) and incubated on a rotary shaker (200 rpm) at 37°C for 10 h to obtain exponentially growing cells. To obtain late stationary growth cells were incubated for 28 h. The yeast was collected and washed by centrifugation (5,000 x g, 10 min). Cells were stored frozen at -20°C

until use.

### Preparation of Cell Walls

A. Preparation of arthrospore walls: Arthrospores were broken by either of two methods. In the initial stages of this study, they were broken by passage through a French pressure cell (Aminco, model 4-3339) pressurized to 35,000 psi (240 MPa) with a French pressure cell press (Aminco, model 5-598-A). Prior to passage the arthrospores were well dispersed by sonication (position 6 [=4.5 amperes] model S-75 Sonifier, Branson Instruments Inc., Stanford, Conn.) for 30 s. Greater than 99% breakage, as determined by phase microscopy, was achieved following 15-25 passages. Temperature was controlled by collecting the effluent into a tube immersed in ice water and by centrifuging the passaged cells (2,600 x g, 10 min, 4°C) after every two passes followed by resuspension in cold water.

Breakage using the French pressure cell was extremely destructive of the cell walls. A more gentle breakage which retained the cell wall shape, was obtained using glass beads according to Van Etten and Freer (1978). Approximately 1-2 g (wet weight) of arthrospores were vortexed with 10 g acid washed glass beads (0.5 mm, B. Braun Melsungen Apparatebau) and 3 ml cold distilled water, in a parafilm sealed 30 ml Corex centrifuge tube (Corning Glass). Six to eight 1 min treatments were sufficient to achieve 99% breakage. Each treatment was followed by a brief wash in cold distilled water by centrifugation (1,500 x g, 5 min) to remove cytoplasmic debris and minimize cell wall degradation by endogenous enzymes.

The arthrospore walls were washed 7-10 times by centrifugation.



To eliminate cytoplasmic particles adhering to the walls, each wash was preceded by mild sonic oscillation (position 2 [=2 A], model S-75 sonifier, Branson) for 10 s.

The cell walls were examined by phase contrast microscopy to ensure removal of cytoplasmic particles. They were then lyophilized and stored in vacuo under  $P_2O_5$  until use.

B. Preparation of hyphal walls: The hyphal walls were also prepared by two methods. Initially, the hyphae were broken by sonication (position 6 [=4.5 A]) for 1 min intervals repeated 20-25 times, (C. Wu, Ph.D. Dissertation, Loyola University, 1976). The temperature was controlled by immersing the tube during sonication in an ice bath and alternating each 1 min sonication with a 4 min cooling period. After every four sonication intervals the cell suspension was centrifuged (1,500 x g, 10 min) and the residue resuspended in ice cold water.

While breakage of the hyphae by sonication was effective, and not overly destructive of the cell walls, it did leave a residue of metal particles which was difficult to remove. In those experiments where enzymatic digestion of the cell walls left a small residue these metal particles would interfere with dry weight determinations. Hyphae were, therefore, also broken with glass beads using the same procedure as was used for breaking arthrospores, the sole difference being the size of the glass beads (1 mm).

The hyphal walls were washed 10 times in distilled water by centrifugation (1,500 x g, 10 min) with occasional mild sonication with a standard tip (position 2, Branson sonifier) used to dislodge adhering

cytoplasmic particles.

The hyphal walls were checked by phase contrast microscopy for the absence of cytoplasmic contamination, lyophilized, and stored in vacuo under  $P_2O_5$  until use.

C. Preparation of *S. cerevisiae* walls: *S. cerevisiae* cell walls were broken with glass beads and purified using the same procedure as was used for preparing arthrospore cell walls.

#### Criteria of Purity of Cell Wall Preparations

The cell wall preparations were considered to be free of cytoplasmic contamination based on the following criteria:

A. Microscopic examination: Cell wall preparations were examined by phase contrast microscopy using an oil immersion lens (DM 100x, numerical aperture 1.25, Nikon). In addition, the absence of cytoplasmic membranes or organelles could be demonstrated by electron microscopy of thin sections of the wall preparations (RCA, model EMU-3).

B. Ultraviolet absorbance: Cell wall preparations were considered to be free of aqueous extractable nucleic acids and proteins when no absorption peaks were detectable at 260 and 280 nm in the washings (Beckman quartz spectrophotometer, model DUR). The absence of an absorption peak at 260 nm in the 6 N HCl hydrolysates of the cell walls was also evidence of the lack of nucleic acids in the cell walls (Bartnicki-Garcia and Nickerson, 1962a).

C. Ultraviolet detection of ergosterol: The absence of ergosterol, a component of fungal cytoplasmic membranes, also indicated the purity of the wall preparations. Cell walls (60 mg, lyophilized) were

suspended in 5 ml chloroform (MC/B, spectral grade) by means of mild sonication for 10 s and extracted for 1 h at room temperature. The chloroform extract was separated by means of vacuum filtration through two Whatman no. 1 filter papers supported on a microanalysis frit support (Millipore). It was analyzed for the presence of ergosterol by comparing the extract's UV spectrum versus that of authentic ergosterol (Sigma) dissolved in chloroform, on a Cary 15 scanning spectrophotometer. This procedure was capable of detecting 5 µg per ml solution.

### Electron Microscopy

To prepare thin sections of cell wall preparations, samples were prefixed with 4% glutaraldehyde (Polysciences, Inc.) in s-collidine buffer (0.1 M, pH 7.3; Polysciences, Inc.; Bennett and Luft, 1959) for 18 h at room temperature. After being washed with buffer four times, the samples were fixed overnight in 1% osmium tetroxide (Polysciences) dissolved in s-collidine buffer. The samples were washed four times with buffer and dehydrated through a graded acetone series. They were then infiltrated and embedded in a mixture of Epon 812 and 815 (1:1, v/v), and polymerized for 24 h at 60°C. Sections were cut with glass knives using a Sorvall MT-2B ultramicrotome and mounted on 300 mesh uncoated copper grids (Ernest F. Fullam, Inc.). Samples were stained for 30 min with uranyl acetate and 10 min with lead citrate (Reynolds, 1963).

Thiery's (1967) method for localizing periodate sensitive polysaccharides was attempted only on those samples that were fixed with glutaraldehyde in veronal acetate buffer (0.1 M, pH 7.2). Sections mounted on nickel grids (200 mesh) were treated with 1% periodic acid for 1 h

at room temperature, followed by 0.2% (w/v) thiocarbohydrazide (Polysciences) in 20% acetic acid for 1 h. The thiocarbohydrazide was dissolved by gentle heating and was membrane-filtered (0.45  $\mu\text{m}$  pore size) before use. Control samples were not treated with periodic acid. However, on adding the thiocarbohydrazide to the control samples the nickel grids unexplainedly dissolved in many cases, directly underneath the sample sections. Thus, this staining procedure could not be used as a cytochemical stain but only as another staining procedure. The sections were finally stained with prefiltered silver proteinate (Polysciences) for 30 min.

For shadowed preparations, wall samples were mounted on formvar coated copper grids and shadowed at an angle of  $35^\circ$  with platinum-carbon in a vacuum evaporator (model HDS-3B, Hitachi, Ltd.).

Samples were viewed with either an RCA EMU-3 (50 kv, 30  $\mu\text{m}$  aperture size) or a Hitachi HU-11A electron microscope (75 kv, 30  $\mu\text{m}$  aperture).

### Photography

Phase contrast micrographs were taken with a Nikon M-35S camera attached to a Nikon Phase Contrast Microscope using panchromatic film (Plus X, Eastman-Kodak). The film was developed with Microdol (Eastman-Kodak).

Electron micrographs were taken using electron microscope film (4489 - Eastman-Kodak) with the RCA EMU-3 microscope or electron image plates (Eastman-Kodak) with the Hitachi HU-11A microscope. These films were developed with Dektol (Eastman-Kodak).

High contrast negatives were made of infrared spectra plots using

Kodalith ortho film 6556 type 3 (Eastman-Kodak). This film was developed with Kodalith developer (Eastman-Kodak).

### Physical Separation of Wall Layers

An attempt was made to separate the arthrospore wall layers by density gradient centrifugation. Linear gradients were prepared with renografin (meglumine diatrizoate, E.R. Squibb, Inc.) as described by Tamir and Gilvarg, 1966. A 57% to 60% continuous gradient (8 ml total volume) was layered over a 76% renografin cushion (0.5 ml) in a 15 ml Corex centrifuge tube. Wall samples were centrifuged at 24,000 x g for 40 min. The bands which formed were withdrawn with a pasteur pipette.

The walls were also separated by differential centrifugation. The cell walls were centrifuged at 2,000 x g for 30 min and the supernatant and sediment separated. The sediment was resuspended in water and centrifuged at 1,500 x g for 10 min and the supernatant was discarded. Five times repetition of this procedure resulted in isolation of two fractions, labeled "sediment" and "supernatant" which were readily distinguishable by phase contrast microscopy.

### Chemical and Enzymatic Fractionation of Cell Walls

The purified hyphal and arthrospore cell walls were fractionated as outlined in Fig. 2. Alkali extraction (left hand branch) allows the separation of the (1 $\rightarrow$ 3)- $\beta$ -glucan and chitin containing fraction from the rest of the cell wall components. The chitinase digestion (right hand branch) was done on a non-alkali treated residue in order to avoid any possibility that alkali treatment deacetylated the chitin, destroying the substrate for chitinase, and thereby artificially creating a chit-


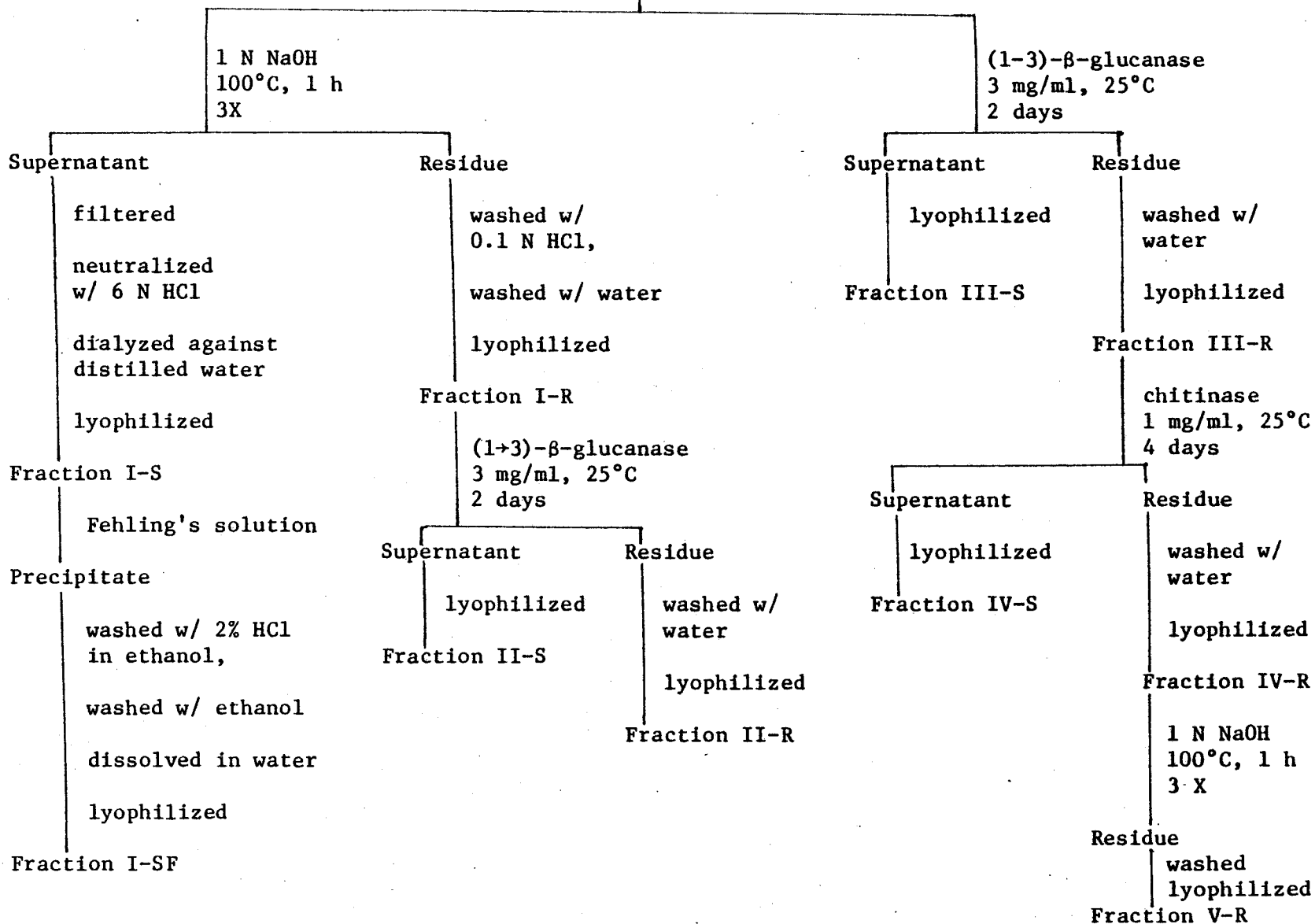


Fig. 2. Procedure for preparing alkali-extracted and enzyme-digested arthrospore and hyphal wall fractions.

Arthrospore or Hyphal Cell Walls



inase-resistant residue.

A. Alkali extraction: Cell walls (50 mg) were extracted with freshly prepared 1 N NaOH (by diluting a stock 50% NaOH solution which is free from carbonates) in capped teflon centrifuge tubes (Nalge) in a boiling water bath. The extracts turned green, then yellow in color, which is an indication of sugar depolymerization and possibly destruction caused by  $\beta$  elimination (Ziderman et al., 1975). This destruction was not prevented by extracting in a nitrogen atmosphere nor by pre-treating the cell walls with sodium borohydride (Bardalaye and Nordin, 1976). Although no color was released if the walls were extracted at 50°C, the lower temperature released only half of the material extracted at 100°C. As there was no apparent chemical difference between the materials extracted at the two temperatures, the walls were extracted at 100°C. After 1 h the alkali soluble fraction was separated by centrifugation (2,600 x g) and the residue was reextracted twice more with fresh 1 N NaOH. The combined soluble fractions were filtered through a teflon filter (Millipore, 0.45  $\mu$ m), neutralized with 6 N HCl, dialyzed against distilled water in dialysis bags with a 1,000 MW cutoff, at 4°C for 48 h, and lyophilized (Fraction I-S). The alkali-insoluble cell wall residues were first washed 5 times with distilled water, then with 0.1 N HCl to remove any residual alkali, and finally with water until the washes were neutral. They were then lyophilized and stored in vacuo over P<sub>2</sub>O<sub>5</sub> (Fraction I-R).

B. Separation with Fehling's solution: The alkali soluble fraction (I-S) was further fractionated by reaction with Fehling's solution



which complexes with and precipitates mannan or galactan-rich polymers, (Jones and Stoodley, 1965). Fraction I-S (15 mg) was solubilized in 1 ml 1 N NaOH to which 0.5 ml Fehling's solution (1 part 0.2 M  $\text{CuSO}_4$  with 1 part solution of 17.3 g sodium potassium tartrate dissolved in 50 ml 3 N NaOH) was added. The mixture was allowed to stand in the cold ( $4^\circ\text{C}$ ) for 2 h after which the bluish white precipitate was separated from the supernatant by centrifugation, and washed twice with 5% (v/v) concentrated HCl in ethanol to dissolve the blue copper salt. The residue was then washed with ethanol until the wash no longer turned yellow upon the addition of acetone, indicating the absence of  $\text{CuCl}_2$ . The residue was then dissolved in water and lyophilized (Fraction I-SF).

C. Disc electrophoresis: The alkali-soluble fraction (I-S) was also separated by disc electrophoresis. The procedure of Davis (1964) was modified by using more alkaline gels and by the addition of potassium tetraborate to enhance the mobility of this fraction. The running gel (13 cm in length) was 5% acrylamide (Bio-Rad Laboratories), 0.375 M Tris([hydroxymethyl]aminomethane) buffer adjusted to pH 9.0 with 1 N HCl, and 0.006 M  $\text{K}_2\text{B}_4\text{O}_7$ . The stacking gel was 2.5% acrylamide, 0.0625 M Tris adjusted to pH 8.8 with 1 N HCl, and 0.0004 M  $\text{K}_2\text{B}_4\text{O}_7$ . The electrolyte buffer was 0.005 M Tris, 0.0006 M  $\text{K}_2\text{B}_4\text{O}_7$ , and 0.002 M glycine which made the pH 9.2. Samples (0.5 to 1 mg) were dissolved in the stacking gel buffer and layered over the stacking gel. When necessary the density was increased by the addition of a drop of 40% sucrose. The current was adjusted to 1 mA per tube and electrophoresis carried out either until the bromphenol blue tracking dye migrated to within 25 mm of the

bottom of the tube or for 4 to 6 h. The gels were fixed with 12.5% trichloroacetic acid (TCA) for 1 h and stained with periodic acid Schiff reagent (Zacharius et al., 1969) for carbohydrates. The gels were washed with water after fixation and immersed in 1% periodic acid for 1 h. They were extensively washed with 10% acetic acid followed by water until no precipitate formed in the wash water upon the addition of  $\text{AgNO}_3$ . They were then stained overnight with Schiff's reagent (2.5 g basic fuchsin, 5 g sodium metabisulfite, 50 ml 1 N HCl, 450 ml water; stir 2 h, add 2 g activated charcoal, and filter, Fairbanks et al., 1971). The gels were destained with 0.5% sodium metabisulfite. The gels were stained with 1% Coomassie blue in 12.5% TCA for protein.

D. Enzymatic digestion of the cell walls: The cell walls were also fractionated sequentially with chitinase (Sigma Chemical Co.) and (1 $\rightarrow$ 3)- $\beta$ -glucanase (kindly provided by S. Nagasaki, Kochi University, Japan). The chitinase, which was used without further purification, was found to be contaminated with (1 $\rightarrow$ 3)- $\beta$ -glucanase as paper chromatographs of chitinase lysates of laminarin ([1 $\rightarrow$ 3]- $\beta$ -glucan, Calbiochem) revealed the presence of glucose. The chitinase was therefore used only after the cell walls had first been digested with (1 $\rightarrow$ 3)- $\beta$ -glucanase. The chitinase also was possibly contaminated with an  $\alpha$ -glucanase since paper chromatographs of the chitinase lysate of the alkali soluble fraction (I-S, which does not contain  $\beta$ -glucan) showed the presence of glucose. This was not considered to be a serious liability since the chitinase was only used to remove chitin from the cell walls and not for the purpose of determining the sugar composition of the residue from the enzy-

matic digestion. The (1→3)- $\beta$ -glucanase was completely free of all chitinase and  $\alpha$ -glucanase activity.

Cell walls were digested with (1→3)- $\beta$ -glucanase (3 mg/ml of distilled water) for 24 h at room temperature. The mixture was centrifuged (5,000 x g, 15 min) and the supernatant was discarded. Fresh glucanase was added to the residue and the digestion continued for 24 h. The residue was collected by centrifugation (5,000 x g, 15 min), washed 5 times with distilled water and lyophilized (Fraction III-R). The residue was then digested with chitinase (1 mg/ml of distilled water) for 48 h at room temperature. The chitinase was replaced with fresh enzyme and the digestion was continued for 48 h. The residue was further digested for an additional week with a mixture of the 2 enzymes. The residue was washed as previously and lyophilized (Fraction IV-R). All enzyme solutions were filtered using single use Millex filters (0.45  $\mu$ m pore size) attached to disposable syringes prior to use, and one or two drops of toluene were added to prevent bacterial growth. In order to ascertain that the enzymatic digestions were complete, a small amount of wall residue was suspended in a fresh enzyme solution in a colorimeter tube (absorbance between 0.20 and 0.30) and the absorbance (540 nm) monitored for a 6 h period. If there was no drop in the absorbance during this period the digestion was considered to be complete. The completeness of the chitinase digestion was further ascertained by the cessation of release of N-acetylglucosamine, as determined by the method of Reissig et al. (1955, see below). The enzyme lysates were checked for the absence of bacterial contamination and lyophilized. For the purpose of identifying their sugar and amino sugar composition, the lyophilized

samples were dissolved in pyridine and separated by paper chromatography as described below.

#### Chemical Analysis of the Cell Walls

A. Dry weight determination: All weights were determined on lyophilized samples which had been further dried over  $P_2O_5$  in vacuo for a minimum of 24 h or until a constant weight was obtained.

B. Total neutral sugars: Total neutral sugars were estimated by the anthrone method (Morris, 1948) with glucose as a standard. Samples (1-3 mg) were well dispersed in distilled water using an ultrasonic bath (Heat Systems-Ultrasonics, Inc.) or, where further dilution was necessary, dissolved in cold concentrated  $H_2SO_4$  (72%). Aliquots of the dissolved/dispersed samples were brought up to a volume of 1.5 ml with water and mixed with 13 ml of 0.2% anthrone in concentrated  $H_2SO_4$  (36 N). The mixture was heated for 10 min in a boiling water bath along with tubes containing the glucose standard solution (5-50  $\mu g/ml$ ). After cooling in an ice bath the absorbances were read at 620 nm (Spectronic 70, Bausch and Lomb). Values obtained by this method were later corrected based on the ratio of monosaccharides actually found in each sample (see below).

C. Individual neutral sugars: For determining the individual neutral sugars the wall samples (1-4 mg) were hydrolyzed with 3 ml of 1 N  $H_2SO_4$  in sealed and evacuated ampules, at  $100^\circ C$ , for 16 h (Wu-Yuan and Hashimoto, 1977). The hydrolysates were diluted to 4 times their volume with distilled water, and neutralized with  $Ba(OH)_2$ . The  $BaSO_4$  was removed by centrifugation and the supernatant was lyophilized. Preliminary experiments showed that if the hydrolysates were not diluted prior

to neutralization, up to 50% of the sugars they contained would be absorbed by the  $\text{BaSO}_4$ , especially if the solutions ever became basic. The length of the hydrolysis was chosen by determining the maximum amount of sugars released from duplicate samples by hydrolysis for times from 1 to 24 h. The sugars remaining in the residue were determined by the anthrone method. The amount of glucose released was determined using glucose oxidase (Worthington) while galactose release was determined using galactose oxidase (Sigma).

1. Glucose oxidase method: Aliquots of each neutralized hydrolysate were brought up to a volume of 2.5 ml and mixed with 2.5 ml of the test reagent (150 units [60 mg] glucose oxidase, 5 mg peroxidase [Sigma], 1 ml of 1% o-dianisidine [Eastman-Kodak] in methanol, dissolved in 100 ml 0.1 M sodium phosphate buffer, pH 7.0). Triton X-100 (0.4 ml) was added to this reagent to prevent precipitation of the reaction mixture. After 2 h incubation at  $37^\circ\text{C}$ , along with the appropriate standard solutions (2.5-50  $\mu\text{g}/\text{ml}$ ), the absorbances were read at 420 nm (Spectronic 70, Bausch and Lomb).

2. Galactose oxidase method: Aliquots of the neutralized hydrolysates were brought up to a volume of 1 ml and reacted with 1 ml of the test reagent (150 [11.5 mg] units galactose oxidase, 10 mg peroxidase, 1 ml of 1% o-tolidine [Eastman-Kodak] in methanol, dissolved in 75 ml 0.1 M sodium phosphate buffer, pH 7.0). Triton X-100 (0.4 ml) was added to prevent precipitation. Absorbances were read at 420 nm after 1 h incubation at  $37^\circ\text{C}$  along with the appropriate standard solutions (2.5-25  $\mu\text{g}/\text{ml}$ ).

The individual monosaccharides in the acid hydrolysates were determined by descending paper chromatography (for 48 h) along with appro-

priate standard sugars (50  $\mu\text{g}/\text{spot}$ ) using Whatman no. 1 chromatography paper. The solvent system was n-butanol-pyridine-HCl (0.1 N) at a ratio of 5:3:2 (v/v). Spots containing neutral sugars and amino sugars were visualized by spraying with aniline hydrogen oxalate (Partridge, 1950) and heating for 10 min at 100°C in a chromatography oven.

The neutral sugars contained in the spots were quantitated by a modification of the methods of Bartnicki-Garcia and Nickerson (1962a) and Caldes and Prescott (1973). The developed spots were cut out of the chromatogram, taking care that the areas cut out for each sugar were equal. Control spots (5-120  $\mu\text{g}/\text{spot}$ ) were also cut out of each sugar. Each spot was then eluted with 0.7% acetic acid in 80% ethanol for 2 h. The absorbances at 410 nm were measured against known standards, which were also chromatographed and eluted from the paper. This procedure was capable of detecting 5  $\mu\text{g}$  glucose.

The ratio of monosaccharides determined by this method was used to correct the value of total neutral sugars obtained by the anthrone method. The ratio of the slopes of the standard anthrone absorbance curves obtained by varying the concentrations of glucose and mannose, were used to create an equation for determining a correction factor, (C.F.):

$$\text{C.F.} = \frac{g + m}{g + \frac{m}{2.15}}$$

where g = parts glucose and m = parts mannose + parts galactose. The value 2.15 is the ratio of the slope of the absorption curve of glucose to that of mannose. This equation was confirmed by actually determining the anthrone absorbance values for each mixture of glucose and mannose. Galactose has essentially the same anthrone absorbances as mannose and

is therefore combined with the mannose value.

D. Total amino sugars: For determining the amino sugars in the intact and digested walls, samples (1-4 mg) were hydrolyzed with 2 ml of 6 N HCl in sealed and evacuated ampules for 6 h at 100°C (Blumenthal and Roseman, 1957). In most cases the HCl was removed by evaporation over NaOH pellets under reduced pressure. However, in some instances it appeared that evaporation adversely affected the detection of the amino sugars. Therefore the HCl was, in separate experiments, neutralized with NaOH and the volume of the hydrolysates brought up to 4 ml. In those instances where neutralization was employed, the hydrolysates were subsequently diluted with 1.5 N NaCl so that all samples had the same salt concentration.

Total amino sugars were determined by two modifications of the Elson-Morgan procedure.

1. Winzler modification (1955): Aliquots of the acid hydrolysates were brought up to a volume of 1 ml with 1.5 N NaCl to which 0.5 ml of 2% (v/v) acetylacetone in 0.5 N Na<sub>2</sub>CO<sub>3</sub> was added, and the tubes were heated in a boiling water bath for 15 min. After cooling in an ice bath, 3 ml of 95% ethanol was added to each tube, followed by 0.5 ml of Ehrlich's reagent (4 parts of 2.5% p-dimethylaminobenzaldehyde in methyl cellosolve, and 1 part concentrated HCl [12 N]). Absorbances were read at 530 nm after standing at room temperature for 30 min.

2. Johnson modification (1971): Aliquots of the acid hydrolysates were brought up to a volume of 1 ml with 1.5 N NaCl and the tubes were heated in a boiling water bath for 30 min with 1 ml of 4% acetylacetone in 1.5% Na<sub>2</sub>CO<sub>3</sub>. After cooling, 5 ml of 95% ethanol was added

followed by 1 ml Ehrlich's reagent (1 part 2.65% p-dimethylaminobenzaldehyde in ethanol with 1 part concentrated HCl [12 N]). Absorbances were read at 530 nm after standing for 1 h at room temperature. This procedure is supposedly less sensitive to differences in the salt concentration than the previous procedure and was used when the hexosamine values of certain fractions, obtained using the Winzler modification, did not appear to account for all the hexosamine expected to be found in those fractions.

