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Chitin Synthesis In Neurospora Crassa Protoplast Systems

Larry Gordon Mcgirr

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CHITIN SYNTHESIS IN *NEUROSPORA CRASSA* PROTOPLAST SYSTEMS

by

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Department of Biochemistry

Submitted in partial fulfillment of

the requirements for the degree of

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ABSTRACT

Protoplasts can be obtained from Neurospora crassa mycelia by treatment with the "snail gut" enzyme preparation. Under appropriate conditions, the protoplasts will revert back to normal mycelia. Protoplast reversion provides an excellent system in which to examine the synthesis of cell wall polymers and their role in fungal germ tube development.

In this study, the reversion of Neurospora crassa protoplasts proceeded in a manner similar to that of protoplasts of other filamentous fungi. Within thirty minutes of incubation in reversion medium, the protoplasts formed aggregates which progressively enlarged in terms of cell number. Following aggregation, the protoplasts encysted by forming a polysaccharide cell wall. Germ tubes emerged from encysted protoplasts in a somewhat synchronous manner twenty-four hours after the start of the reversion process. Sixty percent of the total protoplasts were capable of regenerating a cell wall (encystment) while only twenty percent were capable of forming germ tubes.

An examination of chitin synthesis during protoplast reversion indicated a direct relationship between chitin synthesis and germ tube formation. Polyoxin D, a specific inhibitor of chitin synthetase, inhibited the reversion process and chitin synthesis to the same degree. Similar

polyoxin D concentrations had no effect on conidial chitin synthesis during germination indicating that the lack of effect on conidial germ tube formation was likely a result of the poor transport of the antibiotic.

Chitin synthetase in protoplasts and mycelia was found to exist in an active form and a latent form activated by limited trypsin digestion. Over ninety percent of the total chitin synthetase in protoplasts or mycelia was latent activity. The non-reverting protoplasts had little chitin synthetase activity. This was probably the result of membrane damage due to prolonged exposure to the "snail gut" enzyme. Different degrees of irreversible damage to the plasma membrane depending on the length of time of contact with the snail enzyme could explain the numerous types of protoplasts found in our preparations in terms of reversion capacity.

A "natural" protoplast of Neurospora crassa, the slime mutant, is unable to revert to the hyphal form of growth presumably due to genetic reasons. An examination of chitin synthesis in this organism by [^{3}H]glucosamine incorporation experiments indicated that no chitin was associated with the cell wall or excreted into the growth medium. In agreement with this result, polyoxin D had no effect on the growth of the slime mutant. The major soluble metabolite arising from glucosamine was identified as uridine diphosphate-N-acetylglucosamine indicating a defect at the level of

chitin synthetase. *In vitro* assay of chitin synthetase of the slime mutant indicated that it had some activity which existed as both an active and latent form but the total level of activity was only five percent of that found in mycelia. The lack of chitin synthesizing capacity of the slime mutant may be one of the possible reasons for its protoplast-like form of growth.

These results indicate that chitin synthesis plays an important role in the formation of the germ tube in *Neurospora crassa*.

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I would like to thank first and foremost my supervisor and friend, Dr. A. Vardanis, for keeping me on the "straight and narrow", for encouragement and for insight into the philosophy of science.

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ABBREVIATIONS

butyl-PBD	2-(4'-tert-butylphenyl)-5-(4"-biphenylyl)-1,3,4-oxadiazole
DMSO	dimethylsulfoxide
DNA	deoxyribonucleicacid
EDTA	ethylenediaminetetra-acetate
$GalNH_2$	2-amino-2-deoxygalactose
$GlcNH_2$	2-amino-2-deoxyglucose
GlcNAc	N-acetyl-2-amino-2-deoxyglucose
PMSF	phenylmethyl-sulfonylfluoride
RNA	ribonucleicacid
rpm	revolutions per minute
spm	strokes per minute
TCA	trichloroacetic acid
UDP	uridine diphosphate
UDP-GalNAc	uridine diphosphate-N-acetyl-2-amino-2-deoxygalactose
UDP-GlcNAc	uridine diphosphate-N-acetyl-2-amino-2 deoxyglucose
UMP	uridine monophosphate

INTRODUCTION

Chitin, a homopolymer of β-1,4 linked N-acetylglucosamine is found in invertebrates and in most fungi (Foster and Weber, 1960). The role of this polymer in fungal development appears to be somewhat different in the two major fungal groups, yeasts and filamentous fungi. In budding yeasts, chitin is located in the bud scar (Bacon et al., 1966), where it probably has a distinct function in the separation of the daughter cell from the mother cell during cell division. Polyoxin D, a specific inhibitor of chitin synthetase (Endo et al., 1970a), inhibits the division of yeast cells (Bowers et al., 1974). In filamentous fungi and the fission yeasts, the role of chitin is not as well defined; it is not located in a specific area but envelops the entire germ tube. Its synthesis occurs primarily at the apex of the germ tube and it appears to play a significant role in the extension of the germ tube (Bartnicki-Garcia, 1973).

Although the pathway of chitin biosynthesis is well known (Burnett, 1968), the last step, the chitin synthetase reaction is extremely complex. In general, the enzyme transfers N-acetylglucosamine from uridine diphosphate-N-acetylglucosamine to an endogenous acceptor. The enzyme shows a requirement for magnesium ions and activation by N-acetylglucosamine (for example see Gooday, 1977). In yeast, the enzyme exists mainly in an inactive state which can be activated by limited proteolysis (Cabib and Farkas, 1971; Ruiz-

Herrera and Bartnicki-Garcia, 1974). The latent form is found in the yeast plasma membrane (Duran et al., 1975). A model of the activation of chitin synthetase proposed by Cabib (1975) to explain the discontinuous chitin synthesis during yeast cell division involves control of activity by an endogenous protease as well as an endogenous protease inhibitor. In filamentous fungi, chitin synthetase can also be activated in vitro by protease digestion but the in vivo activation mechanism is not clear (Lopez-Romero and Ruiz-Herrera, 1976; Ryder and Peberdy, 1977).

Fungal protoplasts have been found to be able to regenerate a new wall and subsequently revert to the normal state (Villanueva and Garcia-Acha, 1971). These reverting protoplasts are useful in examining various aspects of fungal growth, particularly the study of cell wall polymer biosynthesis. The reversion process is relatively slow and, therefore, the events of development are easier to examine. These investigations of protoplast cell wall biosynthesis have been primarily restricted to yeast systems (Necas, 1971).

The present investigation examines chitin biosynthesis in Neurospora crassa. This organism was chosen since it is a well studied representative of filamentous fungi where the cell wall structure is relatively well known (Hunsley and Kay, 1976) and it is capable of releasing protoplasts on digestion with "snail gut" enzyme (Emerson and Emerson, 1958). Examination of chitin biosynthesis in reverting protoplasts and the wall-less slime mutant of Neurospora crassa was

undertaken to clarify the role of chitin in the growth of this organism.

CHAPTER I REVIEW OF THE LITERATURE

1.1 Cell walls of fungi

Fungal cell walls are basically eighty to ninety percent polysaccharide with the remaining material consisting of lipid and protein (Bartnicki-Garcia, 1968). Occasionally, substantial amounts of pigment, polyphosphates and inorganic ions are present, but wide departures from the above basic structure are rare. A large number of different monosaccharides have been identified; but, glucose, N-acetylglucosamine and mannose are found in the largest quantities and in the majority of fungi.

A generalized scheme of fungal wall structure has been proposed by Bartnicki-Garcia (1968) who classified fungi on the basis of the two most abundant polysaccharides present in the wall (Table 1). This classification is similar to the conventional classification of fungi based on morphological differences but some groups are classified on data from a single fungal representative (Bartnicki-Garcia, 1969). Five major polymers are found in fungal walls, cellulose, chitin, chitosan (deacetylated form of chitin), glucan and mannan. Cellulose is a linear homopolymer of β-1,4 linked glucose units while chitin is a linear homopolymer of β-1,4 linked N-acetylglucosamine units. Fungal chitin is referred to as α-chitin (Rudall, 1969) where each chitin chain exists as a helix of two residues per turn; numerous chains are held together by hydrogen bonds to form a bundle. In a chitin