Total hexosamines were also determined by adding up the amount of individual amino sugars determined by using the amino acid analyzer (see following). The use of different methods to determine the total hexosamine values was considered to be important to support the thesis of this paper.

E. Individual amino sugars: Glucosamine and galactosamine were differentiated by colorometric means (Wagner, 1978) in the alkali extractable fraction (I-S) where the amount of galactosamine is greater than five times the amount of glucosamine. Total hexosamines were first determined as previously described. Galactosamine was then determined by a modification of the Elson-Morgan reaction in which galactosamine is acetylated at room temperature while glucosamine is hardly acetylated at all. Aliquots of the acid hydrolysates were brought up to a volume of 0.8 ml and mixed with 0.6 ml of acetylacetone reagent (3.5% acetylacetone in 0.5 N potassium tetraborate). After standing at room temperature for two hours, 2 ml Ehrlich's reagent (3.2 g p-dimethylaminobenzaldehyde for 12 N HCl, diluted to 210 ml with 2-propanol) were added and the mixture was heated for 15 min at 50°C in a water bath.



Absorbances were read at 530 nm. Glucosamine concentration was considered to be the total hexosamine concentration minus the galactosamine concentration.

In all other instances where the glucosamine concentration was much higher than the galactosamine concentration, the two amino sugars were differentiated with an automatic amino acid analyzer (Beckman, model 120 C), using a single, acidic cation exchange column (Aminex A-4 Resin, BioRad Laboratories). Amino sugars were eluted with sodium citrate buffer (at start pH 3.44, 0.2 N; at 26 min pH 4.14, 0.2 N; at 75 min pH 6.41, 1.0 N).

F. N-acetylated amino sugars: N-acetylglucosamine was detected in the enzyme hydrolysates either prior to lyophilization or after dissolving the lyophilized samples in water, by the method of Reissig et al. (1955). Aliquots of the enzyme hydrolysates were brought up to a volume of 0.5 ml with water, mixed with 0.1 ml of 0.2 M potassium tetraborate and heated in a boiling water bath for 3 min. After cooling in a water bath, 3 ml of Ehrlich's reagent (1 g p-dimethylaminobenzaldehyde in 1.2 ml concentrated HCl [12 N], diluted to 100 ml with concentrated acetic acid) were added and the mixture incubated at 37°C for 20 min. Absorbances were read at 585 nm after cooling (Spectronic 70).

To determine if the chitinase resistant chitin was acetylated it was necessary to use IR spectroscopy and X-ray diffraction methods (see below).

G. Protein: Protein was quantitated by the method of Lowry et al. (1951) using bovine serum albumin (crystallized and lyophilized, Sigma) as a standard. Cell wall material extracted with 1 N NaOH (see above)

was either diluted 1:10 with distilled water or, neutralized, dialyzed against water, lyophilized, and resolubilized in water (Fraction I-S). Aliquots were brought to a volume of 0.5 ml (with water if neutralized samples were tested, with 0.1 N NaOH if diluted samples were tested) and mixed with alkaline copper reagent (1 ml of a 1:1 mixture of 1%  $\text{CuSO}_4$  and 2% sodium potassium tartrate, added to 50 ml 2%  $\text{Na}_2\text{CO}_3$  in 0.1 N NaOH). After standing for 10 min at room temperature 0.25 ml of Folin-Ciocalteu phenol reagent (Anderson Laboratories Inc.), diluted 1:2 with water, was added. The absorbances were read at 500 nm and at 750 nm after 30 min.

Protein was also quantitated by totaling the amino acids recovered from amino acid analysis (see following).

H. Amino acids: Samples (2-5 mg) were hydrolyzed for 22 h with 6 N HCl at 100°C in sealed, evacuated ampules. The acid was evaporated over NaOH pellets under reduced pressure. The dried hydrolysate was then dissolved in either water or sodium citrate buffer pH 2.2. A two column analysis was performed using an automated amino acid analyzer (Beckman, model 120-C) to determine the amino acids present in the wall fractions. Each amino acid was quantitated by comparing its absorbances at 570 nm (440 nm for proline) after reaction with ninhydrin (3,000 ml methyl cellosolve, 1,000 ml sodium acetate buffer, 4 N, pH 5.51, 80 g ninhydrin, 1.6 g  $\text{SnCl}_2$ ) with those obtained with standard solutions for each amino acid.

I. Lipid: Lipid was determined by the method of Bligh and Dyer (1959). Dried samples (100 mg) were extracted with a chloroform-methanol mixture (1:2 v/v) for 1 h. At intervals the mixture was sonicated. Chloroform was then added until the ratio of chloroform to methanol was

2:1 and the mixture allowed to stand for 3 h. The extractable material was filtered through Whatman no. 1 filter paper into pre-weighted aluminum planchettes and the solvent allowed to evaporate to a constant weight.

J. Phosphorus: Phosphorus was estimated by the method of Chen et al. (1956). Samples (4 mg) were ashed by adding 4 drops of concentrated sulfuric acid and heating in a sand bath until white fumes of  $\text{SO}_2$  appeared. Two drops of 72% perchloric acid were added and the mixture heated until the dark brown solution cleared. The volume was then brought up to 4 ml. Aliquots were brought up to a volume of 1 ml and mixed with 4 ml ammonium molybdate reagent (1 volume 6 N  $\text{H}_2\text{SO}_4$ , 2 volumes distilled water, 1 volume 2.5% ammonium molybdate, 1 volume 10% ascorbic acid). After heating at  $37^\circ\text{C}$  in a water bath the absorbances were read at 820 nm.

K. Ash: Ash was determined according to the method of Smith, (1964). Samples (200 mg) were placed in a tared, covered Vycor crucible (Corning) and heated at  $800^\circ\text{C}$  in a muffle oven (New Brunswick) for 2 h. After cooling the crucible containing the ashed residue was reweighed, the difference in weights was the amount of ash.

L. Other: Sulfur was tested for using the procedure of Lewis et al. (1965). Hexuronic acids were tested for by the method of Dische (1947). Muramic acid was screened for using the method of Hadzija (1974), and with the amino acid analyzer (Beckman 120C).

#### Physical Methods to Determine the Presence of Chitin

A. Infrared spectroscopy: Infrared absorption spectra of hexosamine containing wall fractions were obtained with a Perkin-Elmer, model

377, grating spectrophotometer using KBr pellets containing 2% (w/w) sample material. The degree of acetylation of glucosamine containing polymers was determined according to the method of Moore and Roberts (1980) where degree of acetylation equals:

$$\frac{A_{1655 \text{ cm}^{-1}}}{A_{3450 \text{ cm}^{-1}}} \times \frac{100}{1.33}$$

B. X-Ray diffraction: Powder X-ray diffraction patterns of samples mounted in 0.3 mm in diameter glass tubes, were obtained with a 2 radian Debye-Scherrer camera using Cu radiation filtered through a Ni foil filter. The X-ray generator (North American Philips Co.) was operated at 30 kV and 14 mA. The film was exposed for 7 h.

#### Source of Chemicals

All chemicals were reagent quality and were obtained, unless otherwise specified, either from Mallinckrodt or Sigma Chemical Companies. Laminaran was obtained from Calbiochem. Purified and dispersed chitin was prepared by the method of Skujins et al. (1965). Chitin flakes (Sigma) were dissolved in concentrated HCl (12 N) for 1 h at 4°C. The viscous suspension was centrifuged at 10,000 x g for 20 min and the supernatant poured into 50% ethanol that was vigorously stirred. The precipitate was washed 10 times with water or until the last three washes were neutral, and lyophilized.

Chitosan was prepared according to the method of Bartnicki-Garcia and Nickerson (1962a). A concentrated solution (approximately 25 M) of NaOH was prepared by dissolving 24 g NaOH with 10 ml water (final volume 24 ml) in a teflon screw cap tube. The chitin was added to this solution and heated in an oven for 20 min at 160°C. The residue was washed

until neutral and was then dissolved in 5% cold acetic acid. The chitosan was reprecipitated by adding NaOH until neutrality was attained and then washed five times with water and lyophilized.

## RESULTS

### Appearance and Purity of Cell Wall Preparations

Greater than 99% breakage of the arthrospores was achieved using either the French press or by vortexing with glass beads. However, breakage with the French press was achieved at the expense of the cell wall integrity. The majority of wall fragments were too small to discern the original arthrospore shape (Fig. 3). Breakage with glass beads did retain the original cell shape without sacrificing the high percentage of cell breakage (Fig. 3). Although, some of the initial results in this study were obtained using French press broken cells, all results were rechecked using glass bead broken cells. Furthermore, no chemical difference was observed between the cell walls prepared by the two different methods.

The arthrospore walls were shown to be free of cytoplasmic contamination by a number of methods. Essentially no cytoplasmic debris was evident in either phase contrast or electron microscope micrographs. Ribose was not detected in paper chromatographs of the cell wall hydrolysates, nor was there any absorbance peak at 260 nm in the 6 N HCl hydrolysates, indicating the absence of nucleic acids. Ergosterol, a cell membrane component, could not be detected in the cell wall preparations either (Fig. 4). The cell wall preparations appeared completely white indicating the complete removal of the abundant carotenoid pigment granules that are characteristic of arthrospores of this strain of T. mentagrophytes (Hashimoto et al., 1978).




Fig. 3. Phase contrast micrographs of arthrospore walls prepared by breakage with a French pressure cell (20-25 passages) (A) and with glass beads (10 min) (B) Note that the arthrospore cell shape is retained when the cells are broken with glass beads, but not when broken with the French press.

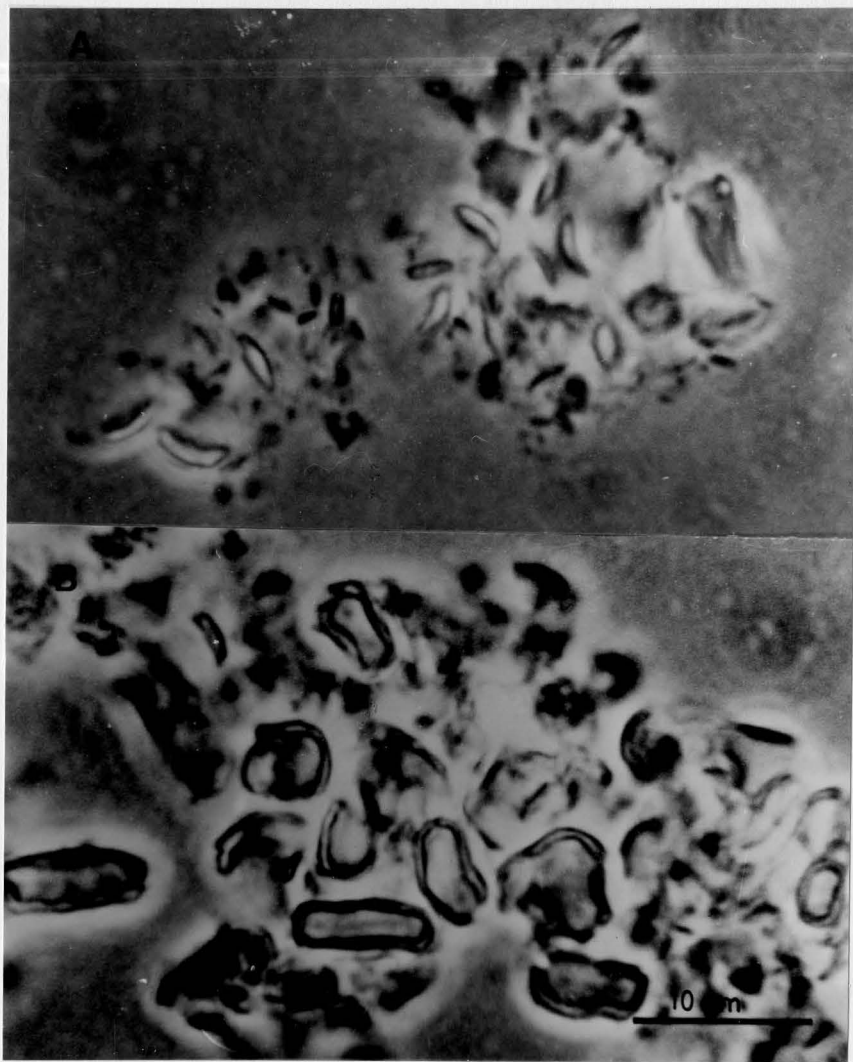
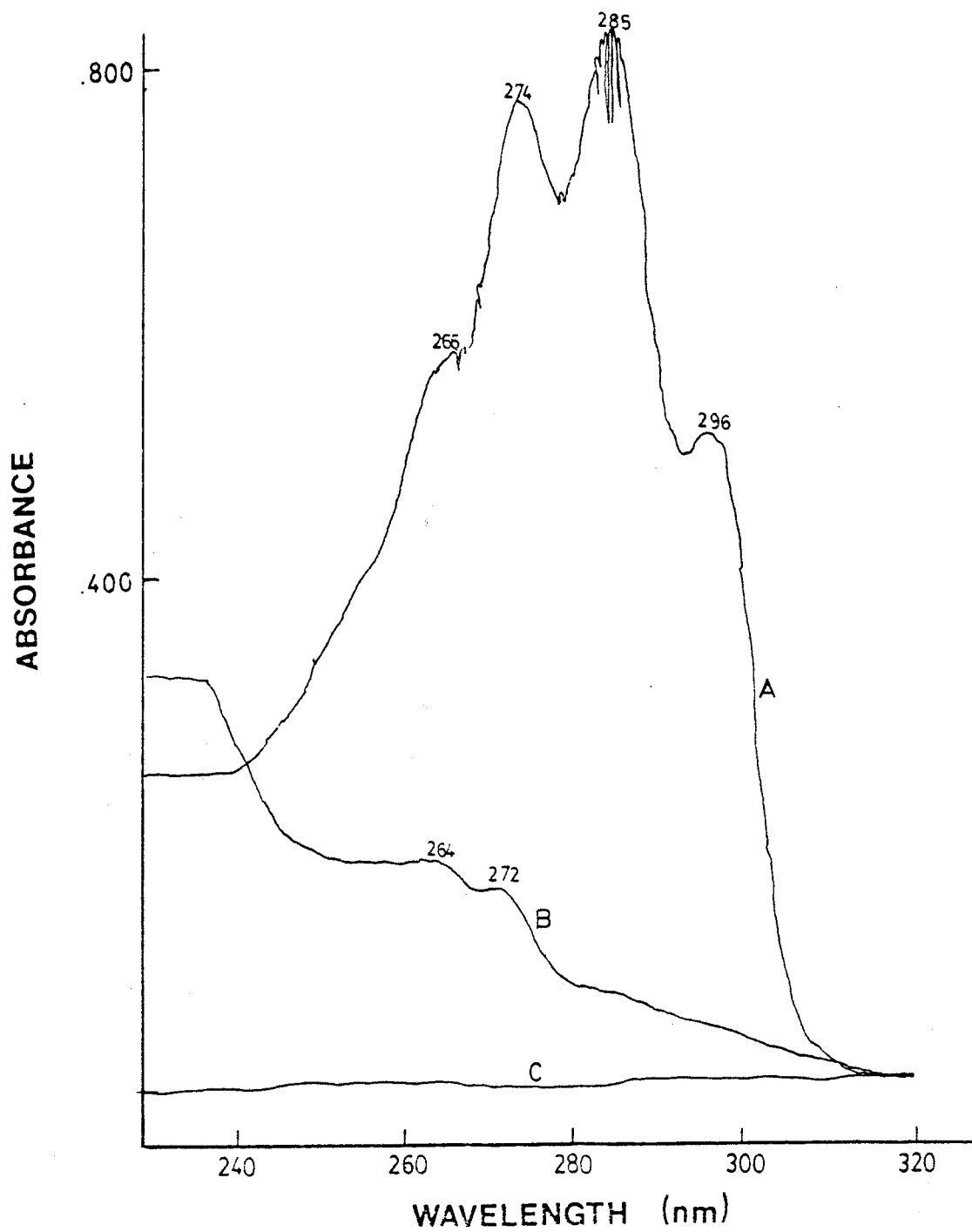




Fig. 4. Ultraviolet light absorption spectra of ergosterol dissolved in chloroform (2.5 mg%, A), arthrospore walls extracted in chloroform (20.8 mg/ml, B), and chloroform (C).



There was no difference in the light microscopic appearance of the hyphal walls prepared either by sonication or with glass beads. The walls retained their structural integrity (Fig. 5). Approximately 2-3% of the wall fragments retained some cytoplasmic debris which was not possible to remove.

Although the arthrospore walls are at least twice the thickness of the hyphal walls, no apparent density difference could be demonstrated by Renografin density gradient centrifugation. Both types of walls banded approximately just below 60% Renografin (results not shown).

#### Mechanical Separation of the Cell Wall Layers

The residual hyphal wall (or outer layer) appeared to be loosely attached to the (inner) arthrospore wall. In many instances, especially under the stress of cell breakage, it was detached from the rest of the wall (Fig. 6). Initial attempts to separate this "exolayer" from the intact arthrospores by immersion in an ultrasonic bath and by vortexing, and to recover it by differential or gradient centrifugation were unsuccessful. Differential centrifugation of the French press prepared cell walls as described in Materials and Methods, resulted in the separation of two wall fractions labeled "supernatant" and "sediment", respectively. These two fractions were also differentiated by Renografin gradient centrifugation. The "supernatant" fraction, which consisted of small, thin fragments, banded at the 76%-60% Renografin interface while the "sediment" fraction, which consisted of larger wall fragments banded at approximately 59% Renografin. However, later chemical analysis failed to show any differences between these two physically different fractions. No further attempts were made to isolate an "exolayer" from cells broken with glass beads.

Fig. 5. Phase contrast micrographs of isolated hyphal walls of T. mentagrophytes prepared by sonication (A) and by breakage with glass beads (B). Note that there are no major differences in the appearances of the two preparations, and that the integrity of the wall fragments is maintained.

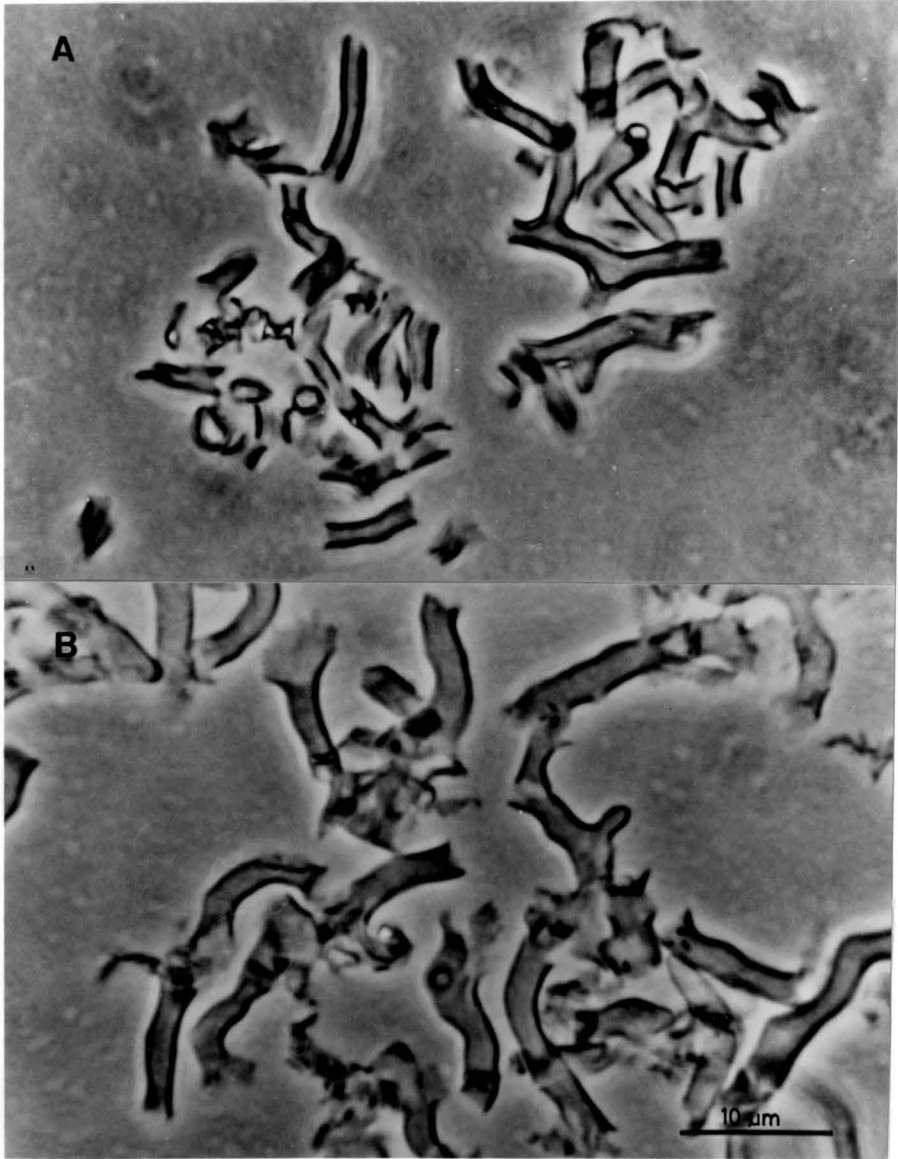


Fig. 6. Electron micrograph of thin sectioned isolated arthrospore wall stained by the method of Thiery. Note that the outer, arthrospore wall (H) is almost completely detached from the inner arthrospore wall (A).



Preliminary Experiments to Chemical Analysis of Cell Walls

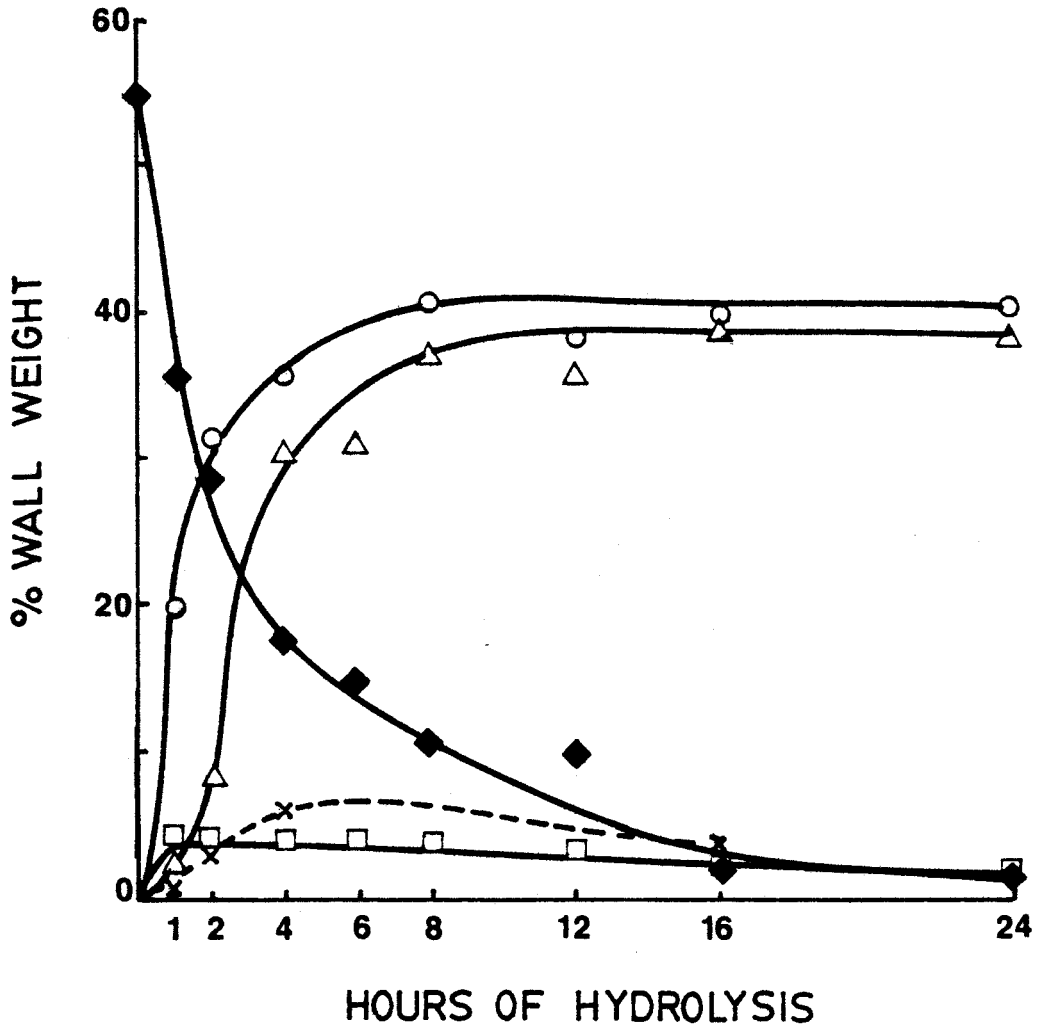
Several experiments were done to determine the optimal conditions for recovery of sugars from hydrolyzed wall preparations.

A. Acid used for hydrolysis: Walls were hydrolyzed with either 1 N HCl (100°C), 1 N trifluoroacetic acid (100°C) or 1 N H<sub>2</sub>SO<sub>4</sub> (110°C) for various periods of time. Hydrolysis with sulfuric acid gave equal or better recoveries of sugars than the other two acids (results not shown). Sulfuric acid also had the added convenience of being quickly removed by precipitation as BaSO<sub>4</sub>. For these reasons it was chosen as the agent for hydrolysis.

B. Length of hydrolysis: Hyphal and arthrospore walls were hydrolyzed for 1 to 24 h and the hydrolysates were analyzed for the amount of individual and total sugars released. As shown in Fig. 7, 96% of the maximal amount of glucose determined by glucose oxidase (37.3% of the wall weight) was released from the arthrospore wall by 8 h. There was little change in the amount of recoverable glucose if the hydrolysis was extended to 24 h. The maximal amount of galactose (3.8% of the wall weight) was released within 1 h. Continued hydrolysis for more than 8 h resulted in a gradual destruction of galactose until only 42% of the maximal value (1.6% of the wall weight) was recovered after 24 h of hydrolysis. The maximum amount of mannose (6.0% of the wall weight) was released by 4 h. By 16 h almost no sugars could be detected in the wall residue. The total amount of sugars detected in the acid hydrolysates (by the anthrone method) was only 69% of the total sugar detected in the wall prior to acid hydrolysis, indicating considerable destruction of



Fig. 7. Release and recovery of total and individual sugars from arthrospore walls during hydrolysis with sulfuric acid (1 N, 100°C) over a 24 h period. Sugar release is expressed as percent of initial wall weight.  $\Delta$ : glucose released as determined by glucose oxidase;  $\square$ : galactose released as determined by galactose oxidase;  $\chi$ : mannose released, determined by computing the ratio of mannose to glucose in the hydrolysates from the eluted paper chromatogram spots as described in Materials and Methods.  $\circ$ : total sugar released as determined by anthrone prior to neutralization;  $\blacklozenge$ : Total sugars remaining in residue, determined by anthrone method.



sugars during hydrolysis. Similar results were obtained for sugar released from the hyphal walls (Fig. 8). Although hydrolysis for 8 h may have been optimal to the recovery of each monosaccharide, it was decided to use a 16 h hydrolysis at which time the walls were more digested and at which time glucosamine could also be detected in the hydrolysates.