Table 1. Cell wall composition and taxonomy of fungi*

Cell wall category	Taxonomic group	Representative genera
I. Cellulose-Glycogen	Acrasiales	Polysphondylium, Dictyostelium
II. Cellulose-β-Glucan	Oomycetes	Phytophthora, Pythium
III. Cellulose-Chitin	Hyphochytridiomycetes	Rhizidiomyces
IV. Chitin-Chitosan	Zygomycetes	Mucor, Phycomyces
V. Chitin-β-Glucan	Chytridiomycetes	Allomyces, Blastocladiella
	Euascomycetes	Aspergillus, Neurospora
	Homobasidiomycetes	Schizophyllum, Fomes, Polyporus
VI. Mannan-β-Glucan	Hemiascomycetes	Saccharomyces, Candida
VII. Chitin-Mannan	Heterobasidiomycetes	Sporobolomyces, Rhodotorula
VIII. Galactosamine-Galactose polymers	Trichomycetes	Amoebidium

* Taken from: Bartnicki-Garcia (1969)

fibril adjacent bundles of chains are antiparallel but all members of one bundle run in the same direction. In contrast, the less stable forms β and γ chitin have bundles of chains arranged in parallel fashion or arranged with two parallel to one antiparallel bundle (Rudall and Kenchington, 1973). Glucans consist of linear β-1,3, glucose chains with β-1,6 branch points (Manners et al., 1973a, 1973b, 1974; Bartnicki-Garcia, 1966; Aronson et al., 1967; Mahadevan and Tatum, 1965) but α-1,3 and α-1,4 glucans have also been reported (Johnson, 1965a, 1965b). Mannan, a polysaccharide-protein complex (Phaff, 1971) makes up the largest proportion of yeast cell wall (Roelofsen, 1953; Northcote and Horne, 1952). The mannose is attached to the polypeptide chain in two ways: 1) short oligosaccharide chains are attached via o-mannosyl bonds to either serine or threonine, and 2) large polysaccharide chains are attached via N-acetylglucosamine to asparagine residues (Sentandreu and Northcote, 1968). The polysaccharide units consist of an α-1,6 linked mannose backbone with short side chains attached via α-1,2 and α-1,3 linkages (Peat et al., 1961; Jones and Ballou, 1969a, 1969b; Kocourek and Ballou, 1969). Some modifications can occur in the side chains (Ballou, 1974).

Electron micrographs of surface views of filamentous fungi and yeasts have led to the generalization that the cell wall is composed of a microfibrillar element embedded in and covered by an amorphous matrix (Aronson, 1965; Burnett, 1968; Matile et al., 1969). In Saccharomyces, it appears that the

amorphous surface layer is composed of mannan as indicated by surface staining procedures (Mundkur, 1960). This is supported by studies on yeast protoplast formation, where a phosphomannase was required to remove phosphomannan complexes before the other polymers could be attacked (Eddy, 1958; McLellan and Lampen, 1968).

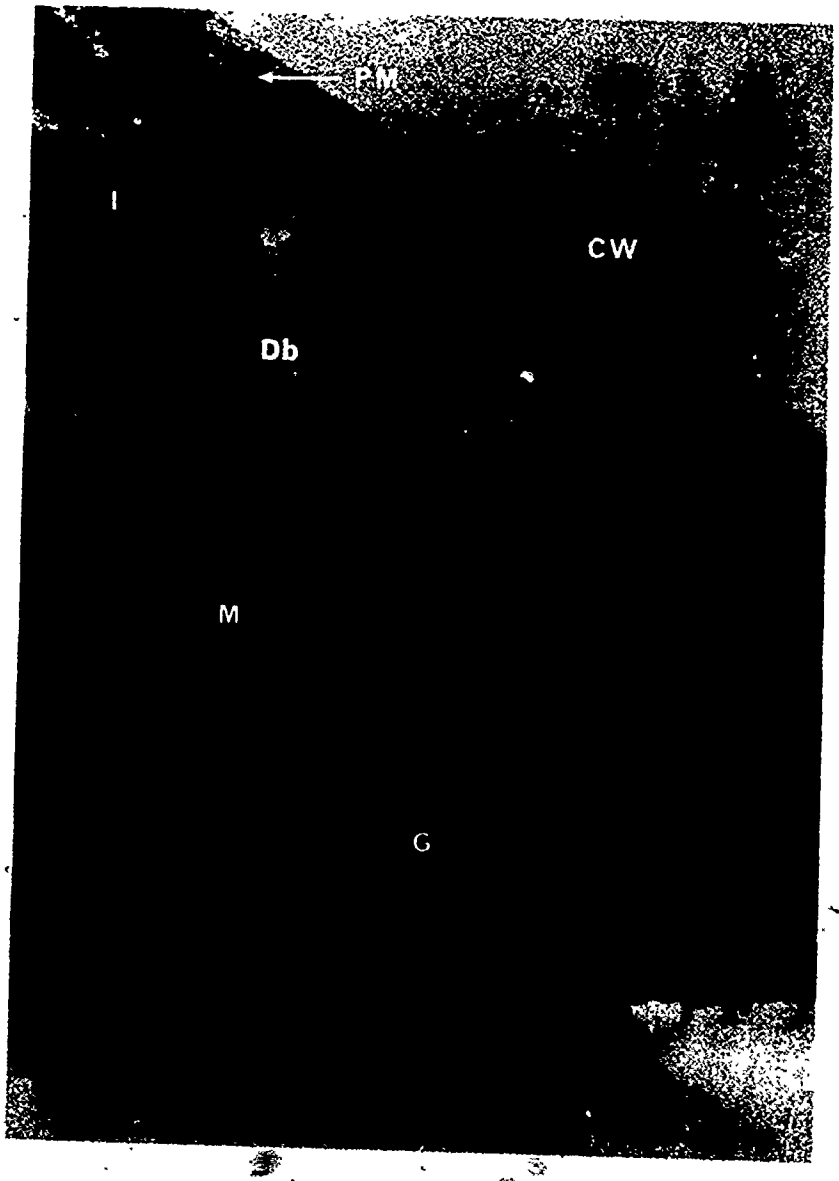
The localization of polymers in filamentous fungi has been established through the use of a combination of enzymatic digestion and electron microscopy (Hunsley and Burnett, 1970; Sietsma et al., 1975; Michalenko, 1976). In the chitin-β-glucan group (Neurospora, Schizophyllum) and the cellulose-β-glucan group (Phytophthora, Pythium), β-glucan covers an inner layer of either chitin or cellulose microfibrils.

(a) Neurospora crassa cell wall structure

The first electron micrographs of mature Neurospora crassa mycelia indicated a cell wall composed of at least two layers (Shatkin and Tatum, 1959; Weiss, 1965). Later, Hunsley and Burnett (1970) showed that mature mycelia had three layers of cell wall material while young mycelia showed a less complex cell wall structure (Figure 1). A detailed chemical study was carried out by Mahadevan and Tatum (1965) who divided the wall into four fractions. Fraction I (soluble in 2N NaOH) contained a galactosamine polymer as well as some glucose and protein. A preliminary examination of the proteins in this fraction indicated some degree of cross linking to sugar residues through an o-glycosyl serine linkage (Wrathall and Tatum, 1973). Fraction II (soluble in 1N H_2SO_4)

Figure 1. Electron micrograph of a Neurospora crassa mycelium. (magnification 50,000 x)

CW	cell wall
PM	plasma membrane
M	mitochondria
G	glycogen
Db	dense body vesicle
l	lipid droplet


PM
I
CW
Db
M
G

consisted mainly of glucose with some mannose. Fraction III (hydrolyzed by Aspergillus niger cellulase) consisted of only glucose (β-1,3 glucan). The remaining fraction, IV, was chitin. Further investigation located Fraction I (glucan-peptide-galactosamine) in the outer layer (Mahadevan and Tatum, 1967) while chitin appeared to be in the innermost layer covered by the β-1,3 glucan. Using a combination of enzymatic hydrolysis and electron microscopy, Hunsley and Burnett (1970) constructed a model for Neurospora crassa cell wall later refined by Hunsley and Kay (1976) by employing immunofluorescent microscopy using antibodies against the four fractions isolated by Mahadevan and Tatum (1965). In this model (Figure 2), the apex wall is much thinner than the more mature regions of the hyphae and consists of only two fractions; an inner chitin layer covered by a layer consisting of a galactosamine-glucan-protein complex. The older regions of the hyphae become progressively thicker and more complex by the addition of protein and β-1,3 glucan. Other evidence for this difference between the apical and the mature wall comes from observed fluorescence differences using the optical brightener Calcofluor White M 2R New (Gull and Trinci, 1974), from exoenzyme secretion studies (Chang and Trevithick, 1974) and from direct electron micrograph observation (Trinci and Collinge, 1975). The septa or cross walls of this organism show strong fluorescence similar to that exhibited by hyphal tips when treated with Calcofluor White M 2R New. They are composed entirely of chitin covered by a thin layer of

Figure 2. Longitudinal model of a Neurospora crassa hypha showing the cell wall structure (Hunsley and Kay, 1976).