C. Neutralization of hydrolysates: Although neutralization of the hydrolysates with  $\text{Ba}(\text{OH})_2$  was quick, it had one major drawback. The  $\text{BaSO}_4$  that was formed adsorbed large amounts of monosaccharides (Table 1). As much as 55% of the sugars in the hydrolysates were adsorbed if in the process of neutralization the solution ever became basic. Even if the hydrolysate never became basic 25% of the sugars were adsorbed. Only galactose was not adsorbed by  $\text{BaSO}_4$ . In order to prevent this adsorption of monosaccharides each hydrolysate was diluted to four times its initial volume with water prior to neutralization. This reduced adsorption of glucose and mannose to less than 6% (results not shown). However, even dilution of the hydrolysates did not always prevent the adsorption of sugar polymers. As shown in Table 2 much of the sugars released by hydrolysis for less than 4 h was not detected following neutralization. This presumably is because the sugars are released as polymers in the early stages of hydrolysis (see Figs. 7 and 8), which eventually are broken down to monosaccharides as the hydrolysis continues. Therefore, the total sugar released by acid hydrolysis was determined by the anthrone method before the hydrolysates were neutralized.

D. Determination of ratio of monosaccharides: The neutralized hydrolysates were separated into individual monosaccharides by paper chro-

Fig. 8. Release and recovery of total and individual sugars from the hyphal walls of T. mentagrophytes during hydrolysis with sulfuric acid (1 N, 110°C) over a 24 h period. Sugar release is expressed as percent of initial initial wall weight.  $\Delta$ : glucose released as determined by glucose oxidase;  $\square$ : galactose released as determined by galactose oxidase;  $\circ$ : total sugar released as determined by anthrone prior to neutralization;  $\blacklozenge$ : total sugar remaining in residue as determined by the anthrone method.

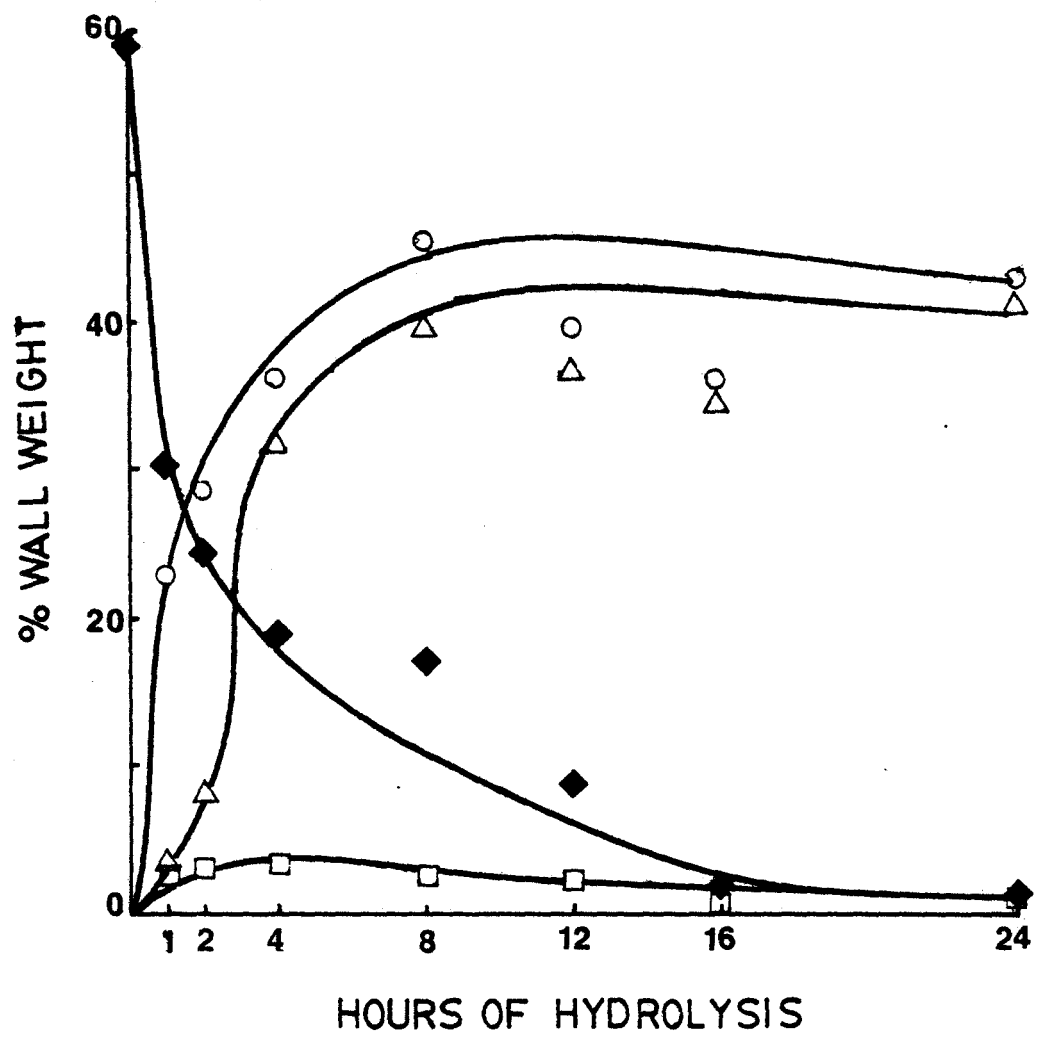


Table 1. Adsorption by  $\text{BaSO}_4$  of monosaccharides during neutralization of 1 N  $\text{H}_2\text{SO}_4$  with  $\text{Ba}(\text{OH})_2$ .<sup>a</sup>

| Treatment of hydrolysate         | Percent sugar recovered |                   |           |
|----------------------------------|-------------------------|-------------------|-----------|
|                                  | glucose                 | mannose           | galactose |
| Neutralized-never basic          | 75.4                    | 76.7              | 86.4      |
| Made basic, then acidic (pH 3.0) | 64.5                    | N.D. <sup>b</sup> | N.D.      |
| Left slightly acidic (pH 3.0)    | 86.3                    | N.D.              | N.D.      |
| Made basic, then neutral         | 45.5                    | 51.8              | 99.8      |

a Monosaccharide solutions (50  $\mu\text{g}/\text{ml}$  1 N  $\text{H}_2\text{SO}_4$ , 2 ml total volume) were neutralized with finely powdered  $\text{Ba}(\text{OH})_2$ . After removing the precipitated  $\text{BaSO}_4$  by centrifugation, the monosaccharide concentration was determined by the anthrone method using the original sugar solutions as standards.

b Not done.

Table 2. Percent sugar not detected in hydrolysates following neutralization with  $\text{Ba}(\text{OH})_2$  as a function of time of hydrolysis.<sup>a</sup>

| Hours of hydrolysis | Percent sugar not detected |
|---------------------|----------------------------|
| 1                   | 43.1                       |
| 2                   | 19.6                       |
| 4                   | 5.6                        |
| 16                  | 4.0                        |
| 24                  | 4.4                        |
| 1 <sup>b</sup>      | 17.2                       |
| 3 <sup>b</sup>      | 12.1                       |
| 12 <sup>b</sup>     | 4.0                        |

a The concentrations of sugars as determined by the anthrone method were determined in the hydrolysates of arthrospore walls before and after neutralization with  $\text{Ba}(\text{OH})_2$ . The value recorded is:

$$\frac{C_b - C_a}{C_b}$$

where  $C_b$  and  $C_a$  are the sugar concentrations before and after neutralization respectively. Hydrolysates were diluted to 4 times their initial volume prior to neutralization.

b Saeman hydrolysis was employed which involved dissolving the wall sample first in cold 72%  $\text{H}_2\text{SO}_4$  for 2 h prior to dilution to 1 N and then heating for the remainder of the hydrolysis.

matography. The developed spots were quantitated after elution with 0.7% acetic acid in 80% ethanol by spectroscopy at 410 nm. As shown in Fig. 9 a straight line absorbance curve was obtained for spots containing between 5 and 100  $\mu$ g. There was no significant absorption differences between the three sugars measured.

E. Determination of total sugar correction factor: The amount of total sugar was determined by the anthrone method using glucose as the standard. However, mannose or galactose yield approximately half the absorbance of an equal amount of glucose. Thus, the values of total sugars obtained by the anthrone method were underestimated. It was not possible to add the individual monosaccharide values obtained from the hydrolysates, since the hydrolysis destroyed approximately 30% of the sample (Figs. 7 and 8). Therefore, in order to compute the actual amount of neutral sugars either the anthrone standard must contain the same ratio of sugars as are present in the wall or a correction factor must be applied. The absorption curves for a number of mixtures of glucose and mannose were determined and compared to that of glucose. As seen in Table 3 the observed ratio of the slopes of these curves were not significantly different from the mathematically predicted ratio, or correction factor. The ratio of the individual sugars in each wall fraction, determined as described above was used to determine this correction factor, which in turn was used to correct the value of total neutral sugars obtained by the anthrone method.

#### Chemical Composition of the Arthrospore and Hyphal Cell Walls

The chemical composition of the arthrospore cell wall was very sim-



Fig. 9. Sample standard calibration curve for sugars eluted from a paper chromatogram sprayed with aniline hydrogen oxalate.  
○: glucose; △: galactose; □: mannose.

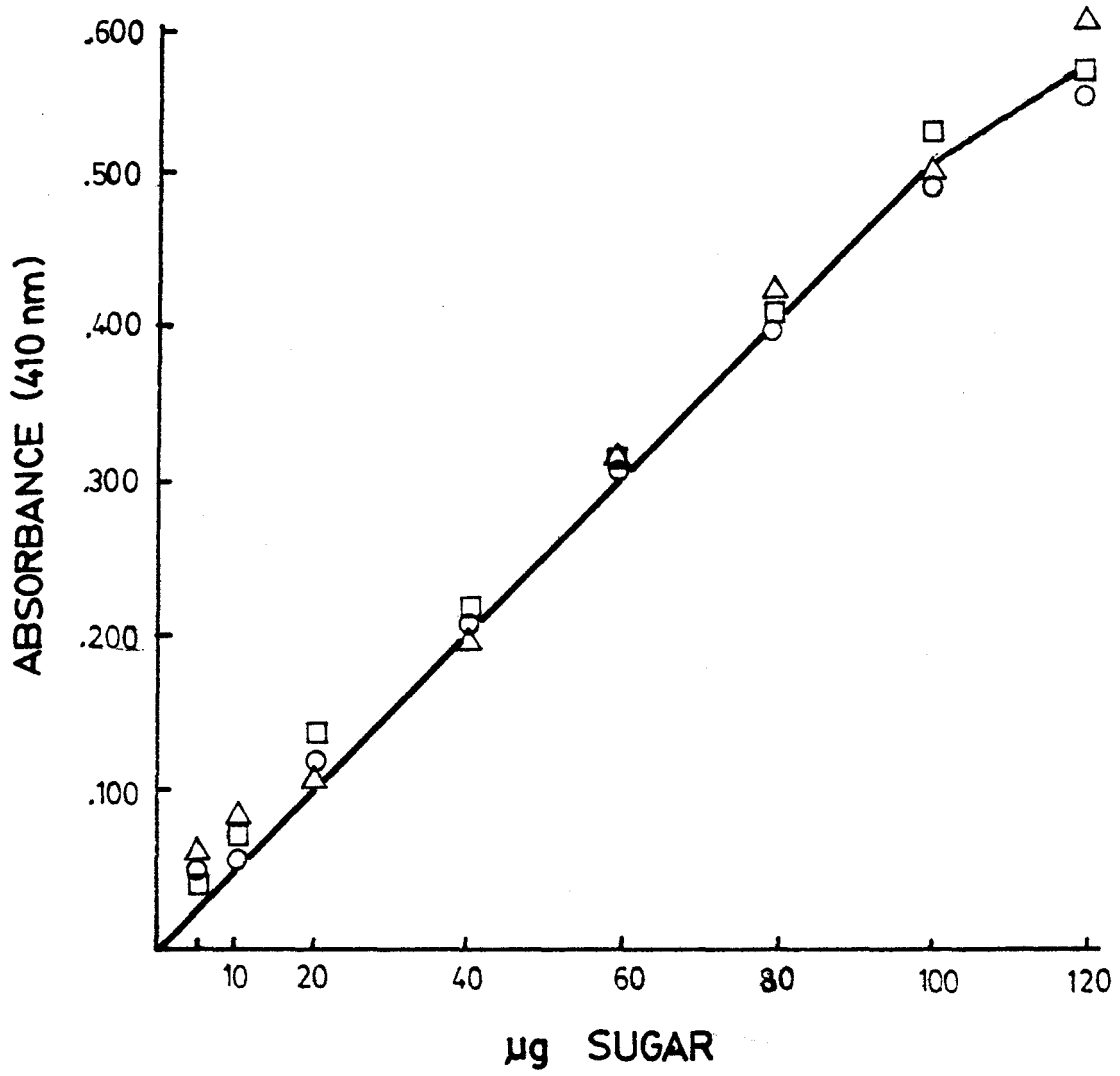


Table 3. Determination of correction factor for total sugar concentration.<sup>a</sup>

| Ratio of<br>glucose:mannose | Observed ratio<br>of slopes <sup>b</sup> | Predicted correction<br>factor <sup>c</sup> |
|-----------------------------|--|---|
| 1:1                         | 1.31                                     | 1.37  |
| 2:1                         | 1.21                                     | 1.22  |
| 3:1                         | 1.12                                     | 1.15  |
| 4:1                         | 1.12                                     | 1.12  |
| 1:2                         | 1.55                                     | 1.55  |
| 1:3                         | 1.71                                     | 1.67  |
| 1:4                         | 1.74                                     | 1.75  |

a The absorbance curves for solutions of glucose and mannose, alone and in combination, were determined by the anthrone method. Each curve was determined on the basis of duplicate readings at concentrations of 10, 30, and 50  $\mu\text{g}$  sugar or combination of sugars per ml water.

b The values recorded are the ratios of the slopes obtained for the sugar mixtures to that obtained for glucose.

c The predicted correction factor is calculated as

$$\text{C.F.} = \frac{g + m}{g + \frac{m}{2.15}}$$

where g equals parts glucose and m equals parts mannose (and/or galactose). The value 2.15 is the ratio of slope the slope of the absorption curve of glucose to that of mannose (or galactose).

ilar to the composition of the hyphal wall from which it was derived. The only neutral sugars found in either wall fraction were glucose, galactose, and mannose. The arthrospore walls contained 65.1% neutral sugars with glucose, mannose and galactose in a ratio of 118:17:10. The hyphal walls were 67.7% neutral sugars with glucose, mannose and galactose in a ratio of 87:14:10 (Table 4). There was little difference in the method used to calculate the monosaccharide ratios. The ratio of glucose to galactose determined using the respective sugar oxidases was 108:10 for the arthrospore wall and 125:10 for the hyphal wall. The same ratios determined by measuring the absorbances of the eluted paper chromatogram spots were 122:10 and 71:10 for the arthrospore and hyphal walls, respectively. The only two amino sugars that could be detected by the amino acid analyzer were glucosamine and galactosamine (Fig. 10). Both hyphal and arthrospore walls contained similar amounts of galactosamine (1.1% and 1.0% respectively). The arthrospore walls contained a slightly higher concentration of glucosamine (23.3%) than did the hyphal walls (20.5%) as determined by averaging the values obtained from the amino acid analyzer and the Elson-Morgan procedure. The hyphal walls did contain significantly more protein (11.2%) and lipid (1.8%) than did the arthrospore walls (3.8% and 0.7%, Table 4). Both types of walls also contained negligible amounts of phosphorus (0.2% and 0.1%). The amino acid compositions of the hyphal and arthrospore walls are presented in Table 5. The amino acids aspartic acid, threonine, serine, glutamic acid, proline, glycine, and alanine accounted for 62.9% of the hyphal wall protein and 72.3% of the arthrospore wall protein.

Table 4. Chemical composition of the arthrospore and hyphal walls of T. mentagrophytes.

| Component                   | Composition (%)  |                  |
|-----------------------------|------------------|------------------|
|                             | Arthrospore wall | Hyphal wall      |
| Neutral sugars <sup>a</sup> | 65.1             | 67.7             |
| glucose                     | 53.1             | 52.9             |
| mannose                     | 7.5              | 8.7              |
| galactose                   | 4.5              | 6.1              |
| Hexosamines <sup>b</sup>    | 24.3             | 21.6             |
| glucosamine                 | 23.3             | 20.5             |
| galactosamine               | 1.0              | 1.1              |
| Protein <sup>c</sup>        | 3.9 <sup>d</sup> | 11.2             |
| Lipid                       | 0.7              | 1.8              |
| Phosphorus                  | 0.1              | 0.2              |
| Ash                         | 1.0              | 1.9 <sup>e</sup> |
| Total                       | 95.1             | 104.4            |

a Values determined by anthrone method corrected for mannose and galactose as described in text.

b Values are the average of values determined by 3 modifications of the Elson-Morgan method and by the amino acid analyzer (see Table 10). The glucosamine value is corrected to reflect 85% acetylation of glucosamine residues (see text). The values obtained by measuring the amount of N-acetylglucosamine released by chitinase using the method of Reissig et al. (1955) were 17.6% and 22.5% for the arthrospore and hyphal walls respectively (see Fig. 15).

c Value determined by the method of Lowry et al. (1951).

d Value determined by amino acid analysis was 3.7%.

## Table 4 (cont'd)

e Not tested. The value was obtained by Wu (Ph.D. dissertation, Loyola University of Chicago, 1976).

Fig. 10. Tracing of elution profile for hexosamines and amino acids separated on the Beckman 120 C amino acid analyzer to demonstrate the presence of galactosamine in the cell walls. A) Profile of acid hydrolysate of Fraction IV-R of the arthrospore wall; B) Standard solution containing glucosamine (glcN), lysine (lys) and histidine (his); C) Standard solution containing galactosamine (galN), lysine and histidine.

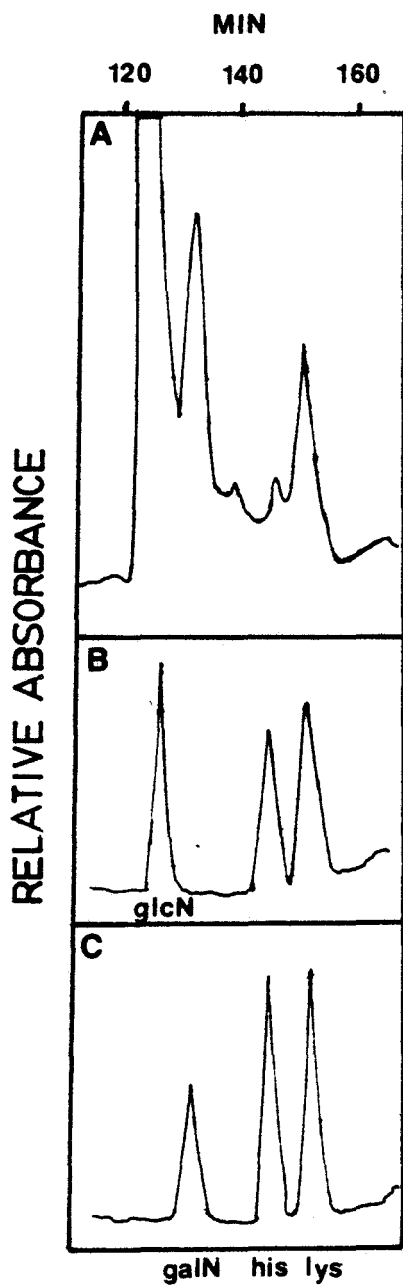




Table 5. Amino acid composition of intact arthrospore and hyphal walls.

| Amino acid                | mole %           |                          |
|---------------------------|------------------|--------------------------|
|                           | Arthrospore wall | Hyphal wall <sup>b</sup> |
| Lysine                    | 3.3              | 5.2                      |
| Histidine                 | 0.6              | Trace                    |
| Arginine                  | 2.2              | 4.5                      |
| Aspartic acid             | 8.4              | 12.6                     |
| Threonine                 | 10.5             | 7.3                      |
| Serine                    | 11.9             | 8.4                      |
| Glutamic acid             | 12.2             | 11.1                     |
| Proline                   | 10.2             | 6.4                      |
| Glycine                   | 13.6             | 8.3                      |
| Alanine                   | 5.5              | 8.8                      |
| Half-cystine              | Trace            | 0.1                      |
| Valine                    | 7.1              | 6.2                      |
| Methionine                | 3.1              | 1.3                      |
| Isoleucine                | 4.4              | 4.7                      |
| Leucine                   | 3.8              | 7.9                      |
| Tyrosine                  | 1.1              | 3.0                      |
| Phenylalanine             | 2.1              | 3.5                      |
| Unidentified <sup>a</sup> | +                | +                        |

a Eluted at 30 ml from acidic column.

b Data cited in C.D.R. Wu, Ph.D. dissertation, Loyola University of Chicago, 1976.

## Chemical Fractionation of the Arthrospore and Hyphal Walls.

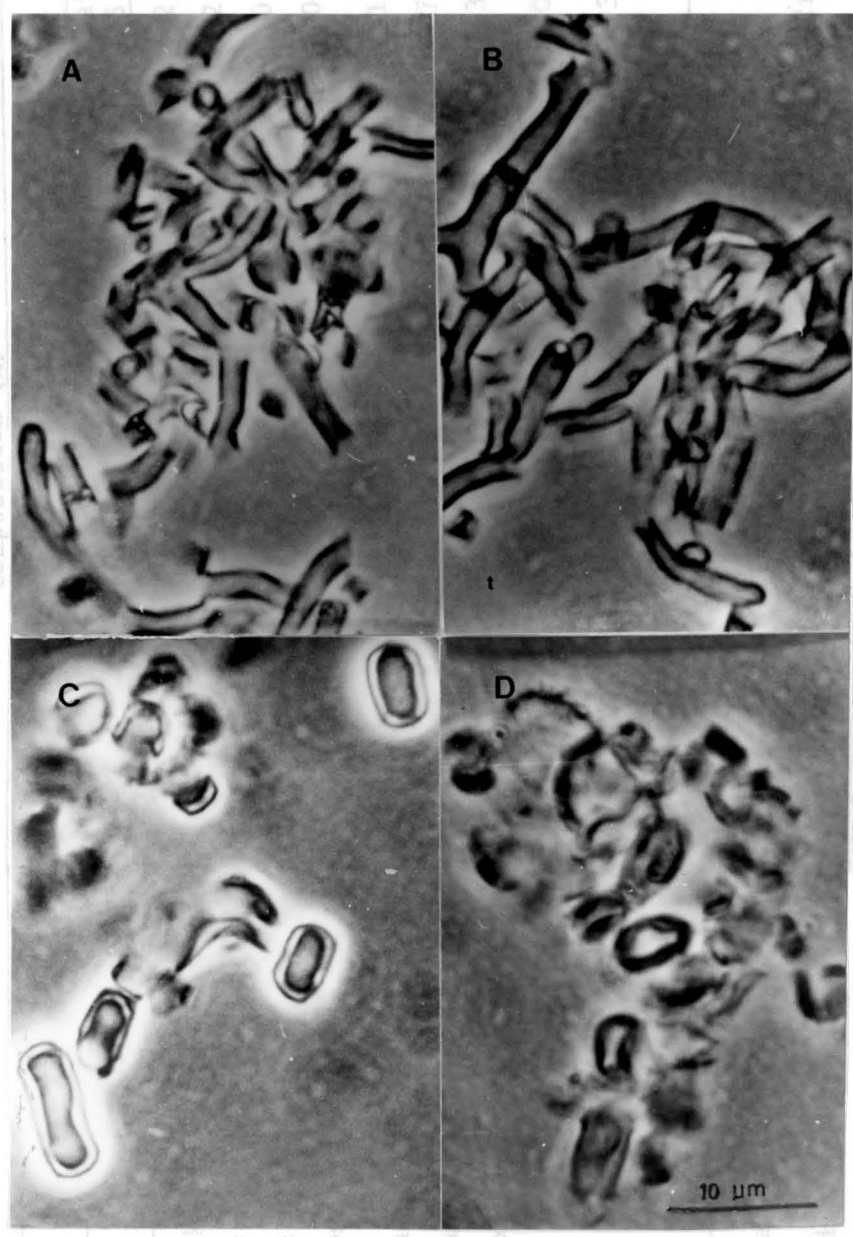
A. Appearance: The arthrospore and hyphal walls were first fractionated with 1 N NaOH at 100°C for a total of 3 h. Although this extraction procedure removed 44.4% and 57% of the dry weights of the hyphal and arthrospore walls, respectively (Table 6), little change other than loss of refractility could be observed in the appearances of either wall preparation using phase contrast microscopy (Fig. 11).

B. Composition: The chemical compositions of the wall fractions resulting from alkali extraction of the hyphal and arthrospore walls are shown in Table 6. Extensive alkali treatment removed all detectable mannose and galactose from both hyphal and arthrospore walls and approximately 50% of the glucose from the hyphal walls and 70% of the glucose present in the arthrospore walls. In addition, essentially all of the protein present in both wall types was removed. However, alkali removed only 2.0% and 2.8% of the hexosamines from the hyphal and arthrospore walls, respectively. Of the hexosamines removed by alkali, less than 1% of the wall glucosamine was removed, while between 30 and 40% of the wall galactosamine was removed. The composition of the residue of the arthrospore wall following alkali extraction was 41.8% glucose, 55.7% hexosamines, of which 3.6% was galactosamine, the remainder glucosamine, and 0.4% amino acids. The hyphal wall residue (I-R) was composed of 52.3% glucose, 41.1% hexosamines, and 0.4% amino acids. The amino acid analyses of the alkali residues are shown in Table 7. Following removal of the majority of the wall protein with alkali, the basic amino acids, lysine and histidine, predominated, accounting for 51.6% and 69.5% of the amino acids of hyphal and arthrospore Fraction I-R, respectively.

Fig. 11. Phase contrast micrographs of intact (A) and alkali extracted (B) hyphal walls, and intact (C) and alkali extracted (D) arthrospore walls of T. mentagrophytes. No change can be observed in the treated hyphal walls. While the alkali extracted arthrospore walls (D) appear less refractile and tend to clump, they do not appear to be much thinner than the intact walls (C).

Table 6. Chemical composition of major wall fractions resulting from alkali extraction of arthrospore and hyphal walls.

|           | Composition (%) |      |
|-----------|-----------------|------|
| Neutral s | 1.51            | 1.3  |
| glucose   | 5.6             | 2.3  |
| mannose   | 1.0             | 1.0  |
| galacto   | 1.0             | 1.5  |
| Hexosamin | 7.79            | 7.79 |
| glucosa   | 3.8             | 3.8  |
| galacto   | 0.41            | 0.41 |
| Protein   | 3.9             | 3.9  |
| Total     |                 |      |
| a         |                 |      |
| b         |                 |      |
| c         |                 |      |
| d         |                 |      |



Values determined by anthrone method corrected for mannose and galactose (see Metarai and Methods).

Values were determined by analyzing the lyophilized sample. Values determined by analyzing reaction.