A. inner chitin layer

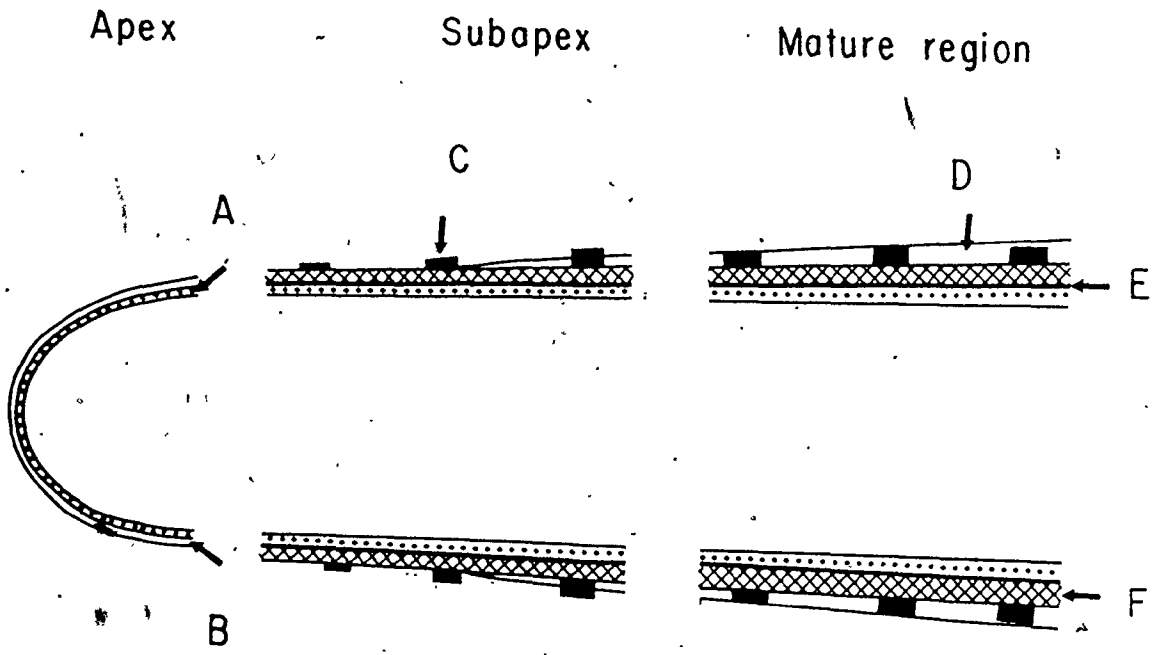
B. glucan-peptide-galactosamine complex

C. reticulate glycoprotein fibers (glucan-peptide-galactosamine)

D. amorphous laminarin-like glucan

E. discrete protein layer

F. easily removed protein


Apex
Subapex
Mature region
A
B
C
D
E
F

protein (Hunsley and Gooday, 1974).

(b) role of cell wall polymers in fungal morphology

It has become evident over a number of years that there is a direct relationship between fungal morphogenesis and cell wall structure (Nickerson and Bartnicki-Garcia, 1964; Bartnicki-Garcia, 1968; Brody, 1973; Schmit and Brody, 1976; Scott, 1976). Neurospora is an excellent organism to investigate the biochemical basis of morphogenesis due to the existence of over one hundred known morphological mutants. Whether cell wall polymer changes are the cause of morphological differences or vice-versa is not known but the first explanation is favored since mutants known to be blocked in cell wall synthesis exhibit large alterations in morphology. Also, all morphological mutants examined have large chemical differences in the composition of their cell walls while the wall-less mutant (slime) and protoplasts are spherical. Morphological mutants of Neurospora crassa in which the cell wall composition has been examined are collectively referred to as colonial mutants due to their compact growth patterns. These strains show increased ratios of glucosamine to glucose in the cell wall fraction due to decreases in glucose polymers rather than increases in chitin (DeTerra and Tatum, 1963). Although enzymatic defects have been found in these mutants (Brody and Tatum, 1966, 1967), the relationship between the defects and cell wall polymer biosynthesis remains obscure.