Table 6. Chemical composition of major wall fractions resulting from alkali extraction of arthrospore and hyphal walls.

|                             | Composition (%)                         |                                    |   |                                    |
|-----------------------------|---|------------------------------------|---|------------------------------------|
|                             | I-S <sup>a</sup>                        |                                    | I-R <sup>a</sup>                        |                                    |
|                             | Arthrospore wall<br>(57.0) <sup>b</sup> | Hyphal wall<br>(44.4) <sup>b</sup> | Arthrospore wall<br>(43.0) <sup>b</sup> | Hyphal wall<br>(55.6) <sup>b</sup> |
| Neutral sugars <sup>c</sup> | 95.3 <sup>d</sup>                       | 92.0 <sup>d</sup>                  | 41.8                                    | 52.3                               |
| glucose                     | 66.3                                    | 59.8                               | 41.8                                    | 52.3                               |
| mannose                     | 20.7                                    | 23.0                               | 0.0                                     | 0.0                                |
| galactose                   | 8.3                                     | 9.2                                | 0.0                                     | 0.0                                |
| Hexosamines                 | 1.2 <sup>e</sup>                        | 1.0 <sup>e</sup>                   | 55.7 <sup>f</sup>                       | 41.5 <sup>f</sup>                  |
| glucosamine                 | 0.4                                     | 0.3                                | 53.7 <sup>g</sup>                       | 37.7 <sup>g</sup>                  |
| galactosamine               | 0.8                                     | 0.7                                | 2.0                                     | 3.8                                |
| Protein                     | 11.3 <sup>h</sup>                       | 20.3 <sup>h</sup>                  | 0.4 <sup>i</sup>                        | 0.4 <sup>i</sup>                   |
| Total                       | 107.8                                   | 113.3                              | 97.9                                    | 93.8                               |

a See Fig. 1 for fraction designation.

b Figures in parentheses represent percent dry weight of intact walls of each fraction.

c Values determined by anthrone method corrected for mannose and galactose (see Material and Methods).

d Values were determined by analyzing the lyophilized sample. Values determined by analyzing

Table 6 (cont'd)

the alkali extract directly without first dialyzing and lyophilizing sample were significantly lower (see text).

- e Determined by the method of Wagner (1979).
- f Values are the average of values determined by three modifications of the Elson-Morgan method (see Table 10).
- g Glucosamine and galactosamine ratio determined by amino acid analyzer following 22 h hydrolysis with 6 N HCl. Glucosamine value is calculated as 85% N-acetylglucosamine.
- h Determined by the method of Lowry (1951).
- i Determined by amino acid analyzer.

Table 7. Amino acid composition of alkali extracted arthrospore and hyphal walls (Fraction I-R).

| Amino acid                | Mole %                            |                              |
|---------------------------|-----------------------------------|------------------------------|
|                           | Alkali-extracted arthrospore wall | Alkali-extracted hyphal wall |
| Lysine                    | 54.3                              | 41.3                         |
| Histidine                 | 15.2                              | 10.3                         |
| Arginine                  | 0                                 | 0                            |
| Aspartic acid             | 4.8                               | 6.9                          |
| Threonine                 | 1.0                               | 1.4                          |
| Serine                    | 4.8                               | 2.8                          |
| Glutamic acid             | 4.8                               | 2.1                          |
| Proline                   | 0                                 | 0                            |
| Glycine                   | 7.6                               | 6.2                          |
| Alanine                   | 1.9                               | 4.1                          |
| Half-cystine              | 0                                 | 0                            |
| Valine                    | 0                                 | 1.4                          |
| Methionine                | 0                                 | 0                            |
| Isoleucine                | 1.0                               | 6.9                          |
| Leucine                   | 1.9                               | 2.1                          |
| Tyrosine                  | 0                                 | 5.5                          |
| Phenylalanine             | 2.9                               | 9.0                          |
| Unidentified <sup>a</sup> | +                                 | +                            |

a Eluted at 30 ml from acidic column.

Analysis of the alkali extractable material (I-S) was complicated by the fact that between 50 and 60% of the expected material (determined by subtracting the weight of the wall residues [I-R] from the initial wall weight) was not recovered following dialysis and lyophilization. Only 3.9% of the wall weight was detected in the dialysate by the anthrone method so it is very likely that much of the alkali extractable material was not lost by dialysis, but rather was depolymerized and destroyed. Analysis of the alkali extract prior to dialysis and lyophilization, and by determining the weight of the extractable material by subtracting the weight of the residue from the initial wall weight, showed that approximately 60% consisted of neutral sugars. However, analysis of the lyophilized extracted material revealed that 92.0% of the hyphal extract and 95.3% of the arthrospore extract were composed of neutral sugars. It appears that more galactose than mannose was destroyed since the ratio of mannose to galactose is much greater in the alkali extractable material (25:10 for both hyphal and arthrospore wall extracts) than in the intact walls (14:10 for the hyphal and 16:10 for the arthrospore walls). After subtracting the amount of glucose that remained in the alkali residue from the initial amount present in the walls, the ratio of glucose to mannose is not significantly different than the ratio in the alkali extractable material suggesting that glucose and mannose are equally affected by alkali.

In addition to the neutral sugars, the recoverable alkali soluble material of the arthrospore walls consisted of 0.4% glucosamine, 0.8% galactosamine, and 11.3% protein. The same fraction of the hyphal walls consisted of 1% hexosamines, 70% of which were galactosamine, the remainder glucosamine, and 20.3% protein.



C. Precipitation with Fehling's solution: The addition of Fehling's solution to fraction I-S of the arthrospore wall dissolved in 1 N NaOH, precipitated 15.3% of the fraction. Analysis of the neutral sugars in the precipitate (I-SF) showed mannose, galactose, and glucose present in the ratio of 25:10:7. The fact that the ratio of mannose to galactose was unchanged from that in Fraction I-S indicated the probable existence of a galactomannan in the arthrospore wall. No attempt was made to further purify this polymer from the glucose present in this fraction by repeated precipitations with Fehling's solution.

D. Disc electrophoresis: Fraction I-S was also separated by disc electrophoresis. Protein was detected only in a band migrating with the front. This band was not reactive with PAS stain. Two bands, which were PAS positive, were detected when the sample was allowed to electrophorese for 6 h (Fig. 12). It was not possible to determine if one of these bands corresponds to the galactomannan due to the impurity of fraction I-SF.

#### Enzymatic Digestion of the Arthrospore Cell Wall and Residues.

A. (1→3)-β-glucanase digestion of Fraction I-R: Digestion of Fraction I-R with (1→3)-β-glucanase removed 50.3% of the fraction's weight and 94%  $(1 - [28.7 \times 0.04] / [41.8 \times 0.43])$  of the fraction's glucose (Table 8). No change was observed in the appearance of phase contrast micrographs of the wall residues as a result of this treatment (Fig. 13). Paper chromatography of the enzyme lysate (Fraction II-S) revealed the presence of glucose and the same glucose oligomers released from laminarin ([1→3]-β-glucan from seaweed) by (1→3)-β-glucanase. In addition N-acetylglucosamine was also detected in the lysate, (data not shown).

Fig. 12. Disc electrophoresis of the alkali extractable material (Fraction I-S) from the arthrospore walls. A. Gel run using the buffer system of Davis (1964). The gel is stained with Coomassie blue for protein. The arrow indicates the location of the lightly stained band and the buffer front. B. Gel run in the more basic Tris-borate buffer system. Gel stained with PAS. Arrows indicate positions of stained bands.

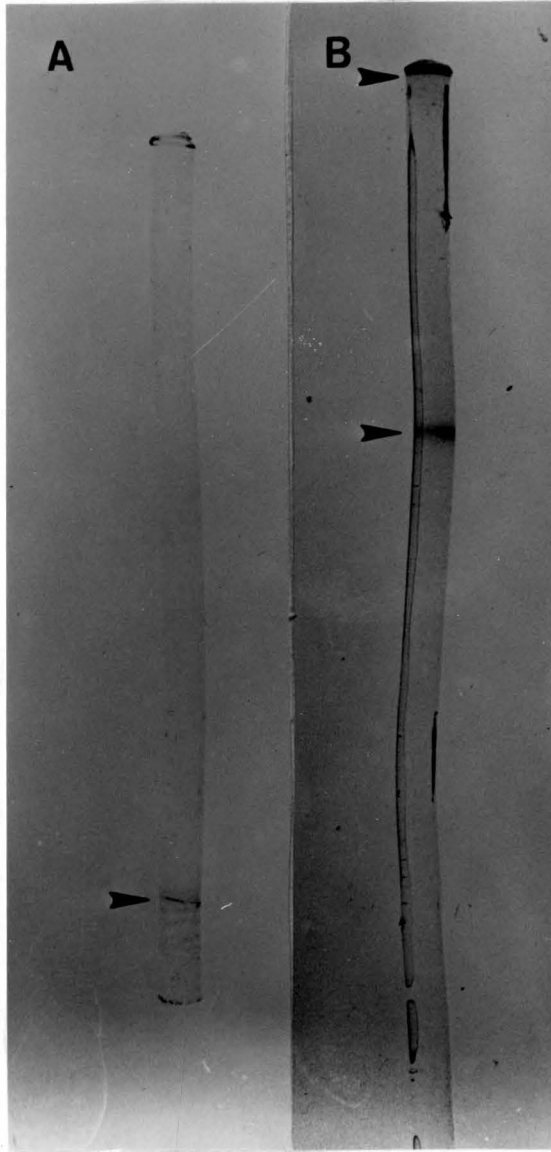


Fig. 13. Phase contract micrographs of (A) alkali extracted, glucanase digested arthrospore walls (Fraction II-R), and (B) glucanase digested arthrospore walls (Fraction III-R). Note that very little, if any, thinning of the arthrospore walls has occurred as a result of these treatments.

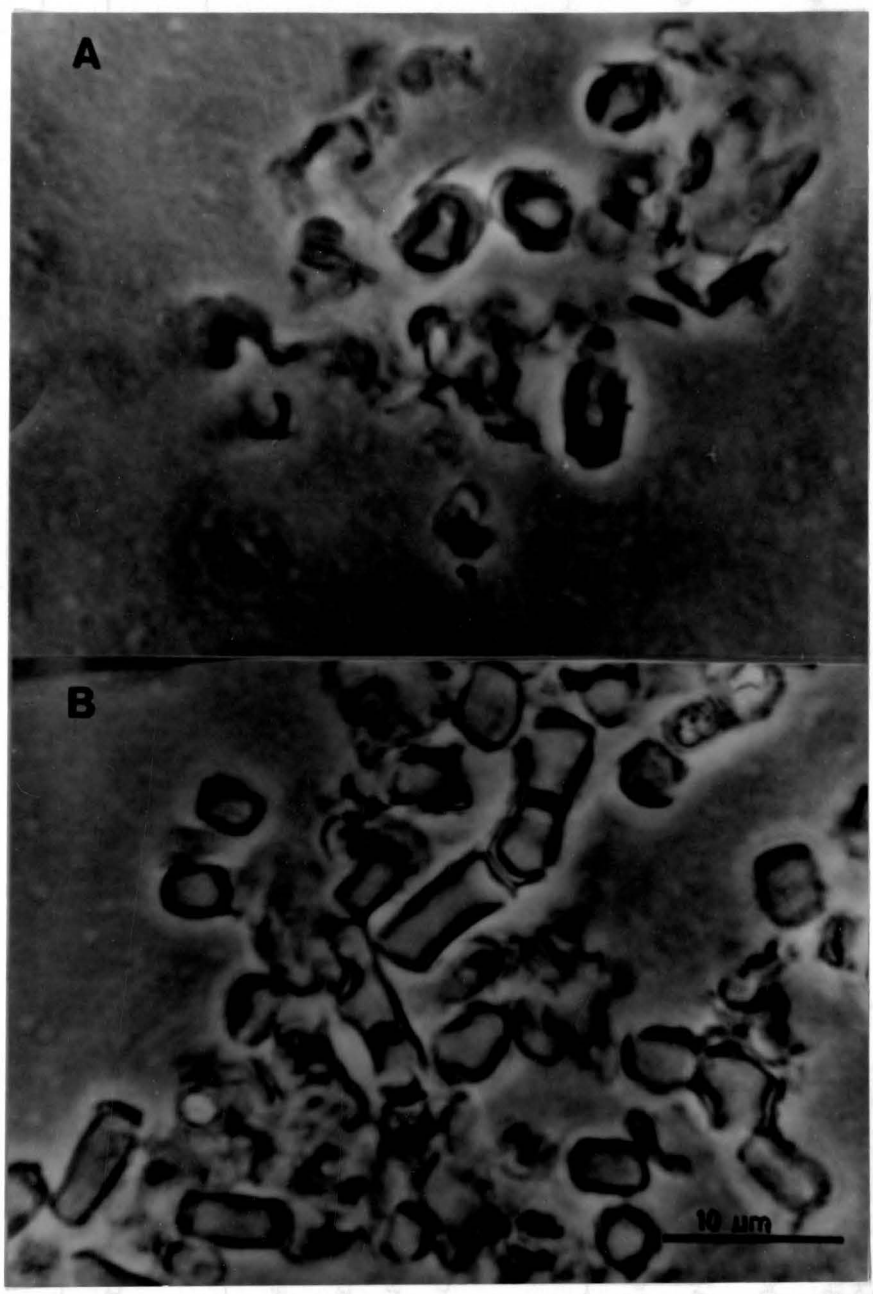


Figure 1. Electron micrographs of the structures showing cross-sectional digestion.

| Component | Neutral | Acidic | Glycosyl | Polysac | Protein |
|-----------|---------|--------|----------|---------|---------|
| a         | S       |        |          |         |         |
| b         | P       |        |          |         |         |
| c         | D       |        |          |         |         |
| d         | V       |        |          |         |         |
| e         | T       |        |          |         |         |

The ratio of glycosylated to unglycosylated was determined by the ratio of the area of the glycosylated to unglycosylated structures determined by the ratio of the area of the glycosylated to unglycosylated structures.

each fraction.  
 in procedure.

Table 8. Chemical composition of the arthrospore wall fractions resulting from enzymatic digestion.

| Component                   | Composition (%)                          |   |  |   |
|-----------------------------|--|---|--|---|
|                             | II-R <sup>a</sup><br>(28.7) <sup>b</sup> | III-R <sup>a</sup><br>(37.3) <sup>b</sup> | IV-R <sup>a</sup><br>(13.4) <sup>b</sup> | V-R <sup>a</sup><br>(10.4) <sup>b</sup> |
| Neutral sugars <sup>c</sup> | 4.0                                      | 9.4                                       | 4.7                                      | 2.5                                     |
| Hexosamines <sup>d</sup>    | 63.3                                     | 58.3                                      | 80.4                                     | 87.0                                    |
| glucosamine <sup>e</sup>    | 62.6                                     | NT  | NT                                       | 85.0                                    |
| galactosamine               | 0.7                                      | NT  | NT                                       | 2.0                                     |
| Protein <sup>f</sup>        | NT <sup>g</sup>                          | NT  | 1.1                                      | 0.4                                     |

a See Fig. 1 for fraction designation.

b Figures in parentheses represent percent of dry weight of intact arthrospore in each fraction.

c Determined by anthrone method using glucose as the standard.

d Values are the average of values determined by 3 modifications of the Elson-Morgan procedure. The glucosamine value is calculated as 85% N-acetylglucosamine.

e The ratio of glucosamine to galactosamine was determined by the amino acid analyzer.

f Determined by the amino acid analyzer.

Table 8 (cont'd)

g Not tested.

The chemical composition of the arthrospore walls sequentially treated with alkali and (1→3)-β-glucanase was 4% neutral sugars, 62.6% glucosamine and 0.7% galactosamine (Table 8).

B. Chitinase digestion of Fraction I-R: Paper chromatography of the chitinase hydrolysate of Fraction I-R revealed the presence of N-acetylglucosamine and a spot which had the same mobility and color when sprayed with aniline hydrogen oxalate, as did N,N-diacetylchitobiose, (data not shown). The fact that this spot was also insensitive to ninhydrin probably indicated that both glucosamine residues were acetylated. Chitobiose was not detected in the chitinase hydrolysates of intact arthrospore walls.

C. (1→3)-β-glucanase digestion of the arthrospore wall: Digestion of the arthrospore cell wall with (1→3)-β-glucanase removed almost 63% of the cell wall (Table 8), despite the fact that the (1→3)-β-glucan present in Fraction I-R accounted for only 16.9% ( $0.94 \times [41.8 \times 0.43]$ ) of the weight of the arthrospore cell wall. As the glucanase showed no activity on the alkali soluble fraction (I-S), this might indicate some type of linkage between the β-glucan and the alkali soluble polymers. Besides releasing glucose and glucose oligomers, the glucanase also released N-acetylglucosamine from the arthrospore wall, (see Fig. 15). The N-acetylglucosamine released by (1→3)-β-glucanase was 0.8% of the arthrospore wall weight (Table 9). However, since the glucanase did not have any chitinase activity, this N-acetylglucosamine was not released from chitin.

The glucanase-digested arthrospore walls (Fraction III-R) consisted of 9.4% neutral sugars (as glucose) and 58.3% hexosamines (Table 8).



Table 9. Quantitative distribution of hexosamines and chitinase-sensitive and -resistant chitins in the arthrospore cell wall.

| Hexosamine type                                  | Hexosamine content          |   |                                       |
|--|-----------------------------|---|---------------------------------------|
|  | Percent initial wall weight | Percent relative to total wall hexosamine | Percent relative to total wall chitin |
| (1→3)-β-Glucanase sensitive <sup>a</sup> (III-S) | 0.8                         | 2.7                                       |                                       |
| Chitinase sensitive <sup>b</sup> (IV-S)          | 17.6                        | 60.3                                      | 67.0                                  |
| Chitinase, alkali resistant (V-R)                | 9.0                         | 30.8                                      | 33.0                                  |
| GlcNAc <sup>c</sup>                              | 8.8                         | 30.1                                      | 33.0                                  |
| GalN <sup>d</sup>                                | 0.2 <sup>f</sup>            | 0.7                                       |                                       |
| Alkali sensitive <sup>e</sup>                    | 1.8 <sup>f</sup>            | 6.2                                       |                                       |
| Total  | 29.2                        | 100.0                                     | 100.0                                 |

- a Released as N-acetylglucosamine by (1→3)-β-glucanase from the intact arthrospore walls. Determined by the method of Ressig et al. (1955).
- b Released as N-acetylglucosamine by chitinase from arthrospore wall fraction III-R. Determined by the method of Ressig et al. (1955).
- c N-acetylglucosamine determined by modified Elson-Morgan reaction (See Table 10).
- d Galactosamine determined with the amino acid analyzer (Beckman 120 C).
- e Determined by subtracting the total hexosamine found in Fraction V-R from that found in fraction IV-R ( $13.4 \times 0.804 - 10.4 \times 0.87$ ) (Table 8). Includes hexosamines lost during extraction and wash

procedures. Only 0.7% of the arthrospore wall hexosamine was released by alkali from the intact arthrospore wall (Table 6).

f Hexosamine not thought to be derived from chitin.

They appear noticeably thinner than the intact arthrospore walls in phase contrast micrographs (Fig. 13).

D. Chitinase digestion of Fraction III-R: Digestion of the arthrospore walls sequentially with (1 $\rightarrow$ 3)- $\beta$ -glucanase and chitinase left only 13.4% of the original arthrospore wall weight (Table 8). Although the walls appeared considerably thinner, the structural integrity of the walls was not impaired, that is, the wall residues still retained their ovoid shape (Fig. 14).

Chitinase removed 47.9% of Fraction III-R (17.6% of the arthrospore wall weight) as N-acetylglucosamine (Table 9). Even so, the arthrospore walls exhaustively digested with glucanase and chitinase (Fraction IV-R) consisted of 80.4% hexosamine and only small amounts of neutral sugars (4.7%) and protein (1.1%) (Table 8). As shown in Fig. 15, essentially no additional N-acetylglucosamine was released by chitinase after digestion for 4 days, indicating completeness of digestion by that time. Extended digestions of this fraction with glucanase and chitinase for as long as 2 weeks had no further effect on either the appearance or composition of this fraction. Treating this fraction with hot 1 N NaOH for 3 h removed approximately one-fifth of the fraction's weight in the form of neutral sugars, protein and some glucosamine. The alkali extracted fraction (Fraction V-R) consisted of 85.0% glucosamine, 2.0% galactosamine, 2.5% neutral sugars, and 0.4% amino acids. Treating Fraction V-R with chitinase was without effect. The percent of chitinase-sensitive and -resistant chitins is shown in Table 9.

#### Determination of Hexosamines in the Wall Fractions.

At first the determination of hexosamine content in Fraction IV-R

Fig. 14. Phase contrast micrographs of (A) arthrospore walls sequentially digested with (1→3)-β-glucanase and chitinase (Fraction IV-R) and (B,C) arthrospore walls treated with the two enzymes followed by hot alkali (Fraction V-R). Note the thinness of the arthrospore side walls and the numerous free septa which are evident (arrows).

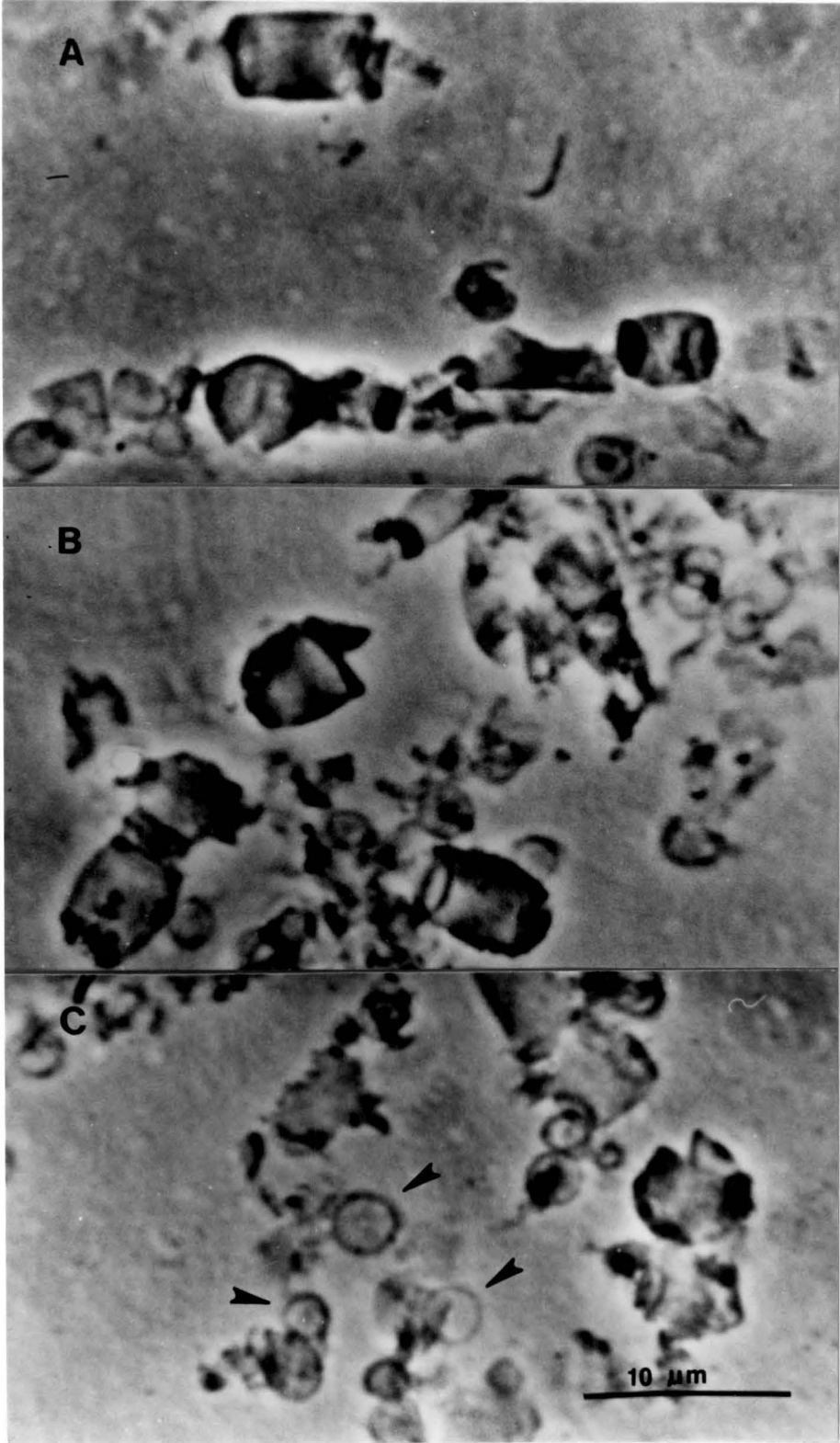
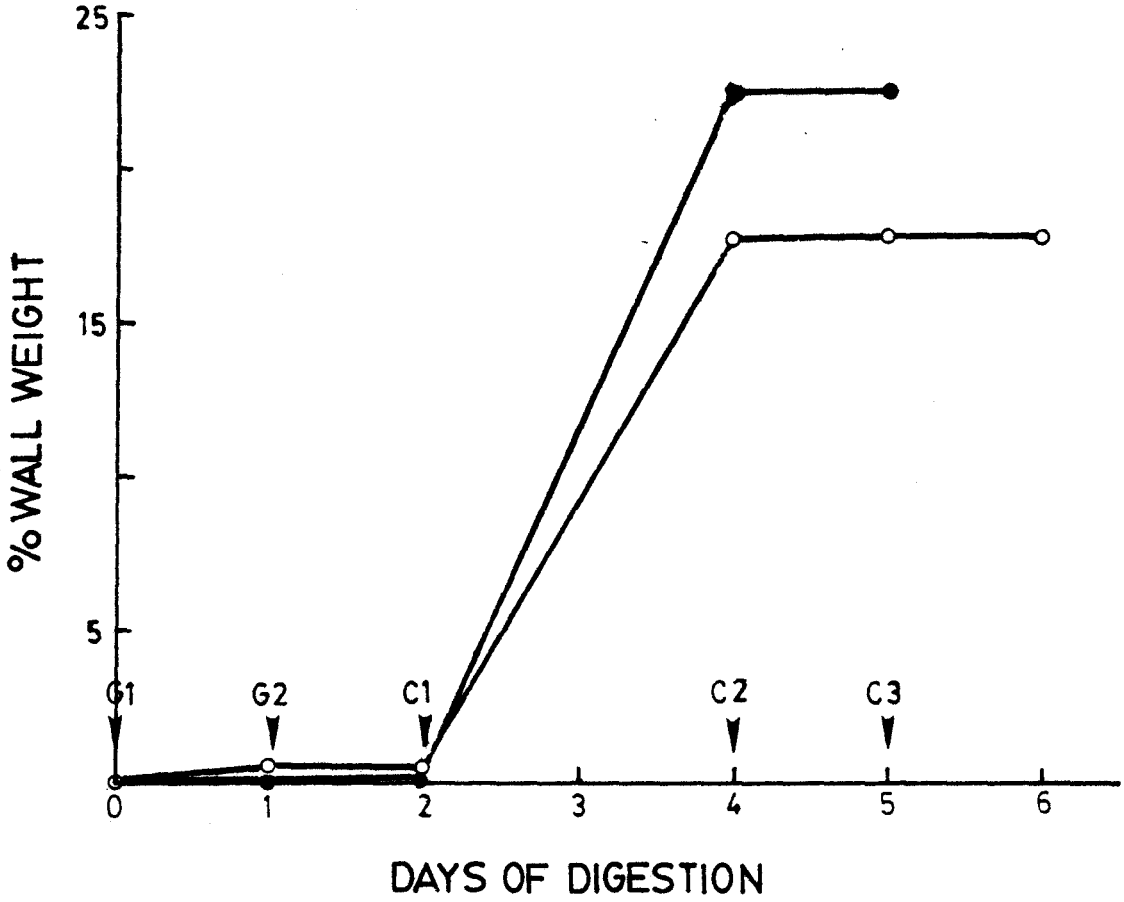


Fig. 15. Release of N-acetylglucosamine from the arthrospore (○) and hyphal (●) cell walls by the action of Streptomyces chitinase and (1→3)-β-glucanase. Samples were digested sequentially with each enzyme. Enzymatic hydrolysates were collected by centrifugation, combined with the three subsequent water washes and analyzed by the method of Reissig et al., (1955) for N-acetylglucosamine. Fresh enzyme was added as indicated by arrows. G1 = 1st glucanase digestion, G2 = 2nd glucanase digestion, C1 = 1st chitinase digestion, etc. N-acetylglucosamine release is expressed as percent of initial wall weight.



appeared to be affected by the method used to remove HCl from the hydrolysate. The average hexosamine content (not corrected to reflect 85% N-acetylation as in previous tables) of Fraction IV was only 46.9% using the method of Winzler (1955), in which the HCl was removed by evaporation under vacuum from the hydrolysate (Table 10). On the other hand, when the hydrolysates were neutralized with 1 N NaOH, the hexosamine content was determined to be 63.0% using the method of Winzler (1955) and 71.0% using the method of Johnson (1971). In later experiments using Johnson's method with evaporated hydrolysates, the hexosamine content was determined to be 65.8% (data not shown), perhaps indicating that hexosamine values are higher using this procedure. A complete table of the hexosamine values for the various wall fractions, determined by the different procedures, is shown in Table 10. No pattern could be detected from the results. Although, the hexosamine values were greater using the Johnson procedure for Fractions I-R, IV-R, and V-R, they were not greater for the other fractions. Likewise, although the hexosamine determination on evaporated hydrolysates were less than those for the neutralized hydrolysates of Fractions I-R-H, III-R, and IV-R, there was no difference in the other fractions.