In addition to these mutations, one can obtain phenocopies of the colonial mutants by adding various chemicals

to the growth media of the wild type strain of Neurospora (Tatum et al., 1949; DeTerra and Tatum, 1961; Scott and Soloman, 1975). Sorbose has been found to be particularly effective in this regard; this compound inhibited β-glucan synthetase as well as glycogen synthetase in Neurospora (Mishra and Tatum, 1972). Since only small amounts of sorbose penetrated the cell (Crocken and Tatum, 1967), the suggestion was made that β-glucan synthetase was located at the cell surface. Another group of compounds which have a similar effect upon morphology (atropine, theophylline, histamine and several quinoline containing antimalarial drugs) lower the endogenous adenosine 3',5'-cyclic monophosphate (cAMP) levels of mycelia. Since the morphological mutants have normal cAMP levels, the effect of these materials on carbohydrate metabolism is indirect (Scott and Soloman, 1975).

The role of specific cell wall polymers like chitin on the morphology of fungi has also been investigated. Polyoxin D, a specific inhibitor of chitin synthetase in Neurospora crassa (Endo and Misato, 1969; Endo et al., 1970a) inhibited the growth of hyphae but it did not effect conidial germination in this organism; possibly this was a result of the lag in chitin synthesis during germination (Schmit and Brody, 1976). This compound inhibits the germination and growth of the fungi Mucor rouxii (Bartnicki-Garcia and Lippman, 1972a) and Trichoderma viride (Benitez et al., 1976). Wheat germ agglutinin which interacts specifically with chitin oligomers (Allen et al., 1973; Lotan and Sharon, 1973), also prevented

germ tube growth in Trichoderma viride (Mirelman et al., 1975).

The investigation of the t_s6 mutant of Aspergillus nidulans has suggested that chitin has an important morphological role in this organism (Cohen et al., 1969). This mutant has a single recessive temperature sensitive mutation in chitin biosynthesis which could be reversed by the addition of GlcNAc or high NaCl (9%) concentrations to the growth medium. The mycelia grown in the presence of NaCl had 15% of the normal chitin levels. The conidia grown at the non-permissive temperature (41°C) normally lysed (Katz and Rosenberger, 1970). The authors initially concluded that chitin was required to maintain the strength of the wall but further investigation indicated that the conidial lysis at 41°C was not due to mechanical weakness but probably autolysin action (Katz and Rosenberger, 1971).

1.2 Chitin biosynthesis

The direct pathway from glucose-6-PO_4 to chitin has been elucidated in fungi (Figure 3) (Blumenthal et al., 1955; Brown, 1955; Davidson et al., 1957; Leloir and Cardini, 1953; Lovett and Cantino, 1960; Reissig, 1956; Ghosh et al., 1960). Glucosamine, as well as N-acetylglucosamine, can enter the main pathway by phosphorylation to give the corresponding 6-phosphates (Davidson, 1960; McGarrahan and Maley, 1965a, 1965b; Bhattacharya et al., 1975). The chitin precursor UDP-GlcNAc can also be the indirect precursor of the galactosamine polymers since it can epimerize at the 4' position of the