#### Determination of the Nature of the Glucosamine Polymer of the Chitinase Resistant Wall Fraction.

The existence of an entire layer of the arthrospore wall, consisting essentially of a glucosamine polymer, even after exhaustive chitinase digestion, appeared to be a unique phenomenon which prompted further investigation of the nature of this structure. The important questions were: 1) Is the glucosamine polymer chitin or a chitin derivative? 2) If the structure is chitin, what differences are there from authentic



Table 10. Percent glucosamine in the wall fractions of *T. mentagrophytes* as determined by the different methods used in this study.

|  | Percent glucosamine |                |                   |                   |                   |                    |                   |                  |
|--|---------------------|----------------|-------------------|-------------------|-------------------|--------------------|-------------------|------------------|
|  | Intact walls        |                | I-R <sup>a</sup>  |                   | II-R <sup>a</sup> | III-R <sup>a</sup> | IV-R <sup>a</sup> | V-R <sup>a</sup> |
|  | H <sup>b</sup>      | A <sup>c</sup> | H                 | A                 | A                 | A                  | A                 | A                |
| Johnson (1971) <sup>d</sup><br>neutralized<br>sample | 18.3                | 21.0           | 46.3              | 52.1              | 50.9              | 49.0               | 71.0              | 76.4             |
| Winzler (1955) <sup>d</sup><br>neutralized<br>sample | 20.9                | 21.7           | 32.9              | 43.7              | 51.7              | 54.6               | 63.0              | 68.6             |
| Winzler <sup>d</sup><br>evaporated<br>sample         | 17.7                | 20.4           | 26.4              | 44.6              | 52.2              | 42.3               | 46.9              | NT               |
| Amino Acid<br>analyzer,<br>evaporated<br>sample      | 15.9                | 18.5           | 24.0 <sup>e</sup> | 35.0 <sup>e</sup> | 56.2              | NT                 | NT                | NT               |

a See Fig. 1 for fraction designation.

b Hyphal wall fraction.

c Arthrospore wall fraction.

d See Materials and Methods section for description of procedure.

Table 10 (cont'd)

e      Sample hydrolyzed for 22 h instead of 6 h.

chitin which might account for its unusual resistance to chitinase?

3) Under what conditions could this fraction be digested?

A. X-Ray Diffraction of Fraction IV-R: The X-ray diffraction pattern of Fraction IV-R was almost identical to that of authentic chitin (Fig. 16). Major reflections were observed at approximately 9.6, 4.7 and 3.3 Å, while a minor reflection was observed at approximately 3.7 Å. Only the minor diffraction line of chitin at 7.0 Å was not observed with Fraction IV-R. The diffraction pattern of Fraction IV-R was not very sharp. However, when the fraction, dissolved in 6 N HCl, was reprecipitated with NaOH, washed until neutral, and lyophilized, the reflections were considerably sharpened. The diffraction line at 7.0 Å did not appear.

B. Infrared spectrum of Fraction IV-R: The infrared spectrum of Fraction IV-R was essentially the same as that of authentic chitin (Fig. 17). The absorbances at  $1655\text{ cm}^{-1}$ , and  $1560\text{ cm}^{-1}$ , which were totally lacking from the spectrum of chitosan, indicated that the wall fraction was acetylated. The degree of acetylation was calculated, as described in Materials and Methods, to be between 85 and 90%. The absorbance peak at  $890\text{ cm}^{-1}$  was evidence of  $\beta$ -glycosidic linkage. The only absorbance peak which was present in the chitin spectrum, but was lacking from the Fraction IV-R spectrum, was at  $1110\text{ cm}^{-1}$ .

C. Appearance of carbon-platinum shadowed preparation of Fraction IV-R: Carbon-platinum shadowed preparations of Fraction IV-R, unlike those of intact arthrospore walls, or walls extracted with alkali or digested with (1 $\rightarrow$ 3)- $\beta$ -glucanase, showed no evidence of chitin microfibrils. Instead, the surface of the chitinase digested fractions appeared rough and amorphous (Fig. 18).

Fig. 16. X-ray diffraction patterns (left) and density scanning of the patterns (right) of (A) arthrospore walls sequentially digested with glucanase and chitinase (Fraction IV-R), (B) chitin, and (C) Fraction IV-R following precipitation from 6 N HCl. Numbers are the  $d$  values in  $\text{\AA}$  of each reflection.

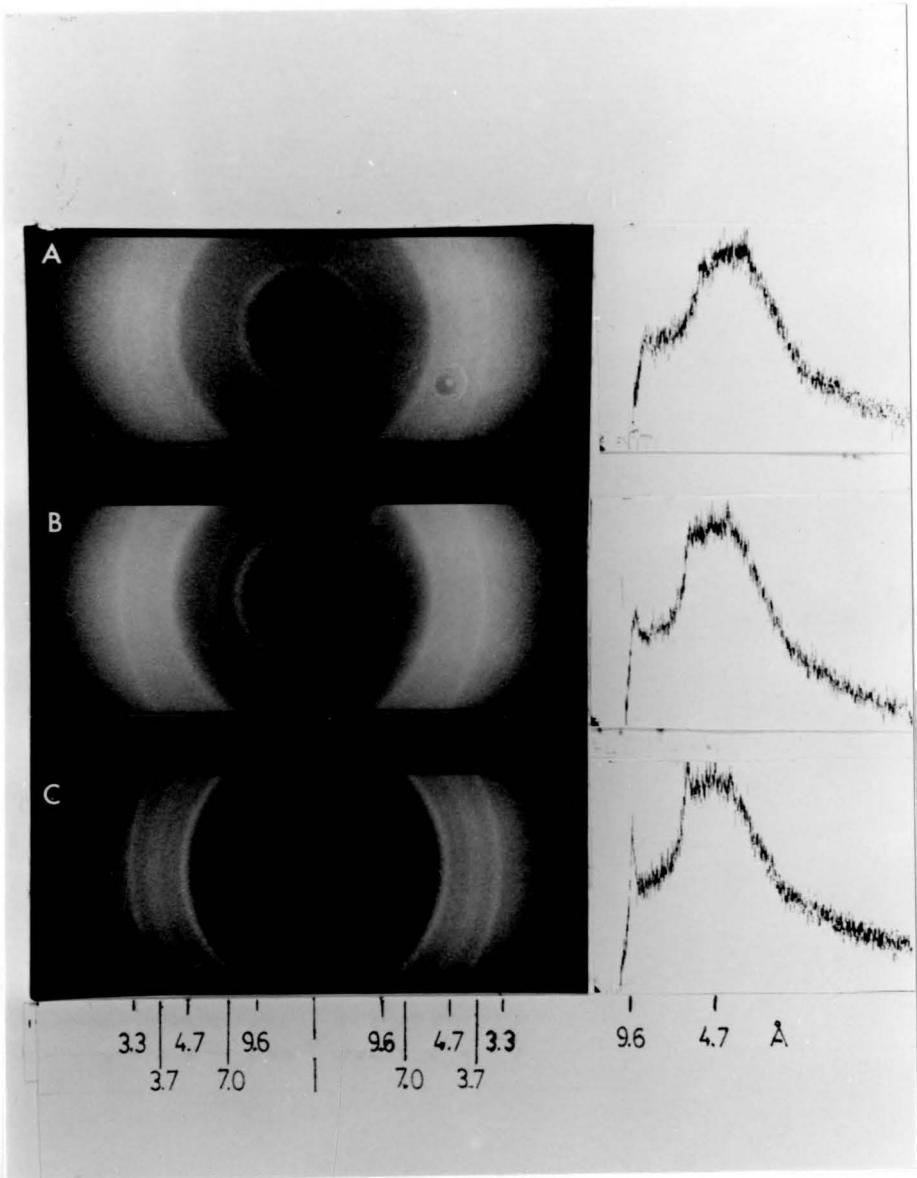


Fig. 17. Infrared spectra of (a) arthrospore wall Fraction IV-R, (b) Fraction IV-R precipitated from 6 N HCl, (c) arthrospore wall residue following alkali extraction (Fraction I-R), (d) chitin, and (e) chitosan.

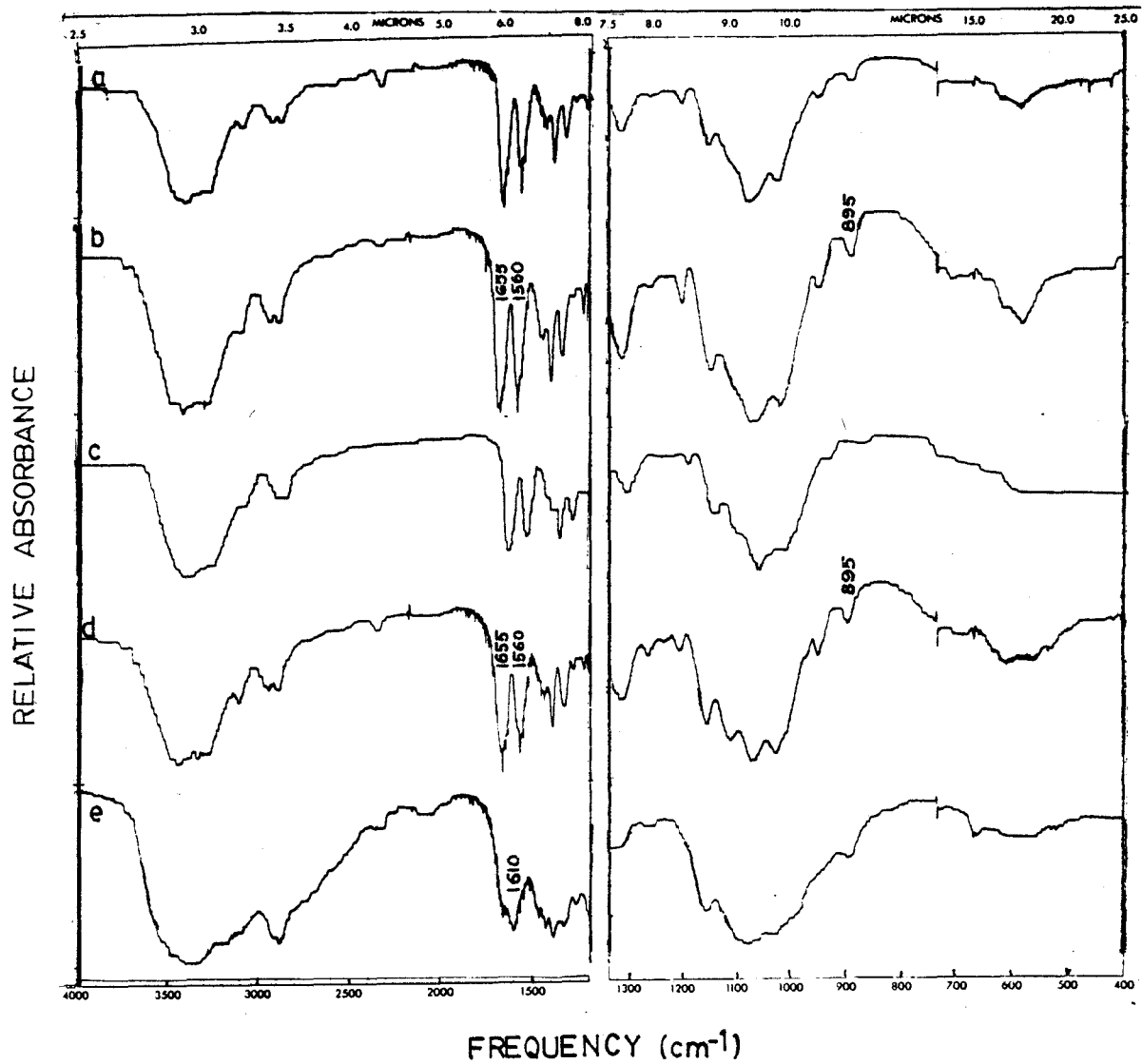
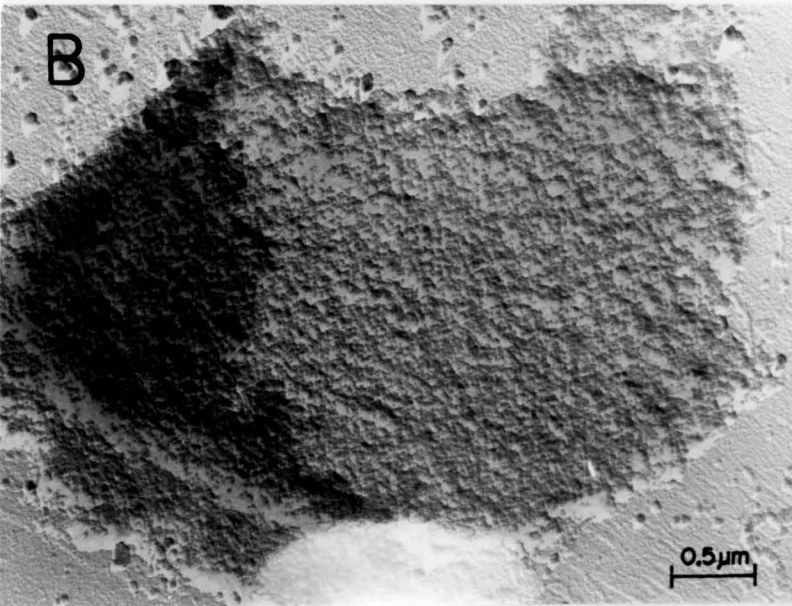
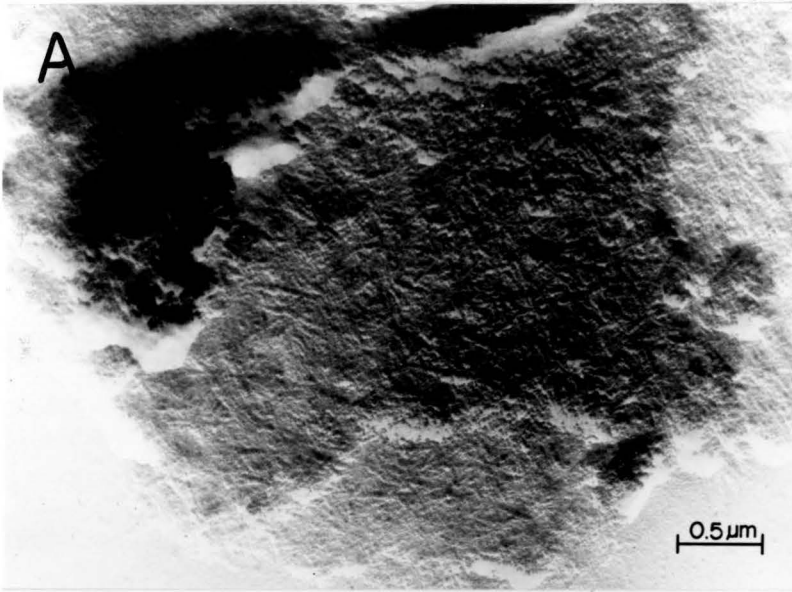


Fig. 18. Electron micrographs of platinum-carbon shadowed preparations of (A) an intact arthrospore wall and (B) an arthrospore wall digested with (1→3)- $\beta$ -glucanase and chitinase.





aromatic acid and none of these were detected.

Enzymatic Digestion of the Hyphal Cell Wall

Digestion of the hyphal cell wall with either (1-3)-D-glucanase or

D. Amino acid compositions of Fractions IV-R and V-R: The amino acid composition of the chitinase-resistant arthrospore wall fractions IV-R and V-R are shown in Table 11. As was seen following alkali extraction of the intact arthrospore wall (Table 7), alkali extraction of the enzyme-digested wall fraction also removed most of the neutral and acidic amino acids. Of the amino acids found in Fraction V-R (0.4% of the fraction's weight, Table 8) 82.9% of them consisted of the basic amino acids lysine and histidine.

E. Chemical and enzymatic resistance of Fraction IV-R: Arthrospore wall Fraction IV-R was tested for its resistance to various chemical and enzymatic agents known to be effective in removing chitin or chitin-like residues in other organisms (Table 12). Pretreatment of Fraction IV-R with the reducing agents mercaptoethanol or dithiothreitol, alone and in combination with pronase, was ineffective and did not facilitate digestion with chitinase. Alkaline protease and trypsin were also ineffective pretreatments. The wall fraction was resistant to lysozyme. Attempts to digest the wall residue with acetic acid or nitrous acid were also without effect indicating that the residue was not composed of chitosan (deacetylated chitin). The only two agents which dissolved Fraction IV-R were cold 6 N HCl and 30% NaOH, neither of which have an effect on authentic chitin.

F. Miscellaneous tests: Fraction IV-R was tested for the presence of combined inorganic and organic sulfur, hexuronic acids, and muramic acid and none of these were detected.

#### Enzymatic Digestion of the Hyphal Cell Wall.

Digestion of the hyphal cell wall with either (1→3)-β-glucanase or

Table 11. Amino acid composition of intact arthrospore walls and chitinase-resistant arthrospore wall fractions.

| Amino acid                | Mole %            |   |  |
|---------------------------|-------------------|---|--|
|                           | Arthrospore walls | Chitinase resistant arthrospore wall fraction IV-R <sup>a</sup> | Chitinase resistant arthrospore wall fraction V-R <sup>a</sup> |
| Lysine                    | 3.3               | 20.0  | 74.0   |
| Histidine                 | 0.6               | 1.4   | 8.9  |
| Arginine                  | 2.2               | 0   | 0  |
| Aspartic Acid             | 8.4               | 8.1   | 2.4  |
| Threonine                 | 10.5              | 8.8   | 0.8  |
| Serine                    | 11.9              | 8.8   | 3.3  |
| Glutamic Acid             | 12.2              | 7.8   | 1.6  |
| Proline                   | 10.2              | 9.0   | 0  |
| Glycine                   | 13.6              | 12.3  | 3.3  |
| Alanine                   | 5.5               | 8.1   | Trace  |
| Half-cystine              | Trace             | Trace   | 0  |
| Valine                    | 7.1               | 0   | 0  |
| Methionine                | 3.1               | 0.5   | 0  |
| Isoleucine                | 4.4               | 3.8   | Trace  |
| Leucine                   | 3.8               | 5.0   | Trace  |
| Tyrosine                  | 1.1               | 5.7   | 2.4  |
| Phenylalanine             | 2.1               | 0.7   | 3.3  |
| Unidentified <sup>b</sup> | +                 | +   | +  |

a See Fig. 2 for fraction designation.

b Elutes at 30 ml on Beckman Model 120 C Amino Acid Analyzer.

Table 12 Effect of selected chemicals and enzymes on chitinase resistant arthrospore wall fractions IV-R and V-R.

| Treatment   | Effect <sup>a</sup>   |
|---|-----------------------|
| 3 N HCl, 25°C, 1 h  | N.C. <sup>b</sup>     |
| 3 N HCl, 100°C, 2 h   | Partial destruction   |
| 6 N HCl, 25°C   | Immediate dissolution |
| 2.5 N NaOH, 100°C, 3 h  | N.C.                  |
| 5.0 N NaOH, 25°C, 3 h   | N.C.                  |
| 7.5 N NaOH, 25°C, 1 h   | Gradual dissolution   |
| 1 N Acetic acid, 25°C, 3 h  | N.C.                  |
| 3.9 N HNO <sub>2</sub> , 25°C, 3 h  | N.C.                  |
| 3.9 N HNO <sub>2</sub> , 3 h + DMSO <sup>c</sup> , 3 h                                  | N.C.                  |
| Mercaptoethanol (1%, v/v), 25°C, 2 h  | N.C.                  |
| Chitinase, 24 h, after:   |                       |
| Pronase (1 mg/ml) + DTT <sup>d</sup> (50 mM) +<br>EDTA <sup>e</sup> (5 mM), pH 8.9, 2 h | N.C. <sup>f</sup>     |
| Pronase (1 mg/ml, pH 7.8), 24 h   | N.C. <sup>f</sup>     |
| Alkaline protease (1 mg/ml, pH 7.5), 24 h   | N.C. <sup>f</sup>     |
| Trypsin (1 mg/ml, pH 8.0), 24 h   | N.C. <sup>f</sup>     |
| 0.025 M NaIO <sub>4</sub> , 25°C, 2 h + chitinase 6 h                                   | N.C.                  |
| (1→3)-β-glucanase + chitinase, 2 weeks  | N.C. <sup>f</sup>     |
| Lysozyme (1 mg/ml, pH 8.3), 24 h  | N.C. <sup>f</sup>     |

a Determined by phase contrast microscopy

b No change was observed

c Dimethylsulfoxide

d Dithiothreitol

e Ethylenediamine tetraacetic acid

f Also determined by change in turbidity

chitinase considerably thinned the side walls (Fig. 19). Sequential or combined digestion of the hyphal walls with the two enzymes completely removed the side walls leaving only the septa. The septa appeared to be resistant to further degradation with hot 1 N NaOH.

#### Enzymatic Digestion of *Saccharomyces Cerevisiae* Cell Wall.

In order to determine whether the enzymatic resistance of the arthrospore cell wall was unique, and to ascertain the activity of the enzyme preparations, cell walls from *Saccharomyces cerevisiae* were digested with (1 $\rightarrow$ 3)- $\beta$ -glucanase and chitinase. Digestion of walls from exponentially growing cells with glucanase left only the faint outline of the cell shape (Fig. 20). Both enzymes together completely digested the walls. However, sequential or simultaneous digestion of the walls from stationary phase cells with glucanase and chitinase was ineffective unless the walls were pretreated with dithiothreitol (DTT) (50 mM), ethylenediamine tetracetic acid (EDTA) (5 mM), and pronase (1 mM/ml) in a 0.1 M Tris-HCl buffer (pH 8.9) (Torres-Bauza and Riggsby, 1980). Following the pretreatment they were degraded completely by the two enzymes. The identical pretreatment of the arthrospore walls did not facilitate their digestion by the two enzymes (Table 12).

#### Appearance of Thin-Sectioned *T. mentagrophytes* Arthrospore Wall and Localization of the Chitinase-Resistant Layer.

Electron micrographs of thin sectioned cell walls of dormant arthrospore showed the walls to consist of essentially a single layer of intermediate electron density, approximately 300 to 450 nm thick (Fig. 21). Occasionally, the outer arthrospore wall, of slightly greater electron density, approximately 150 nm thick, was observed still at-

Fig. 19. Hyphal walls of T. mentagrophytes digested with (A) (1→3)-β-glucanase, (B) chitinase, and (C) with both (1→3)-β-glucanase and chitinase. Notice that only the septa remain after digestion with both enzymes. Fig. 19A and 19B are the same magnification.

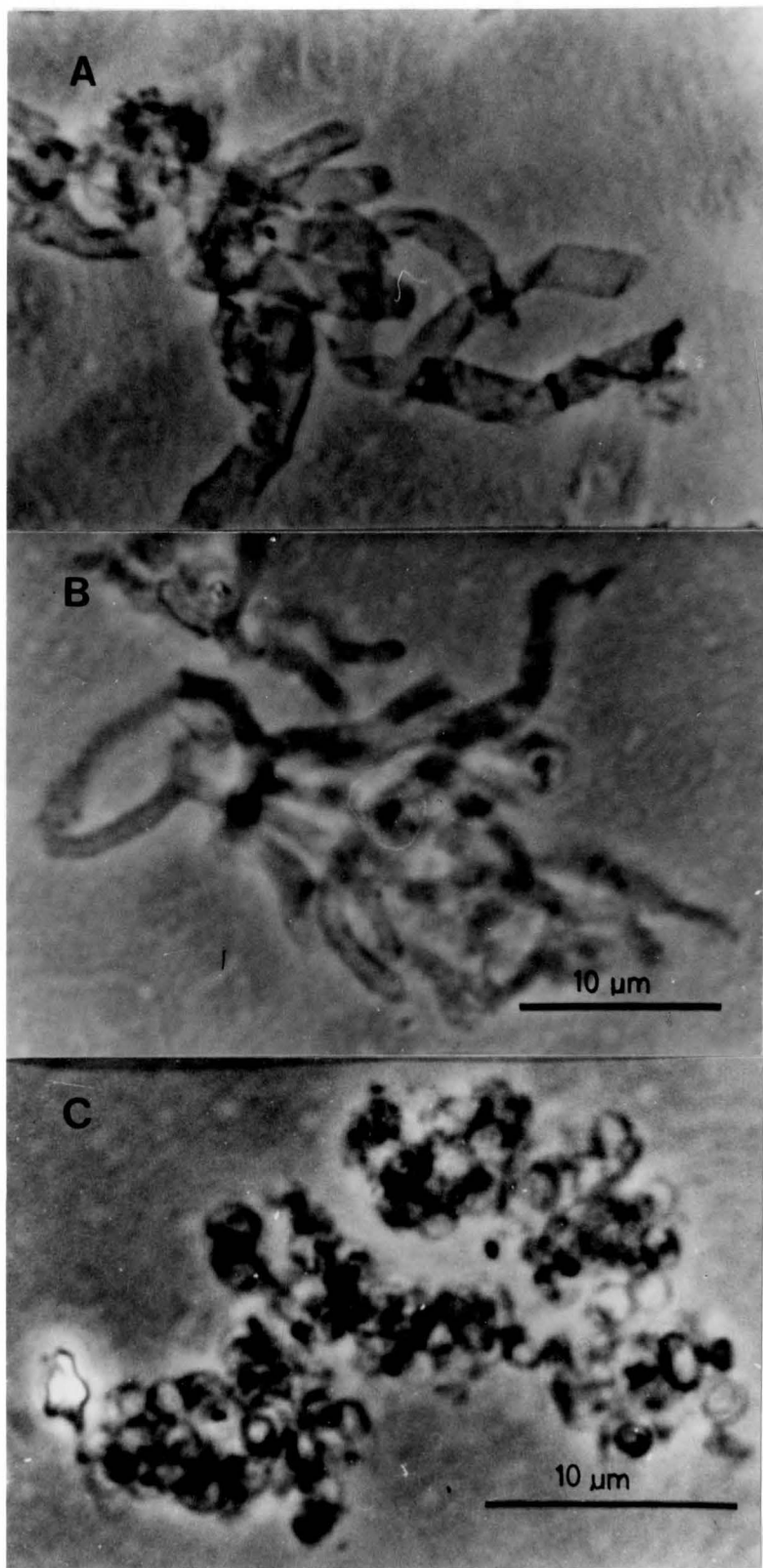


Fig. 20. Phase-contrast micrographs of (A) isolated walls of Saccharomyces cerevisiae, and (B) S. cerevisiae walls from exponentially-growing cells digested with (1→3)-β-glucanase.



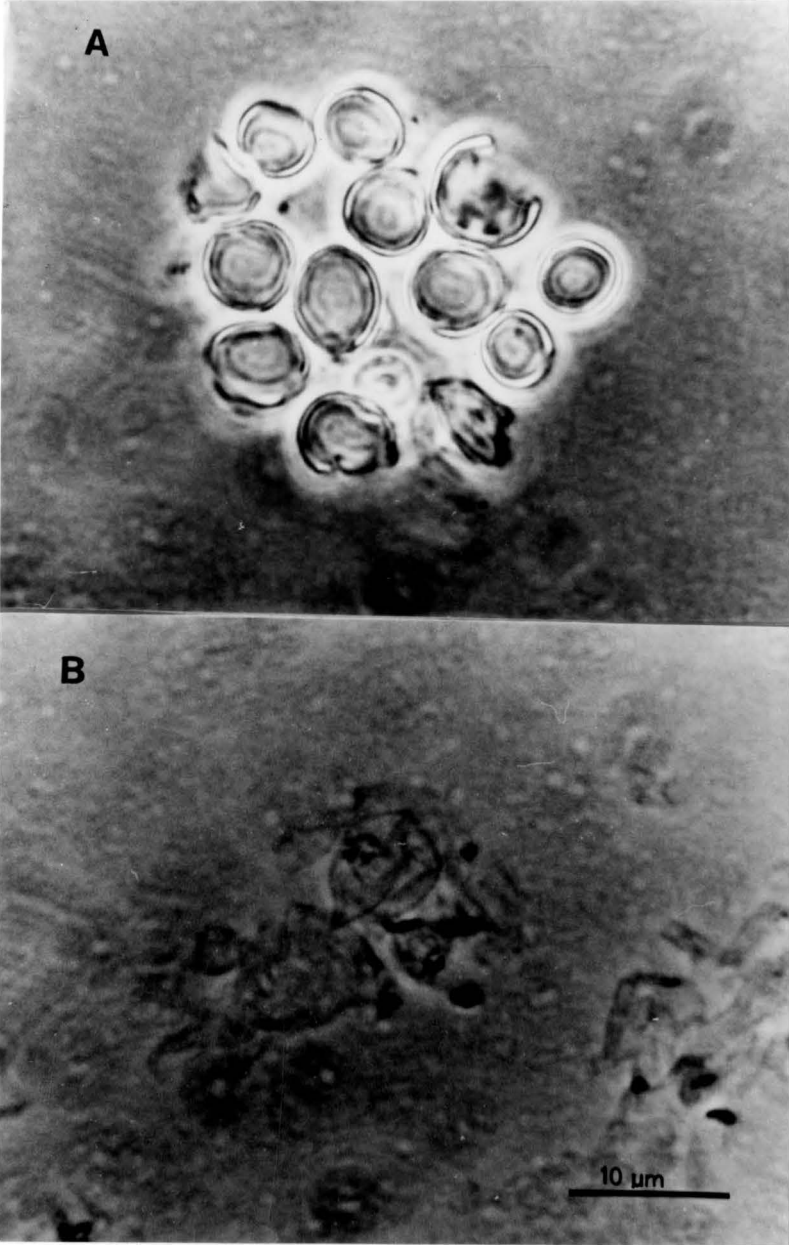
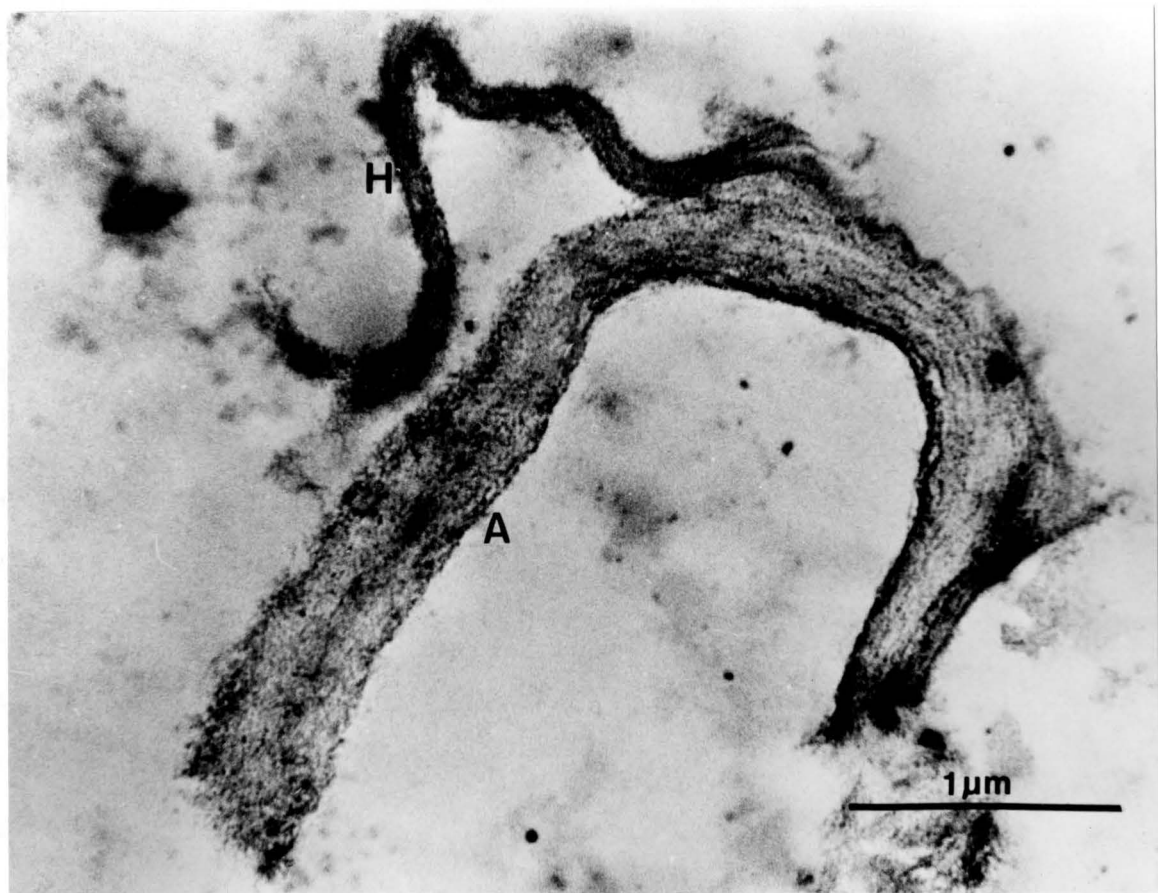


Fig. 21. Electron micrograph of a thin sectioned arthrospore wall. The arthrospore wall (A) is a single layer of intermediate electron density. Note the denser outer wall (H) still attached to the inner arthrospore wall.

attached to the spore wall (Fig. 2). In some instances a thin, slightly darker region, was observed, especially in the center of the outer arthrospore wall, which varies in thickness from about 30 to 100 nm (Fig. 12).

Digestion of the wall with (1-5)- $\beta$ -glucosase, appeared to remove much of the material surrounding this factory-like region, leaving it



thickness varied between 150 nm and 400 nm (Fig. 3).

tached to the spore wall (Fig. 21, 22). In some instances a thin, slightly darker region, was observed, generally in the center of the inner arthrospore wall, which varied in thickness from about 30 to 100 nm (Fig. 22).

Digestion of the wall with (1 $\rightarrow$ 3)- $\beta$ -glucanase, appeared to remove much of the material surrounding this faintly dark region, making it stand out more clearly. In many instances this gave the arthrospore wall the appearance of consisting of two electron lucid layers divided by a thin (30-50 nm thick) electron dense layer (Fig. 23a). In other instances this electron dense layer appeared to vary in thickness from 30 to 300 nm covering almost the entire width of the wall. In the thicker areas this layer had a stratified appearance (Fig. 23b, 23c).

Digestion of the wall with both (1 $\rightarrow$ 3)- $\beta$ -glucanase and chitinase and extraction with alkali, resulted in the chitinase-resistant layer (Fraction V-R). In many instances the residue appeared to be identical with the stratified electron dense material observed in the glucanase digested residue (Fig. 24). In almost all cases there was a large gap in the center of the septa separating the chitinase-resistant layers of adjacent arthrospores. In a number of cases there was an apparent separation of the resistant layer into two parallel layers, whose total thickness varied between 150 and 400 nm (Fig. 25).

Fig. 22. Electron micrograph of thin sectioned arthrospore wall. Note that the outer arthrospore wall (H), which is of greater electron density than the inner arthrospore wall (AW), is still attached. Also visible is the faint, darker region in the center of the arthrospore wall (arrows), which is thought to correspond to the chitinase-resistant layer.

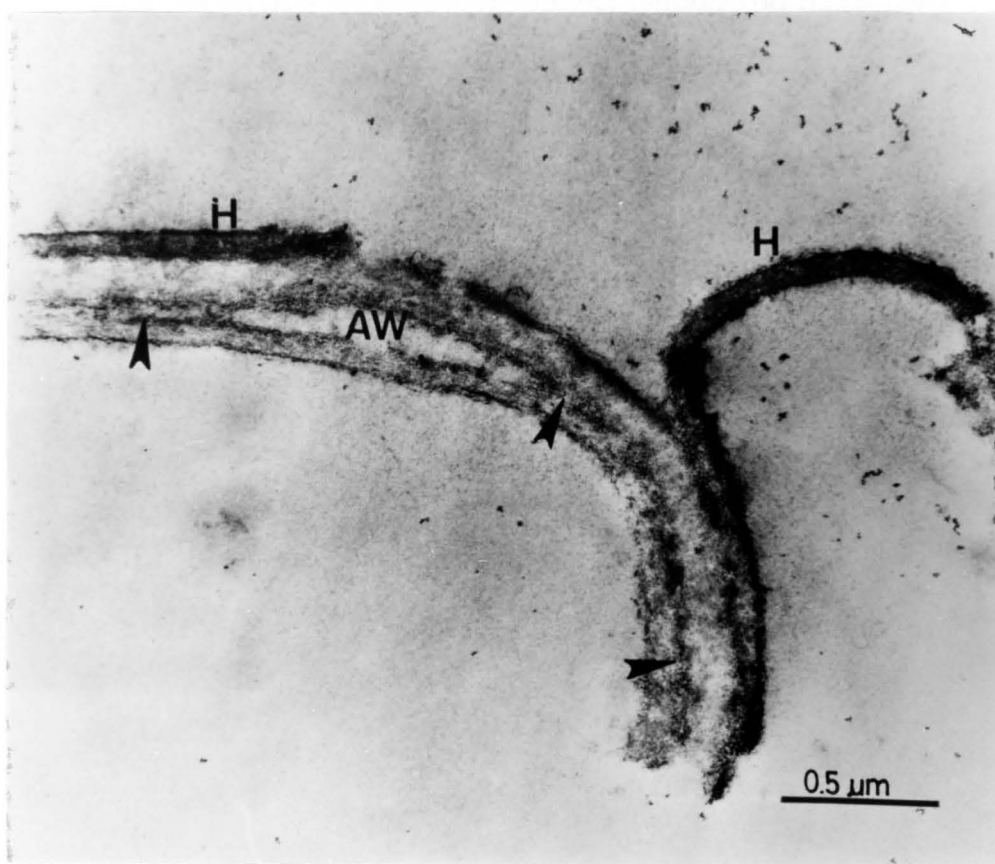


Fig. 23. Electron micrograph of thin sectioned glucanase-digested arthrospore walls. A) Notice that the faintly darker region barely visible in the intact walls now is very obvious (arrows). B) In many cases this electron dense region (CR) is of variable thickness (arrows). This is thought to correspond to the chitinase-resistant layer. Surrounding this layer is a weakly stained layer (CS) thought to correspond to the chitinase-sensitive chitin layer. C) Note the stratified appearance of the electron dense layer. (H): Outer arthrospore wall.

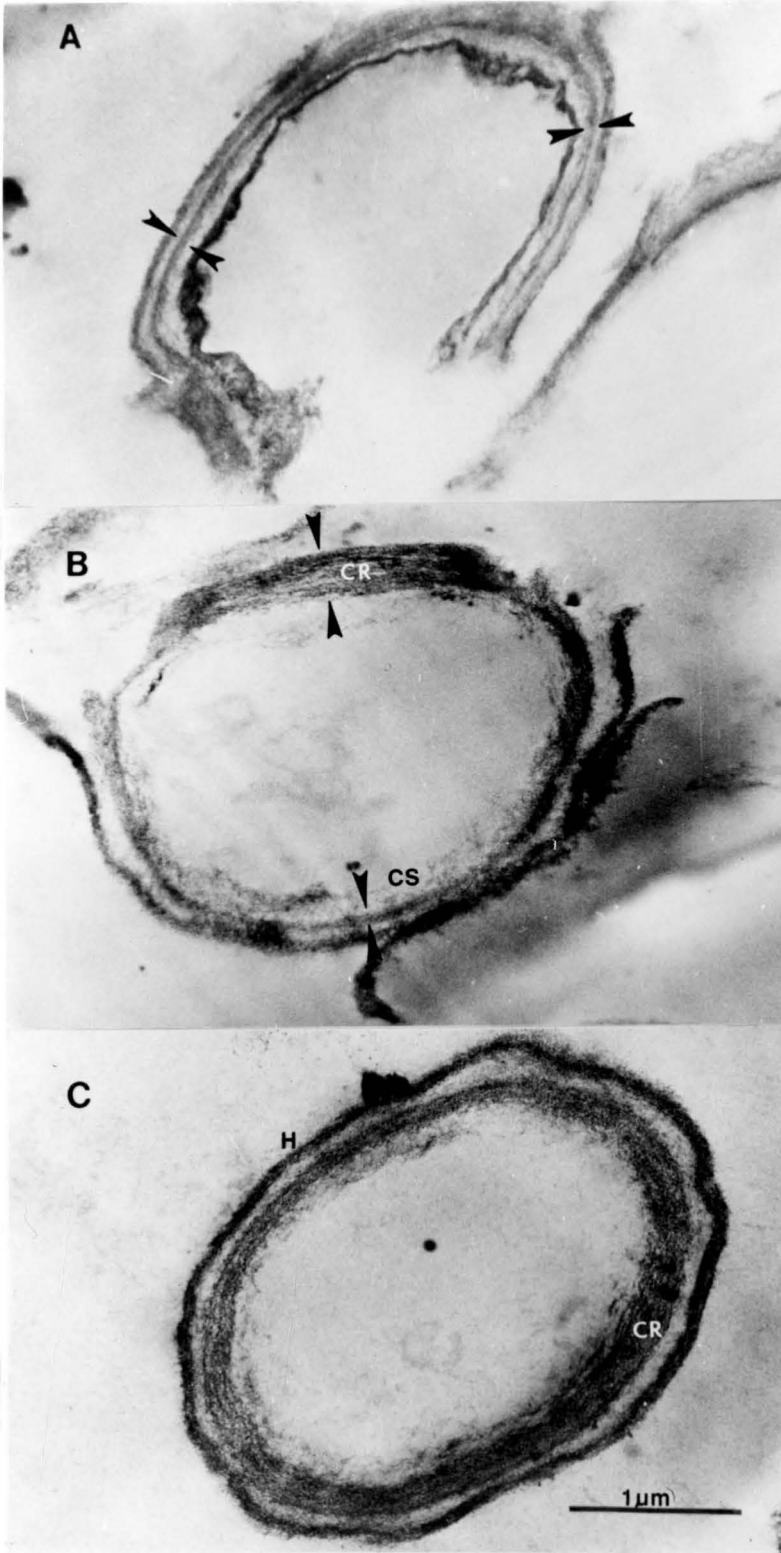




Fig. 24. Electron micrographs of thin sectioned arthrospore walls digested with both (1→3)-β-glucanase and chitinase. Note the variable thickness of the wall residue (A). There is a large gap in the center of the septum where chitinase sensitive material has been digested away (S) (B). Note that the residue is stratified as is the electron dense material found in the walls digested with (1→3)-β-glucanase alone.

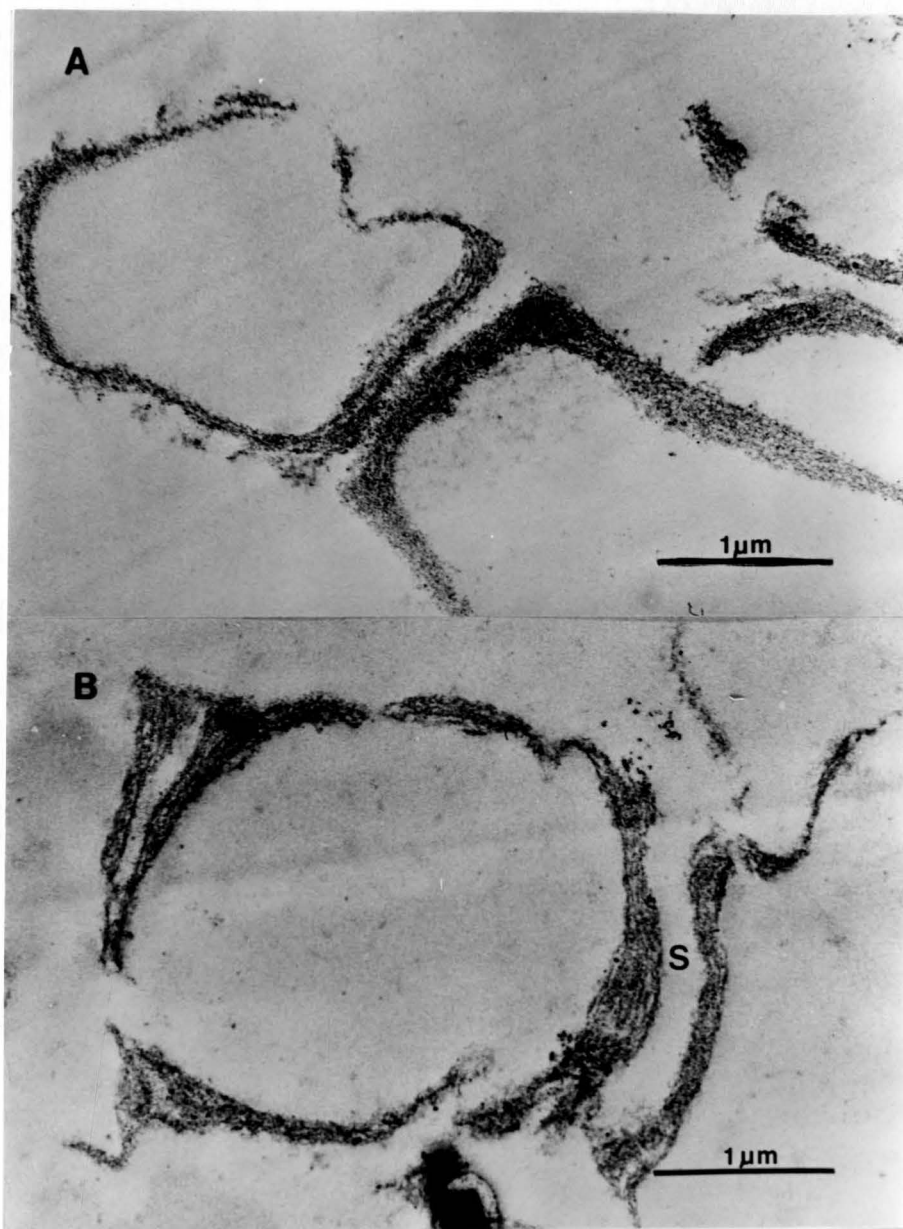
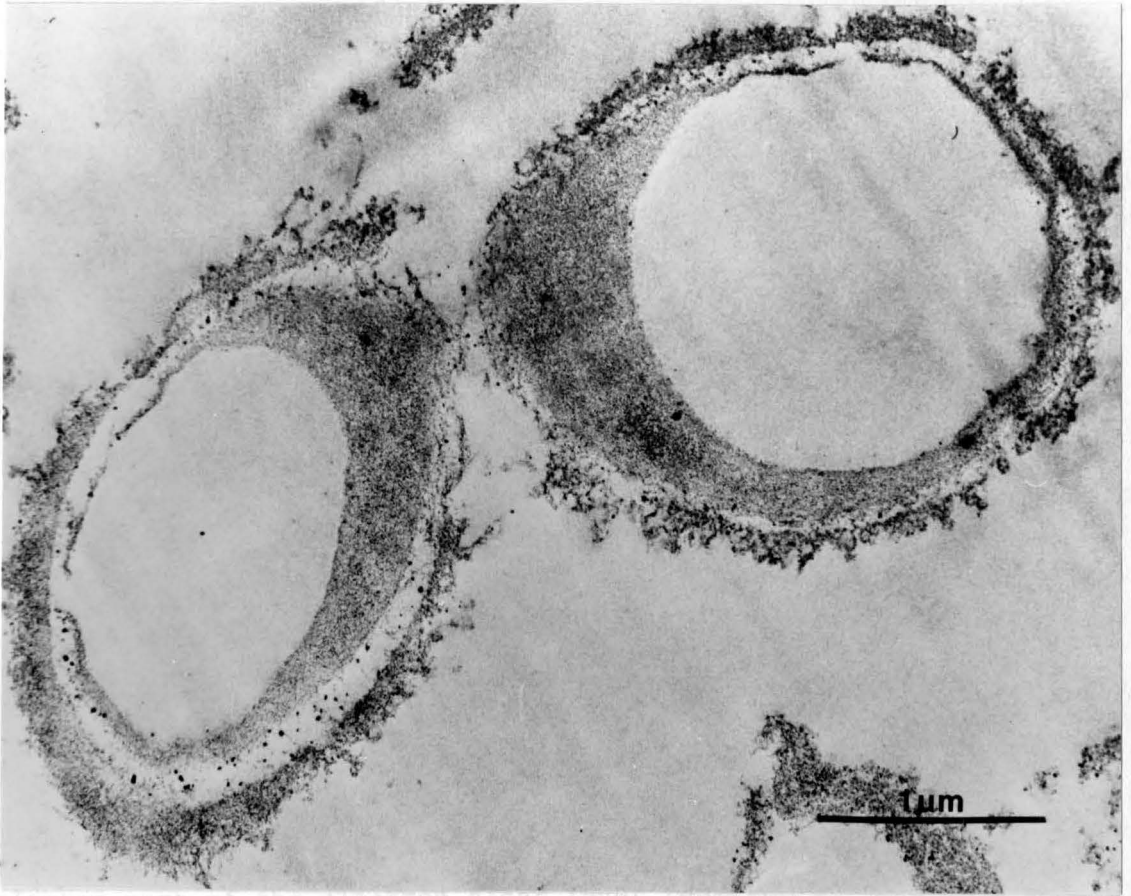


Fig. 25. Electron micrograph of thin sectioned arthrospore walls digested with (1→3)-β-glucanase and chitinase. In a number of cases this residue consisted of two concentric layers. The reason for this separation is not clear.

## DISCUSSION

The present study is the first to describe the structure of the prospore walls of *T. aestivum*. The major findings are:

A. With the exception of chitin there were only two major differences in the types of polymers found in the



prospore walls of *T. aestivum* (see also the electron micrograph in the

literature, 1978) to be deposited during arthropod development.

B. Some physical properties of this chitin appear to be different from either those of chitin from other insects or from chitin isolated from *T. aestivum*.

A few technical problems had to be solved in order to obtain

## DISCUSSION

The present study is the first to chemically characterize the arthrospore walls of T. mentagrophytes. The major findings were:

A. With the exception of chitin there were only minor quantitative differences in the types of polymers found in the cell walls of arthrospores and hyphae of T. mentagrophytes.

B. The major difference between the arthrospore and hyphal walls is the presence in the arthrospore walls of a nonfibrillar, chitinase-resistant chitin layer, which retains the original shape of the arthrospore. A similar chitinase-resistant chitin can only be found in the septal rings of the hyphae. This nonfibrillar, chitinase-resistant chitin is present along with the more common, fibrillar, chitinase-sensitive chitin in the arthrospore wall, and constitutes approximately one-third of all chitin present in the cell wall.

C. This unique chitin appears to be localized as an electron dense "layer" within the arthrospore wall, but its thickness is variable, ranging from one-tenth to almost the entire thickness of the arthrospore wall. It is probably covered by electron lucid glucan and so it constitutes part of the electron clear layer reported by Emyanitoff (Ph.D. dissertation, 1978) to be deposited during arthrospore formation.

D. Some physical properties of this chitinase-resistant chitin appear to be different from either those of chitin isolated from crab shells or from chitin isolated from fungal walls.

A few technical problems had to be addressed in order to analyze

the arthrospore cell walls. The first was to find an efficient method for breakage of the cells. Emyanitoff (Ph.D. dissertation, 1978) had reported the arthrospores to be extremely resistant to breakage by glass beads and sonication and that they could only be partially broken by grinding lyophilized spores. We initially were able to get greater than 99% breakage by passage of the spores through a French pressure cell. However, more than twenty passages were required to obtain this high degree of breakage, which in most cases mascerated the cell walls so that their oval shape was no longer recognizable. This made any study of wall architecture impossible. We later found that the cells could also be broken with glass beads whose diameters were 0.5 mm, as described by Van Etten and Freer (1978). This procedure had the advantage of not altering the spore cell shape, and of being fairly rapid, thus decreasing the chances for autolytic enzymes to act. Thus, this became the procedure used to obtain cell walls.

The other problems dealt mainly with determining the most efficient methods to hydrolyze the cell walls and to recover the sugars from the hydrolysates. Under all acid hydrolysis conditions there is much destruction of sugars. Noguchi et al. (1971, 1975) determined the neutral sugar composition of T. mentagrophytes hyphae after hydrolysis with 10% HCl in anhydrous methanol for 4 h at 100°C. These conditions degraded 20% of a standard glucose solution and 33% of a standard mannose solution (Nozawa et al. 1969). Although an effort was made to correct for the degradative effects of hydrolysis it is very likely that hydrolysis of cell walls is more destructive of their sugars than is hydrolysis of a standard sugar solution. Wu (Ph.D. dissertation, 1976) reported that hydrolysis of glucose with 1 N H<sub>2</sub>SO<sub>4</sub> for 12 h destroyed only

4% of the glucose while hydrolysis in the presence of albumin destroyed almost 20%. The present study, using the same procedure as Wu-Yuan and Hashimoto (1977), showed that the total neutral sugar present in the sulfuric acid hydrolysates was only 69% of the value obtained measuring the total sugar present prior to hydrolysis (Fig. 7 and 8). Attempts to hydrolyze the walls with trifluoroacetic acid and hydrochloric acid were not less destructive of the neutral sugars (data not shown).

Following hydrolysis with 1 N sulfuric acid it was also noticed that much sugar in the hydrolysates could be lost by adsorption to the  $\text{BaSO}_4$  formed on neutralization (Table 1). Fortunately this effect was countered by 3- to 4-fold dilution of the hydrolysates with water prior to neutralization.

Since destruction of sugars is an inevitable consequence of acid hydrolysis, it was necessary to develop a method to correct for this destructive effect. In the present study the total sugar present in the cell walls was determined prior to acid hydrolysis using the anthrone method with glucose as the standard. In order to correct this value to reflect the amount of mannose and galactose present in the cell wall (since the absorbance of the color produced by anthrone on each of these sugars is approximately one-half that produced on glucose), the ratio of all three sugars was determined, though necessarily after hydrolysis, and the ratio was used to determine a correction factor to the anthrone value (Table 3). The ratios of the neutral sugars were determined colorimetrically after eluting the aniline oxalate-developed sugars separated by paper chromatography. The ratios of glucose to galactose obtained by this method were similar to those obtained using glucose and

galactose oxidases directly on the neutralized hydrolysates (which was thought to be a more accurate procedure, but incapable of determining the amount of mannose present). This was true even though the ratios using the former procedure were determined with the sugars found in 16 h hydrolysates, while the ratios using the latter procedure were determined from the maximum amount of each sugar released following a series of hydrolyses ranging from 1-24 h. It was felt, though, that the procedure used gave a fairly accurate indication of the total neutral sugar content of the cell walls, and best corrected for the destructive effects of hydrolysis.

Noguchi et al. (1971), Kitazima et al. (1972) and Wu (Ph.D. dissertation, 1977) could detect only trace amounts of galactose in the hyphal walls. The present study found galactose to be present in much greater than trace amounts in the hyphal walls. On average the amount of galactose detected was 70% that of mannose (Table 4). Since the percent of galactose in the hyphal walls was determined by both quantitative elution of sugars separated by paper chromatography and by a colorimetric method utilizing galactose oxidase, the value obtained was considered to be a fairly accurate indication of the amount of galactose present in the walls.

The only other hyphal wall component detected in this study, which was reported previously only in the study of Shah and Knight (1968), was galactosamine (Fig. 10).

The most critical determination in this study was that of hexosamine content. The initial results seemed to indicate that there was not much difference in the amino sugar contents of the hyphal or arthrospore walls, determined using the Elson-Morgan procedure following 6 h



hydrolysis with 6 N HCl. However, the value of 24.3% hexosamines for the arthrospore wall (Table 4), was lower than the sum of hexosamines found in the various wall fractions. Digestion of the walls with (1→3)- $\beta$ -glucanase released 0.8% of the initial wall weight as N-acetylglucosamine, determined by the method of Reissig et al. (1955). Digestion of the subsequent wall residue (III-R) with chitinase released 17.6% more of the initial wall weight as N-acetylglucosamine (Table 9). The wall residue, which was 13.4% of the original wall weight, consisted of 80.4% hexosamines, which would account for an additional 10.8% ( $0.134 \times 80.4\%$ ) of the wall as N-acetylglucosamine (Table 8). Thus the total hexosamine content of the arthrospore wall would actually be 29.2% ( $0.8 + 17.6 + 10.8$ ). This value would be significantly higher than the 21.6% hexosamines found in the hyphal wall using the Elson-Morgan procedure (or 22.5% N-acetylglucosamine released by chitinase [Table 4]).

It is possible that this higher hexosamine determination was due to the non-destructive nature of the procedure of Reissig et al. (1955), which depends on the release of N-acetylglucosamine by enzymatic digestion. Only 37% ( $10.8/29.2$ ) of the hexosamine content had to be determined by the Elson-Morgan procedure prior to which the remaining hexosamines were released by hydrolysis with 6 N HCl. Such a hydrolysis for 4-6 h has been shown to destroy 10-15% of glucosamine or galactosamine in a standard solution (Noguchi et al., 1974). Although, there were differences found in the hexosamine values obtained using different modifications of the Elson-Morgan procedure, and by removing the HCl used for hydrolysis by evaporation or by neutralization (Table 10), no consistent pattern could be found for these differences.

With the exception of the chitin content, the hyphal and arthrospore walls seemed to be remarkably similar in composition (Table 4). In order to determine the nature of the different wall polymers, the walls were subjected to alkali extraction. While 55.6% of the hyphal walls remained after alkali extraction, only 43% of the arthrospore wall remained. In both cases the only detectable neutral sugar in the alkali resistant residue was glucose (Table 6).

As discussed in the Introduction, alkali-resistant glucan is generally  $\beta$ -linked. That this was the case was tested in the arthrospore alkali-resistant fraction (I-R). Digestion of this residue with (1 $\rightarrow$ 3)- $\beta$ -glucanase removed 94% of the fraction's glucose. Paper chromatography of the sugars released by the enzyme revealed glucose and the same glucose oligomers that are released from laminarin, a known (1 $\rightarrow$ 3)- $\beta$ -glucan. These results indicated that the majority of the linkages, and certainly those of the "backbone" glucan, were  $\beta$ -(1 $\rightarrow$ 3). Some branches may also have  $\beta$ -(1 $\rightarrow$ 6) linkages. Since it is probably very difficult to distinguish gentiobiose (a  $\beta$ -[1 $\rightarrow$ 6] disaccharide) from the other glucose oligomers released by glucanase, this was not verified by paper chromatography in this study. However, Kitazima et al. (1972) have identified  $\beta$ -(1 $\rightarrow$ 6) linkages in the identical fraction of T. mentagrophytes hyphal wall using the Smith degradation procedure, which is based on the recovery of the appropriate borohydride reduction products of periodate oxidation.

Following (1 $\rightarrow$ 3)- $\beta$ -glucanase digestion of Fraction I-R, one would expect to detect essentially chitin alone. However, repeated attempts by different variations of the Elson-Morgan procedure, and by chromato-

graphy using the amino acid analyzer (Table 8), showed only 63.3% of this fraction (II-R) to consist of hexosamines. However, since no other component could be identified, one explanation is accelerated destruction of hexosamines during acid hydrolysis. Why this fraction, and also Fraction III-R, seemed to be more susceptible to this destructive effect than Fractions IV-R and V-R (Table 8) is not known. As will be discussed shortly, Fractions IV-R and V-R probably consist of a different type of chitin, which might explain our accounting for more of the composition of these fractions.

Treatment of the arthrospore walls with glucanase released not only glucose and glucose oligomers, but also N-acetylglucosamine equal to 0.8% of the arthrospore wall dry weight (Table 9). This result is not unique, as it has been reported previously (Troy and Koffler, 1969; Davis et al., 1977; Sietsma and Wessels, 1977, 1979) and perhaps indicates the existence of N-acetylglucosamine linked to the anomeric carbon atom of the  $\beta$ -linked glucan. The existence of N-acetylglucosamine in fungal wall polymers other than chitin has also been reported in S. cerevisiae, where a diacetylchitobiose unit serves as the linkage between wall mannan and protein, (Nakajima and Ballou, 1974; Ballou, 1976).

Since hot alkali is known to deacetylate chitin, alkali-extracted walls were not digested with chitinase. Instead the walls were sequentially digested with (1 $\rightarrow$ 3)- $\beta$ -glucanase and chitinase. What was expected to result was an alkali-soluble fraction similar to the "exo-layer" found in Epidermophyton floccosum (Kitajima and Nozawa, 1975). Instead this fraction (IV-R) was resistant to alkali-extraction. What was even more unexpected was the fact that 80.4% of Fraction IV-R and

85.0% of Fraction V-R consisted of N-acetylglucosamine (Table 8). After exhaustive digestion with chitinase we expected to find very little glucosamine in the wall residue. Kitajima and Nozawa (1975) found only 17% hexosamine in the "exolayer" of E. floccosum.

A number of possibilities might have accounted for the resistance of the wall to chitinase. One possibility is that the resistant layer is not chitin, but perhaps a substituted or deacetylated chitin (chitosan). Chitosan is soluble in nitrous acid or in dilute acetic or hydrochloric acid (Bartnicki-Garcia and Nickerson, 1962a; Shively and Conrad, 1970). As seen in Table 12 none of these acids had any degradative effect on the resistant wall fraction. Thus it is unlikely that this fraction contains large stretches of deacetylated chitin chains. This conclusion is supported by the results of X-ray diffraction (Fig. 16) and infrared absorption spectroscopy (Fig. 17) of this fraction. Both techniques showed that the main polymer of Fraction IV-R was identical with commercially available chitin from crab shells. The only differences were the absence of a minor X-ray diffraction ring at 7.0 Å and the absence of an IR absorbance peak at  $1110\text{ cm}^{-1}$ . The difference in the X-ray pattern may have been due to the pattern's lacking sufficient sharpness. Infrared absorption at  $1110\text{ cm}^{-1}$  has been tentatively assigned to the ability of the  $C_1-O-C_5$  and  $C_2-C_3-C_4$  bonds of the glucosamine rings in the chair conformation to stretch antisymmetrically, in-phase (Liang and Marchessault, 1959; Pearson et al., 1960; Galat and Popowicz, 1978). The lack of such stretching ability might imply some constraints on the conformation of the glucosamine polymer which are not normally found in chitin.

That the glucosamine polymer of Fraction IV-R is acetylated is demonstrated by the infrared absorbances at  $1655\text{ cm}^{-1}$  and  $1560\text{ cm}^{-1}$ , which are lacking in the spectrum of chitosan (Fig. 17). Using the method of Moore and Roberts (1980), the degree of acetylation was calculated as between 85 and 90%, or approximately the same as commercial chitin. Another procedure to estimate the degree of acetylation, using the ratio of transmissions at  $1550\text{ cm}^{-1}$  and  $2878\text{ cm}^{-1}$ , has been reported (Muzzarelli et al., 1980). Their standard curve determines the degree of acetylation only until 50%. Extrapolation of their curve however would also result in a high degree of acetylation for Fraction IV-R.

A number of substitutions of N-acetylglucosamine occur in bacteria, some of which are resistant to lysozyme, a N-acetylglucosaminidase similar to chitinase. In fact, chitin is a substrate for lysozyme, although bacterial murein is not a substrate for chitinase (Salton, 1964; Muzzarelli, 1977). It is possible that similar substitutions of chitin may render it resistant to chitinase.

The most common substitution of N-acetylglucosamine is that of an O-lactyl group on C<sub>3</sub> forming muramic acid. (Salton, 1960, 1964; Ghuyssen and Shockman, 1973; Tipper and Wright, 1979). The appearance of lactyl groups in hydrolysates of fungal walls has been reported (Pine and Boone, 1968; San Blas and Carbonell, 1974), although there has not been any evidence that the lactyl groups were attached to N-acetylglucosamine. In the present study it is possible that some lactyl groups are also present (not more than one for every fifty glucosamine residues). The procedure of Hadzija (1974), which was used to test for the presence of muramic acid, is based on the detection of lactate released by acid

hydrolysis. However, the amount of lactyl substitution possibly present is too small to be responsible for enzymatic resistance. Furthermore, the lactic acid previously reported in fungal walls is alkali soluble. That alkali treatment does not change the arthrospore walls' resistance properties has already been noted.

Another common substitution is an O-acetyl on C<sub>6</sub> of muramic acid forming N,O, diacetylmuramic acid. This substitution has been reported to be resistant to lysozyme (Ghuysen and Strominger, 1963; Tipper et al. 1965). An analagous substitution of chitin also decreases the rate of hydrolysis by chitinase (Hirano and Yagi, 1980b). One method used to detect this O-acetyl is the presence of absorption maxima at 1723 cm<sup>-1</sup> and 1230 cm<sup>-1</sup> in the infrared region. No absorption was detected at those wave numbers in the infrared spectrum of Fraction IV-R (Fig. 17).

The N-acetyl group of muramic acid of some bacteria is replaced by a N-glycolyl group (Azuma et al., 1970). Although O-glycolyl (hydroxyethyl ether) chitin is sensitive to chitinase (Muzzarrelli, 1977), it has been reported that substituents other than acetyl on the amino group decrease chitin's susceptibility to chitinase (Hirano and Yagi, 1980a, 1980b). Similar results have been reported for N-substituted glucosamine residues of murein being less susceptible to lysozyme (Amano et al., 1980). No evidence for the presence of N-glycolyl chitin was found in T. mentagrophytes arthrospores in the present study.

The cortex of Bacillus subtilis spores consists of peptidoglycan in which 50% of muramic acid residues have been converted to muramyl lactam (Warth and Strominger, 1969, 1972). These muramolactam glycosidic bonds were also resistant to hydrolysis by lysozyme. The lactum was

detected by reduction with sodium borohydride which converted the muramyl-lactam residues to either a cyclic secondary amine or an acyclic amino alcohol. No test to detect the presence of such a lactam was attempted. However, it was noted that when the cell walls were treated with  $\text{NaBH}_4$  in an attempt to prevent destruction of the alkali extractable material (see Materials and Methods), subsequent determinations of the hexosamines in the alkali resistant residue (II-R) showed that only 66-71% of hexosamines found in the non-reduced walls could be detected (results not shown). It is not known whether a cyclic derivative of N-acetylglucosamine is present in the arthrospore walls. However, it is known that under acidic conditions, N $\rightarrow$ O acyl migration occurs, during which an unstable cyclic intermediate is formed (Hough and Richardson, 1967). It is possible that such an intermediate could be stabilized by enzymes which would link it to some other wall compound. Such a proposal would have to be tested. The only previous report of an unusual glucosamine derivative in fungal cell walls (Applegarth, 1966) apparently was due to an artifact of hydrolysis (Applegarth and Bozoian, 1967).

The possible effect of the small amount of galactosamine in Fraction V-R should not be discounted, although it is not known how such a small percent (2%) of the fraction (Table 8) could affect the ability of chitinase to digest the rest of the fraction. It does appear to be significant that the galactosamine in Fraction V-R is alkali resistant. Previous reports of a galactosamine-containing polymer in N. crassa (Mahadevan and Tatum, 1965) and galactosaminogalactan in A. niger (Bardalaye and Nordin, 1976) have found these polymers to be alkali-soluble. A little less than half of the galactosamine in the arthro-

spore and hyphal walls of T. mentagrophytes also was alkali-soluble (Table 6). In Physarum polycephalum spherules, where 88% of the wall consists of a (1→4)- $\alpha$ -galactosaminoglycan (Farr et al., 1977), approximately 50% of the polymer consists of spindle shaped crystalline fibers resistant to alkali (Zaar et al., 1979).

If the chitinase-resistant wall layer consists of chitin, as the evidence suggests, the resistance may also be caused by the existence of a wall layer, or layers, impervious to chitinases.

Protoplast formation in yeasts, which is rapidly accomplished by treatment of exponential phase cells with glucylase (snail gut juice, containing glucanases and chitinase) is difficult, if not impossible to achieve with stationary phase cells (Brown, 1971; Deutch and Perry, 1974). In order to obtain protoplasts with stationary phase yeasts, the cells must first be pretreated with thiol reducing agents (preferably dithiothreitol) and proteases (Partridge and Drewe, 1974; Chattaway et al., 1976; Schwenke et al., 1977; Scott and Scheckman, 1980; Torres and Rigsby, 1980). The DTT has been shown to remove saccharide and protein material from the outer cell surface (Chattaway et al., 1974). Following this pretreatment, protoplasts can be rapidly obtained by digestion with glucylase or a mixture of (1→3)- $\beta$ -glucanase and chitinase.

The possibility that a similar protective layer exists in T. mentagrophytes arthrospores was tested. However, pretreatment of the arthrospore walls with DTT and proteases under the conditions tested, did not improve the cells sensitivity to chitinase (Table 12).

Protective layers have also been found in other organisms. For example S. commune is unaffected by treatment with a mixture of laminar-



inase, chitinase, and pronase (Hunsley and Burnett, 1970), or snail gut juice (deVries and Wessels, 1972, 1973). However, once the S-glucan (S=alkali soluble) is removed, either with NaOH or with S-glucanase, the cell wall is susceptible to enzymatic degradation. A similar (1→3)- $\alpha$ -glucan layer has also been found in Aspergillus nidulans (Polacheck and Rosenberger, 1977).

There is a difference between the protective layer found in yeasts and that found in S. commune. Isolated walls of S. commune are sensitive to enzymatic degradation without further removal of S-glucan. In the case of S. cerevisiae, as shown in the present study (Fig. 20), isolated walls remain resistant to lysis until the carbohydrate-protein layer is removed by DTT. Apparently the S-glucan of S. commune is located only on the surface of the cells (van der Valk et al., 1977), whereas the protective carbohydrate-protein complex in S. cerevisiae, may be found throughout the cell wall (Fig. 20).

The possibility of such an alkali-soluble protective layer in the arthrospore walls was investigated by treating Fraction V-R with a mixture of glucanase and chitinase; however, this treatment was not effective (Table 12).

Resistance to chitinase digestion due to protective layers has been reported for a number of soil fungi. Hyphal walls of Sclerotium tuliparum are not lysed by chitinase and are only slightly affected by snail juice (Jones et al., 1972). Zygothrychus vuilleminii call walls are resistant to a mixture containing chitinase, glucanase, and hemi-cellulase (Ballestra and Alexander, 1971). It is not clear which wall component protects the chitin from the action of chitinase. Some pos-

sible structures include glucuronic acid- or fucose-containing polymers or possibly a lignin-like material. Another chitin-containing organism which shows high resistance to chitinase is Mortierella parvispora, which has a high fucose content (Pengra et al., 1969).

One of the most common causes of resistance to lysis in fungi is the presence of melanin or melanin-like pigments in the surface layers (Potgieter and Alexander, 1966; Bloomfield and Alexander, 1967; Kuo and Alexander, 1967; Bull, 1970; Chu and Alexander, 1972). However, although the microconidial walls of T. mentagrophytes contain melanin associated with the rodlet layer (Hashimoto et al., 1976), the arthrospore walls lack the pigment.

Another compound found in plant and fungal walls, and which is considered to be "the most resistant organic material known" is the highly acid- and alkali-resistant carotenoid, sporopollenin (Brooks and Shaw, 1977, 1978). It is unlikely that sporopollenin is responsible for the resistance of T. mentagrophytes arthrospore walls since the resistant fraction is very soluble in 6 N HCl (Table 12).

Protective layers are not the only causes of chitinase resistance. In Fusarium solani (1 $\rightarrow$ 3)- $\beta$ -glucanase or chitinase alone effect little cell wall lysis. However, in combination there is an apparent synergistic effect in which 94% of the walls are rapidly lysed. The final 20% of the walls are lysed only by chitinase, and not by glucanase, suggesting that only the residue is free of  $\beta$ -glucan, and is protected by glucan from lysis by chitinase (Skujins et al., 1965). However, since (1 $\rightarrow$ 3)- $\beta$ -glucanase alone can release only a small amount of glucose from the wall, there must be an intimate relationship between the glucan and

chitin in the remainder of the wall.

A similar synergistic effect has been noted by Wessels and Marchant (1974) in S. commune. They reported that R-glucanase (R=alkali-resistant) and chitinase separately removed 8.7% and 20.8% of the alkali insoluble hexosamine, respectively, but together they removed 42.8%. More telling was the observation that septal dissolution, which occurred after successive treatments with the two enzymes irrespective of sequence, was as effective as using the enzymes in combination. It is highly unlikely that enzymatic resistance in the case of T. mentagrophytes arthrospores is due to protection of chitin by an impervious glucan layer, since degradation of the fibrillar chitin by chitinase occurred even without glucanase treatment (data not shown). The evidence rather suggested a close association between the two polymers.

In order to eliminate the possibility of similar  $\beta$ -glucan protection of chitin, or glucan-chitin interactions preventing lysis in T. mentagrophytes, the resistant layer (IV-R) was repeatedly treated with (1 $\rightarrow$ 3)- $\beta$ -glucanase and chitinase, successively and in combination, for periods up to 2 weeks. Such treatments were ineffective in digesting this fraction (Table 12).

Stagg and Feather (1973) have also presented evidence for the close association of glucan and chitin in A. niger. They found that after treatment of the alkali-insoluble material with nitrous acid, a procedure which deaminates amines and depolymerizes glucosamine polymers, the residue is soluble in dimethylsulfoxide. This procedure was tested without effect on Fraction V-R of the arthrospore wall (Table 12). It should be noted that Stagg and Feathers (1973) found their alkali-resistant glucan to be a heteropolymer containing  $\alpha$ -(1 $\rightarrow$ 4) and  $\beta$ -(1 $\rightarrow$ 3) link-

ages in a 4:1 ratio. As mentioned previously, it appears that the arthrospore alkali-resistant glucan contains mostly  $\beta$ -linkages.

Hirano and Yagi (1980a) have reported that N-acetylchitosan, after oxidation with  $\text{NaIO}_4$ , was hydrolyzed by *Streptomyces* chitinase fifteen times as fast as natural chitin. Such a treatment of Fraction IV-R was without effect (Table 12).

The evidence thus far presented is consistent with the idea that Fractions IV-R and V-R consist almost entirely of a chitin which is resistant to chitinase. There is no evidence that the chitin is substituted or is protected by, or linked to, any other wall polymer, which would account for its resistant properties. However, further results showed that this chitinase-resistant chitin is physically different from other chitins.

Chitin is a polymer highly resistant to chemicals (Muzzarrelli, 1977; Stirling et al., 1979; Austin et al., 1981). Chitin is resistant to concentrated NaOH, although it is deacetylated to chitosan by this treatment. It is also resistant to dilute acids. It is reported to dissolve rapidly in hydrochloric acid when the normality reaches 8.5 (Clark and Smith, 1936). The only report of chitin soluble at a lower normality apparently refers to a previously acid denatured chitin (Zimmerman, 1953). However, Fractions IV-R and V-R were quickly dissolved in cold 6 N HCl and gradually dissolved in 30% NaOH (Table 12).

These unusual solubility properties could not be readily explained. The possibility that the chitin was phosphorylated, as is galactosamine in *N. crassa* (Harold, 1962), or sulfated, as are the mucopolysaccharides chondroitin sulfate and keratosulfate (Hunt, 1970), was examined, but no phosphate or sulfate was found in the resistant fraction. The  $\alpha$ -galac-

tosaminoglycan found in P. polycephalum was found to dissolve in 6 N HCl (Farr et al., 1977). However, it is not likely that there are any alpha linkages in the resistant fraction since the IR spectrum showed no absorption peaks at 484 and 820  $\text{cm}^{-1}$ , which are diagnostic for alpha linkages (Fig. 17). There was an absorption peak at 895  $\text{cm}^{-1}$  which indicates beta-linkages (Michell and Scurfield, 1967; Kanetsuna and Carbonell, 1970; San Blas and Carbonell, 1974).

The most unusual property of the chitinase-resistant chitin was its non-fibrillar nature (Fig. 18). There have been suggestions that native fungal chitin which has not been released from surrounding matrix polymers is not fibrillar, as evidenced by the intact walls lacking clear X-ray diffraction patterns. Abundant microfibrils and clear X-ray diffraction patterns are reported to occur only in walls treated with (1 $\rightarrow$ 3)- $\beta$ -glucanase or acid, which remove the  $\beta$ -glucan (Sietsma and Wessels, 1977), or in regenerating protoplasts where the delay in  $\beta$ -glucan synthesis allows chitin to be synthesized alone (Van der Valk and Wessels, 1976; Wessels and Sietsma, 1981). Microfibrils, however, have been reported to be visible on the inner wall surface of T. mentagrophytes arthrospores even without  $\beta$ -glucanase digestion. After chitinase treatment these fibrils are completely removed (Hashimoto et al., Abstr. Annu. Meet. Amer. Soc. Microbiol., 1979, J 23, p. 92).

Microfibril organization is closely associated with the crystallinity of the polysaccharide chain. Chitin microfibrils are thought to consist of "piles of chains" of poly-N-acetylglucosamine linked together by hydrogen bonds between CO and NH of the acetyl-amino groups of adjacent chains. These chains have direction ( $\text{C}_1 \rightarrow \text{C}_4$  linkages). Three

types of chitin have been identified, depending on the orientation of the poly-N-acetylglucosamine chains. In alpha chitin the neighboring piles have opposite chain direction, and are said to be anti-parallel, having one chain "up" and one chain "down" ( $\uparrow\downarrow, \downarrow\uparrow$ ) (Rudall, 1963; Rudall and Kenchington, 1973). This is the orientation of chitin found in fungi (Rudall, 1969; Stirling et al., 1979). In insects and crustaceans two other orientations of chitin are found. In beta chitin the chains are all parallel ( $\uparrow\uparrow$ ), while in gamma chitin two chains are "up" and one "down" ( $\uparrow\uparrow\downarrow, \downarrow\uparrow\uparrow$ ). Another way of looking at the different chitins is that, respectively, in beta, alpha, and gamma chitin the chains are grouped in sets of one, two, and three chains (Rudall, 1963). One estimate is that 21 chains make up one microfibril (Bade and Stinson, 1980). However, many microfibrils can associate to form larger fibrils containing 350-2000 chains (Herth, 1979).

Is all chitin microfibrillar? Comparisons have been made with cellulose microfibrils. Cellulose, (1 $\rightarrow$ 4)- $\beta$ -glucan, has been shown to consist of crystalline (or crystallite) and amorphous regions, although there is disagreement on just which regions are amorphous. It has been variously thought that amorphous regions are: (1) Poorly organized regions which alternate with crystalline regions along with microfibril; (2) A sheath of unordered glucan surrounding the crystalline microfibril; or (3) The folded regions of the cellulose chains (Rodgers and Perkins, 1968; Ward and Seib, 1970).

In order to measure the percent amorphous cellulose, one measures the uptake of deuterium oxide by the specimen with densitometry. Since in crystalline regions the molecules are closely packed compared to the amorphous regions, small molecules cannot enter as fast. Therefore the

rate of D<sub>2</sub>O uptake is a measure of crystallinity (Rodgers and Perkins, 1968).

Such a procedure was used to compare alpha chitin from insects, and beta chitin from squids and cuttlefish (Hackman and Goldberg, 1965). They found that alpha chitin consisted of one-third to one-quarter highly crystalline regions, while beta chitin was almost completely "non-crystalline". (Non-crystalline here refers only to compactness of the chain structure. "Crystallinity", as measured by X-ray diffraction still was present. In fact, Pearson et al. (1960) has shown chitoheptaose to have the same X-ray diffraction pattern as does native chitin). Beta chitin was also hydrolyzed by chitinase and by acid at a much faster rate than was alpha chitin. This, of course, is consistent with the idea that less crystalline structures are more accessible to chemical reagents and enzymes. Part of the amorphous nature of cuttlefish beta chitin could be attributed to its containing large amounts of non-acetylated glucosamine. In fact, 25% of the beta chitin consisted of a chitotriose which contained two free amino groups. In centric diatoms, though, a highly crystalline, microfibrillar beta chitin has been isolated (Herth and Barthlott, 1979). Thus while there may be differences in the rate of digestion of the different chitins by chitinase, they are all still susceptible to chitinase action.

Other than chain direction, which affects the degree of hydration, the different chitins are similar. Both beta and gamma chitin can be converted to the more stable alpha form by placing them in 6 N HCl, or by reprecipitating them from solutions in concentrated acid (Rudall, 1963). It was not possible to determine if the chitin in Fraction IV-R is alpha chitin or not, from the X-ray diffraction patterns we obtained,

as they were not sharp enough. In any event, even if Fraction IV-R was beta or gamma chitin it would not explain its resistance to chitinase.

It should be mentioned that cellulose I and cellulose II, which also differ in chain direction, show different susceptibilities to endo- and exo-cellulases. These two enzymes can act synergistically to digest either cellulose (Kanda et al., 1980). Whether a group of enzymes (or enzyme complex) is necessary to digest chitin, as is necessary to digest cellulose, is not clear. It is thought that a  $C_1$  component of cellulase is necessary to degrade fibrillar or crystalline cellulose, while non-fibrillar cellulose (carboxymethylcellulose or swollen cellulose) can be digested by the other  $C_x$  components of the complex (Ward and Seib, 1970; Stirling et al., 1979). The  $C_1$  component is thought by various authors to be: (1) an oxidizing enzyme which can insert uronic acid moieties into the cellulose chains, breaking hydrogen bonds and causing the cellulose to swell, thus making the crystalline parts more accessible to  $C_x$  components (Eriksson et al., 1974); (2) an exo-(1 $\rightarrow$ 4)- $\beta$ -glucan cellobiohydrolase (Halliwell and Griffin, 1973; Bergham and Petterson, 1973); or (3) an endo-(1 $\rightarrow$ 4)- $\beta$ -glucanase which acts randomly over the chain, opening up chain ends so exo-(1 $\rightarrow$ 4)- $\beta$ -glucanase can act (Streamer et al., 1975). The  $C_1$  component acts synergistically with the  $C_x$  components, (1 $\rightarrow$ 4)- $\beta$ -endoglucanases and cellobiase to digest fibrillar cellulose (Bergham et al., 1976; Shoemaker and Brown, 1978).

Monreal and Reese (1969) have reported a  $CH_1$  factor in the chitinase complex may be necessary to hydrolyze crystalline chitin, but were not able to purify such a factor. Although it is possible to suggest that the Streptomyces chitinase used in this study consisted only of a  $CH_1$  factor unable to digest noncrystalline chitin, this seems very un-



likely since the crystalline chitin was broken down to N-acetylglucosamine and not into large polymers (Fig. 15).

From the above discussion it is clear that amorphous regions tend to be more susceptible to enzymatic action. However, perhaps it is possible that there are highly crystalline regions which are not fibrillar.

Chitin fibrils are produced in fungi by chitin synthetase-containing particles (or granules) called "chitosomes" (Ruiz-Herrera et al., 1975; Bracker et al., 1976; Bartnicki-Garcia et al., 1978). Isolated chitosomes when incubated with UDP-N-acetylglucosamine can be observed by electron microscopy to contain exceedingly fine fibrils only 1-2 nm in diameter (Bracker et al., 1976). These fibrils apparently coalesce to form thicker fibrils 12-18 nm in diameter, which can be seen extruding from the chitosomes for lengths up to 2  $\mu$ m (Ruiz-Herrera et al. 1975). These observations have led them to propose that polymerization of chitin chains and their crystallization into microfibrils are not independent and sequential processes, but rather are more or less simultaneously occurring events which are controlled by a "large multienzyme aggregate" associated with the chitosomes.

On the other hand, Herth (1980) has presented evidence that the two processes are separate. Congo red and calcafluor are dyes which bind to both crystalline polymers and nascent polymer chains. These dyes apparently do not inhibit the formation of short rods or "extremely thin subelementary" chitin fibrils (15-20 Å) in the alga Poteroioochromonas, but do prevent the lateral associations of these elementary fibrils into fibers of greater length and width by blocking hydrogen bonding

This could occur only if one assumes a "gap" between polymerization and crystallization, but not if they were simultaneous occurring events. A similar dissociation of those two events is also proposed for the production of beta chitin fibrils in centric diatoms (Herth, 1979).

If there is a gap between polymerization and microfibril formation it would be possible that a non-fibrillar but highly crystalline chitin intermediate could exist. There is some evidence that such a chitin can be found in regenerating protoplasts of S. cerevisiae. Chitin in normal walls of S. cerevisiae is not very crystalline, as measured by X-ray diffraction (Beran et al., 1972). The chitin of regenerating protoplasts is extremely crystalline (Kreger and Kopecka, 1976), possibly because there is little association with amorphous components which either diffuse into the medium (Peberdy, 1979a, b), or are not synthesized in the early stages of regeneration (Van der Valk and Wessels, 1976). Yet, the fibrillar net of the regenerating protoplasts does not consist of chitin, but of  $\beta$ -glucan (Kreger and Kopecka, 1976). Farkas and Svoboda (1980) have suggested that the chitin is present as granular material, which can be observed on the fibrillar glucan nets. It is of great interest that the chitin of regenerating protoplasts has been reported to be highly resistant to chitinase (Beran et al., 1972). Kreger and Kopecka (1976), though, have removed crystalline chitin from regenerating protoplasts with chitinase.

The next question which can be asked is why shouldn't the chitin in Fraction IV-R polymerize as does normal chitin? One possible hint may be found in the results of the amino acid analysis (Table 11). Alkali removes almost all of the protein from both the hyphal and arthro-

spore walls. However, what protein is left contains large amounts of lysine and histidine. The amino acids of Fraction I-R of the hyphal walls consisted of 41.3 mole % lysine and 10.3 mole % histidine while the same fraction of the arthrospore wall contained 54.3 and 15.2 mole %, respectively (Table 5). The alkali treatment of Fraction IV-R of the arthrospore wall resulted in the residual amino acids being 74% lysine and 8.9% histidine (Table 11).

This observation of a high basic amino acid content in wall residues has been observed by others. Wang and Bartnicki-Garcia (1970) found the acid and alkali resistant fraction of Verticillium albo-atrum, which was predominantly chitin, to contain only two amino acids (less than 0.8% of the fraction's weight), lysine (35.4 mole %) and histidine (64.6 mole %). Maret (1972) found the amino acids of the pronase-treated, alkali-resistant fraction of Chaetomium globosum to be 30% lysine. In these cases the amino acids were thought to be involved in the linkages between chitin and protein. Evidence for covalent linkages between N-deacetylated residues of chitin and protein has been found in Mucor mucedo (Daetma et al. 1977). Amino acid linkages have also been proposed for insect and crustacean chitin where protein makes up more than 50% of the chitinous tissues (cuticles) in some cases (Hackman and Goldberg, 1965; Hunt, 1970). Hackman (1960) reported that the alkali-resistant residue of beetle larval cuticle contains 2 histidines and 1 aspartic acid for each 400 glucosamine residue. No other amino acids were found. These results have been questioned (Hunt, 1970).

A more recent proposal by Sietsma and Wessels (1979, 1981; Wessels and Sietsma, 1981) is that lysine, along with citrulline, links chitin

to  $\beta$ -glucan. This conclusion is based, in part, on the isolation of an oligomer, following digestion of the alkali-resistant residue of S. commune with glucanase and chitinase, which consisted of glucose, glucosamine, lysine and citrulline.

The results presented here are compatible with this proposal. However, there is a difference between S. commune hyphal walls and T. mentagrophytes arthrospore walls. In the latter case, the alkali- and chitinase-resistant residue contained lysine. In S. commune the lysine was released by the chitinase treatment. It is therefore tempting to suggest that the lysine and histidine serve as bridges between chitin chains similar to the peptide bridges in bacterial peptidoglycan. This would, perhaps, also explain the non-fibrillar nature of the chitin chains since the lysine may be located in such a manner as to prevent interchain hydrogen bonding. This might also explain the difference in solubility between the chitin of Fraction IV-R and fibrillar chitin. The weak point in this suggestion is that lysine is only a small fraction of this residue. There would only be 1 lysine for every 190 N-acetylglucosamine residues. Lysine might also be present as a polylysine much as polyphenylalanine is associated with the galactosamine polymer of P. polycephalum. However, in that case there is almost equivalent amounts of amino acid and amino sugar (Zaar et al., 1979).

There was also present in the acid hydrolysates of the arthrospore walls an unknown ninhydrin positive substance which appeared to be resistant to alkali extraction and enzymatic digestion (Table 11). This compound eluted before the other acidic amino acids in the same position where urea would elute (although it was not urea). It was unusual in

that it had a high absorbance at 440 nm, although its major absorbance was still at 570 nm. Its structure was not analyzed further. There has been a report of an unusual amino acid, 3,4-dihydroxyproline, which was isolated from diatom cell walls (Nakajima and Volcani, 1969). Some have suggested that the 3,4-dihydroxyproline serves to link chitin to some other wall polymer, much as hydroxyproline links cellulose in Oomycetes (Aaronson, 1970; Rosenberger, 1976; Burnett, 1979). Whether the unknown compound found in T. mentagrophytes arthrospores also serves as a linkage between chitin and other wall polymers is unknown.

This author is aware of only one other case in fungi where, following chitinase digestion, a residue remains which is free of chitin fibrils but still contains a large amount of chitin. After 7 days of incubation of Paracoccidioidis brasiliensis yeast phase walls with chitinase, 41.4% of the chitin remained but no fibrils were observed. After 14 days digestion, 14.4% of the total chitin still remained (Carbonell et al., 1970). However, it is possible that the fibrils were only hidden by the other wall polymers which were not removed by the chitinase.

Others have reported the presence of wall residues containing high amounts of glucosamine polymer(s) after extensive chitinase digestion. Kanetsuna et al. (1972) found that following extraction of P. brasiliensis mycelial phase walls with acetic acid, sodium hydroxide, and chitinase, the residue (2% of the initial wall weight) contained 74.5% amino sugars. Whether this residue is possibly the same as the non-fibrillar chitin residue reported by Carbonell et al. (1970) for yeast phase walls, was not discussed.

What is most unusual about Fraction IV-R is that it is localized as a single, continuous wall layer. The amorphous regions of chitin and

cellulose fibrils appear to be non-crystalline areas associated with crystalline fibers. But removal of arthrospore wall fibrillar chitin with chitinase, along with the alkali-soluble polymers and  $\beta$ -glucan, left a wall layer which retained the original wall shape. Thus its existence appears not to be dependent on the presence of the microfibrils.

Localized regions of cell walls concentrated with chitinase resistant chitin have been reported. Mahadevan and Tatum (1967) found that subsequent to digestion of N. crassa with (1 $\rightarrow$ 3)- $\beta$ -glucanase and chitinase, and extraction with NaOH, only the septal rings remained. Acid hydrolysis of these structures released primarily glucosamine, so they were assumed to consist of "very compact" chitin.

Hunsley and Gooday (1974) also found the septal rings of N. crassa to contain chitin. Light and electron microscopic autoradiography of N. crassa grown in the presence of N-acetyl (1- $^3$ H) glucosamine (the acetyl is labeled), showed the septal rings to have the heaviest distribution of silver grains. This result was also supported by ultraviolet fluorescent microscopy of calcofluor stained cells, which revealed fluorescent rings around the periphery of the septa.

van der Valk et al. (1977) found the septal rims (or annuli) of S. commune hyphae to be resistant to attack by an enzyme preparation from Trichoderma harzianum (viride), which contains S-glucanase, R-glucanase, and chitinase, and which virtually removed all R-glucan, chitin and 70% of the S-glucan from the cell walls. Shadowed preparations of these isolated septal rims, following extraction with KOH, showed that they contain microfibrils embedded in a matrix. This matrix could be removed with hot 0.5 N HCl, exo (1 $\rightarrow$ 3)- $\beta$ -glucanase, or by Smith degradation. Since the septal rings were stained by Thiery's method, it was

assumed that the matrix consisted of R-glucan which contained  $\beta$ -(1 $\rightarrow$ 6) linkages. For some unknown reason this association of chitin and R-glucan was resistant to the enzyme preparation. No attempt was made to see if the chitin fibrils could be digested after the residual matrix was removed by acid or glucanase.

The only previously reported instance of chitinase-resistant chitin in a structure other than the septal rings was the "mural core" of the yeast form of Histoplasma capsulatum (Reiss, 1977; Reiss et al., 1977). Following serial digestion with (1 $\rightarrow$ 3)- $\alpha$ -glucanase, (1 $\rightarrow$ 3)- $\beta$ -glucanase, pronase, and chitinase, a "mural core" remained which comprised 18.7% of the initial wall weight of chemotype 1 cells and 14.8% of chemotype 2 cells. Electron microscopy of negatively stained cores showed them to be very thin and to have a pocked, cratered appearance. But, at least in the case of chemotype 1 cores, they maintained the elliptical shape of the original cells. Shadow cast preparations showed these cores to contain microfibrils. The chemotype 1 mural cores contained 34.9% N-acetylglucosamine, and the chemotype 2 cores, 25.4%.

It is thus apparent that when this chitinase-resistant chitin is present, it is localized in the septal rings of hyphal forms (N. crassa, S. commune) but is in an entire layer in yeast forms (H. capsulatum). Subsequent testing also found resistant chitin in the septal rings of T. mentagrophytes hyphae (Fig. 19).

This distribution of chitinase-resistant chitin in hyphal septa and in a wall layer in yeasts and arthrospores may have implications for understanding fungal morphogenesis, especially if these results are found to be of a more general occurrence. One possible explanation for

yeast development is that it is the result of uniform deposition of wall material over the entire cell surface, as opposed to localized deposition in hyphal formation (Bartnicki-Garcia, 1973a). In many instances the dimorphic transformation to yeasts is preceded by arthrospore formation, which is the result of increased septal formation (Howard, 1959, 1961; Howard and Herndon, 1960; Bartnicki-Garcia, 1962b; Restrepo, 1970). There is thus a possibility that the septal material, which is high in chitin content, is the substance being deposited. The results from the experiments with T. mentagrophytes arthrospores seem to confirm this view, since the septal chitin has similar properties to the arthrospore side wall chitin, in that both are chitinase-resistant, but is different from the hyphal side wall chitin, which is chitinase-sensitive. Another example of delocalization of chitin deposition has been demonstrated in bud initiation mutants (CDC24) of S. cerevisiae, which instead of depositing chitin only in the bud scar region, displayed chitin deposition over the entire cell surface at the nonpermissive temperature (Sloat et al., 1981).

It is clear that all of the chitinase-resistant chitins are not identical. Chitin fibrils were observed in both S. commune and H. capsulatum chitinase-resistant residues. Only in P. brasiliensis yeast phase walls was a fibril-free residue found. The chitinase-resistant chitin of T. mentagrophytes is the only one shown to have solubility properties different from "common" chitin. One other instance of a change in chitin characteristics is the observation of Barathova et al. (1975) that the chitin in N. sitophila grown in the presence of rami-hyphin A loses its crystalline X-ray diffraction pattern (see Introduction).



The appearance of the arthrospore walls of T. mentagrophytes (Fig. 21) is fairly similar to that obtained by Bibel et al. (1976) and Emyanitoff (Ph.D. dissertation, 1978) for mature cells. They reported the walls of younger, developing arthrospores appeared to be bilayered, with an electron transparent inner layer and electron dense outer layer. However, as the cells mature the outermost layer, which consisted mainly of the original hyphal wall, tended to slough off, leaving the single layered arthrospore wall observed in the present study.

Localization of the non-fibrillar, chitinase-resistant arthrospore wall layer was complicated by the almost total absence of any apparent discrete structure in the intact arthrospore wall. While some of the wall sections showed the presence of a faintly darker zone, varying in thickness from 30-100 nm (Fig. 22) in the middle of the arthrospore wall, it was totally lacking in most sections. It was, therefore, not possible to unequivocally demonstrate the localization of a structure in the intact wall which remained after the enzymatic digestion and chemical extraction. Such an approach, as was used by Wu-Yuan and Hashimoto (1977) to demonstrate the location of the rodlet and glycoprotein-lipid layers in the microconidia of T. mentagrophytes, would have been preferred. However, following digestion of the arthrospore wall with (1 $\rightarrow$ 3)- $\beta$ -glucanase, an electron-dense zone was observed which varied in thickness from 30-300 nm, and in location from the center of the arthrospore wall to practically covering the entire thickness of the wall (Fig. 23). This appearance of a darker layer following digestion with glucanase probably is due to the removal of a poorer staining  $\beta$ -glucan, revealing a more highly stainable material, which was inaccessible to the stain in the presence of the glucan. Poulain et al. (1975)

has noted that the cell walls of old *T. mentagrophytes* hyphae, stained using Thiery's procedure (1967) to detect periodate sensitive polymers, do not have an innermost reactive layer found in the walls of younger cells. Presumably, deposition of glucan and chitin in the older cells prevented the staining of the reactive polysaccharides.

The appearance of darker layers, where none were seen previously, has also been noted to occur during activation and germination of arthrospores of Streptomyces (Sharples and Williams, 1976; Hardisson et al., 1978). In Streptomyces arthrospores, the normally electron-lucid inner layer becomes electron-dense upon germination and separates from the outer wall layer. Since (1 $\rightarrow$ 3)- $\beta$ -glucanase is known to be released during autolysis of a number of fungi (Fleet and Phaff, 1974; Polacheck and Rosenberger, 1975; Santos et al., 1979; Flores-Carreón et al., 1979; Reyes et al., 1981), and is probably necessary for germination of fungal spores (Fevre, 1979a, b), it would not be unexpected to find a similar occurrence in the arthrospore walls treated with (1 $\rightarrow$ 3)- $\beta$ -glucanase. In a number of cases darker inner zones were observed in the walls of intact arthrospores of *T. mentagrophytes* (data not shown). It is very possible that this occurrence was due to the arthrospores being activated prior to germination, as immersion in water is sufficient to activate these spores (Hashimoto and Blumenthal, 1977).

The strongest evidence that the chitinase-resistant layer is the electron-dense material observed after digestion with (1 $\rightarrow$ 3)- $\beta$ -glucanase, is its stratified appearance. Both the electron-dense material in the glucanase-digested wall residues and in the residues following digestion with both glucanase and chitinase had the same stratified or laminar

appearance (Fig. 24c). The electron-dense material in both residues also appeared to vary in thickness even in the same cell (Figs. 24a, 24b). The variation in thickness, however, can only be verified by serial sections of the arthrospore walls.

Frequently the chitinase-resistant residue appeared to consist of two concentric layers (Fig. 25). This was not observed when the walls were treated only with (1 $\rightarrow$ 3)- $\beta$ -glucanase. This is, perhaps, the appearance of a transverse section through a septum. Since the entire arthrospore (inner) wall layer, including the fibrillar chitin, contribute to the formation of the septum, it is very possible that the chitinase digests away the fibrillar chitin (or other sensitive substrate since the chitinase is contaminated with other glucanases, as mentioned in Materials and Methods) leaving both a chitinase-resistant annulus and a second chitinase-resistant central septal area. This would also account for the observation of the many septa in phase-contrast micrographs of the chitinase-treated wall fraction (Fig. 14c). The possibility that the outer layer is really the residual hyphal wall is not to be discounted. However, this possibility seems highly unlikely, unless the outer layer was somehow modified by the interposition of resistant material during arthrosporulation, since similar digestion of the hyphal lateral walls left no discernible layers. At the present we have no certain explanation for this observation.

The arthrospores of T. mentagrophytes have been found to be much more resistant than the hyphae to killing by antifungal drugs and chemicals (Hashimoto and Blumenthal, 1978). It is possible, that the non-fibrillar, chitinase-resistant chitin deposited in the arthrospore wall is, at least in part, responsible for this resistance. The resistance

of the ellipsoidal arthrospores (yeast-like cells) of G. candidum to enzymatic lysis has also been attributed to a "new synthesizing process" and to a structural rearrangement of the wall polymers (Duran et al., 1973).

This unusual, nonfibrillar, chitinase-resistant chitin which is deposited in the lateral walls of T. mentagrophytes only during arthrosporulation will perhaps help us to more fully understand the mechanics of wall formation and its importance to the sporulation process. A number of questions remain to be answered. The primary question remains: Is this "non-fibrillar" chitin synthesized by the same enzymes as "fibrillar" chitin, after which it is somehow modified as is chitosan (Araki and Ito, 1975), or is it synthesized by a totally different enzyme system? Initial experiments could be done using the chitin synthetase inhibitor polyoxin D to ascertain if it would inhibit the synthesis of this "non-fibrillar" chitin during arthrosporulation.

While it appears that this "non-fibrillar" chitin is synthesized along with "fibrillar" chitin, since they both are present in the arthrospore wall, it is not clear whether they are synthesized simultaneously or sequentially. In an apparently analogous case, McVittie (1974) characterized a sporulation-defective mutant of Streptomyces coelicolor whose wall thickening is blocked at a stage corresponding to the wall thickness of immature spore chains in the wild type. This conceivably indicated "that the first part of wall thickening involves the deposition of material of the same composition as the presporulation hyphal wall while the further thickening is due to the addition of material of a different composition". This explanation may be true in part for the arthrospores of T. mentagrophytes. However, it is apparent from a num-

ber of thin sections of (1→3)- $\beta$ -glucanase digested walls that "fibrillar chitin which supposedly appears to be electron-lucid (Pearlmutter and Lembi, 1978) is present internally to the "non-fibrillar" electron-dense zone. This is also apparent from platinum-gold shadowed preparations of intact and glucanase digested walls which show the presence of chitin fibers on their inner surface (T. Hashimoto, J.H. Pollack, H.J. Blumenthal, Abstr. Annu. Meet. Amer. Soc. Microbiol., 1979, p. 92, J 23). It is thus very likely that fibrillar chitin is synthesized subsequent to the non-fibrillar chitin. It is necessary to study the kinetics of formation of these two chitins during the early stages of arthrosporulation possibly, using radiolabeled N-acetylglucosamine. If both types of chitin are synthesized simultaneously, it would be interesting to understand why the non-fibrillar chitin associates to form a discrete layer. Associated with this problem is why the deposition of this non-fibrillar chitin layer is not uniform. It is apparently heavy in some regions but light in other regions.

This leads to the key question of why the non-fibrillar, chitinase-resistant chitin is synthesized in large quantities only during arthrosporulation. During saprophytic growth it is found only in small amounts in the septal rings, but during arthrosporulation it is deposited throughout the arthrospore wall. The answers to these questions may lead to a better understanding of this unique sporulation process.

## SUMMARY

The cell walls of Trichophyton mentagrophytes arthrospores and hyphae were characterized chemically and ultrastructurally in order to explain what changes occur during the conversion of the hyphae to arthrospores.

T. mentagrophytes arthrospore and hyphal walls were obtained by vortexing the cells with glass beads. Cytoplasmic debris was removed by a combination of sonication and differential centrifugation.

T. mentagrophytes arthrospores were found to contain 65.1% neutral sugars (53.1% glucose, 7.5% mannose, and 4.5% galactose), 29.2% amino sugars (28.2% glucosamine and 1.0% galactosamine), 3.8% protein and small amounts of lipid (0.7%), phosphorus (0.1%), and ash (1.0%). The hyphal walls contained 67.7% neutral sugars (52.9% glucose, 8.7% mannose, and 6.1% galactose), 21.6% amino sugars (20.5% glucosamine and 1.1% galactosamine), 11.2% protein and small quantities of lipid (1.8%), phosphorus (0.2%), and ash (1.9%).

Approximately 17% of the arthrospore wall consisted of alkali resistant (1→3)- $\beta$ -glucan, while 29% of the hyphal wall consisted of alkali resistant glucan. The arthrospore wall contained 57% alkali soluble material, part of which may be a galactomannan. Only 44% of the hyphal wall was alkali soluble.

The major difference between the arthrospore and hyphal walls appeared to be the existence of a nonfibrillar, chitinase-resistant chitin wall layer in the arthrospores which was absent from the hyphal walls.

Digestion of the arthrospore wall with chitinase, after first digesting the walls with (1 $\rightarrow$ 3)- $\beta$ -glucanase, removed 17.6% of the arthrospore wall weight as N-acetylglucosamine, which accounted for 67% of all chitin found in the walls. Electron microscopy of carbon-platinum shadowed wall preparations showed that the chitinase removed all visible chitin microfibrils present in the intact arthrospore walls. Thus the arthrospore wall contained the typical, microfibrillar, chitinase-sensitive chitin. The residue following chitinase digestion (Fraction IV-R), which accounted for 13.4% of the initial arthrospore wall weight, still consisted of 80.4% hexosamines and only small amounts of neutral sugars (4.7%) and protein (1.1%). Further extraction of Fraction IV-R with 1 N NaOH removed approximately 20% of its dry weight, leaving a residue consisting of 85% N-acetylglucosamine (33% of all wall chitin), 2% galactosamine, 2.5% glucose and 0.4% amino acids, of which 74% were lysine and 8.9% histidine. Even though Fraction IV-R was completely devoid of microfibrils normally associated with chitin, its X-ray diffraction pattern and infrared absorption spectrum were essentially identical to those of crab chitin. The resistance of this nonfibrillar arthrospore chitin to chitinase was not due to the presence of melanin, protective layers of protein or sugar polymers, or to the close association of  $\beta$ -glucan and chitin. Besides its nonfibrillarity and resistance to chitinase, this fraction also differed from authentic chitin in its being soluble in 6 N HCl and 7.5 N NaOH.

Phase contrast micrographs showed that following digestion of the arthrospore wall with (1 $\rightarrow$ 3)- $\beta$ -glucanase and chitinase, and extraction with alkali (Fraction V-R), an entire wall layer remained which re-

tained the original shape of the intact arthrospores. Similar treatment of the hyphal walls completely degraded the lateral walls leaving only an annulus from each septum.

Electron micrographs of thin sections of this unusual chitin layer showed that it was electron-dense and had a stratified appearance. It was not distinguishable in electron micrographs of intact arthrospore walls, probably because it was covered by poorly stained  $\beta$ -glucan. This layer varied in thickness, even within the same cell, from 10% to almost the entire thickness of the arthrospore wall.

Possible modifications of this unusual chitin, which might account for its resistance to chitinase and for its nonfibrillarity, were discussed.

The occurrence of this nonfibrillar, chitinase-resistant chitin layer only in the arthrospores of *T. mentagrophytes* may have implications to our understanding of the morphogenesis of arthrospores and perhaps of other fungal organisms. Furthermore, there may be a relationship between this unique layer and the high resistance of these arthrospores to antifungal agents.



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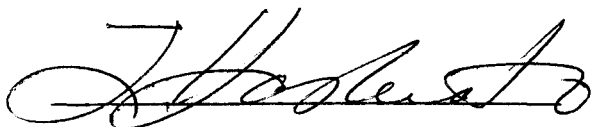
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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

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