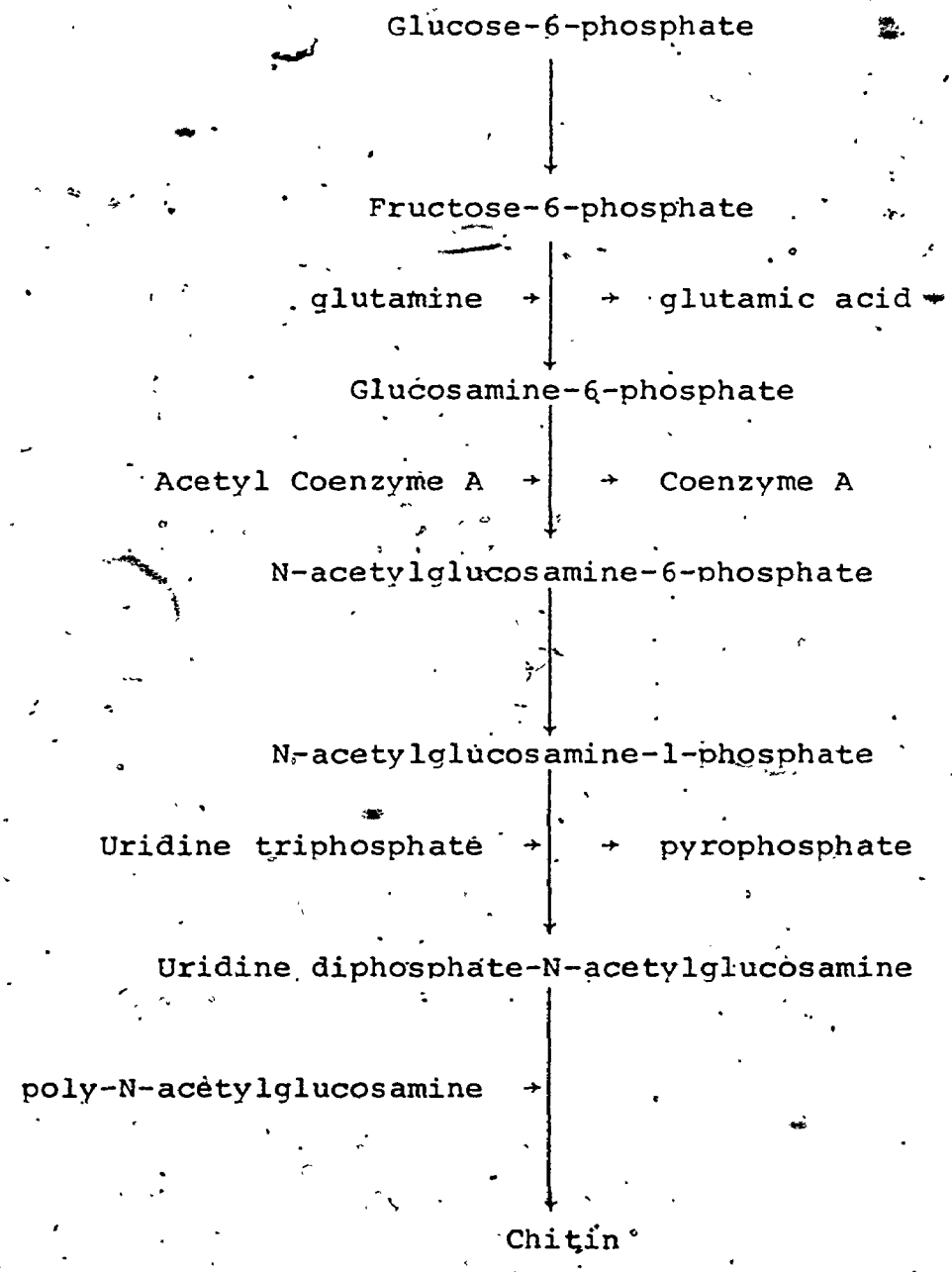


Figure 3. Chitin biosynthetic pathway in Neurospora crassa.

sugar moiety in the presence of the appropriate enzyme in Neurospora crassa to give the corresponding galactosamine compound (Edson and Brody, 1976). An osmotic mutant of Neurospora crassa has equivalent quantities of galactosamine polymers and chitin (Trevithick and Metzenberg, 1966), whereas normal mycelium contain very small amounts of the galactosamine polymers (Schmit et al., 1975). Hence, the epimerase reaction probably does not compete with chitin synthetase for the UDP-GlcNAc pool. UDP-GlcNAc was found to be a potent feedback inhibitor of the L-glutamine fructose-6-PO_4 transamidase reaction (Endo et al., 1970b; Selitrienníkoff et al., 1976).

All enzymes in this scheme are "soluble", with the exception of chitin synthetase, which is found in the particulate fraction. Using Saccharomyces cerevisiae protoplasts, Duran et al. (1975) showed that chitin synthetase activity was located within the plasma membrane. The enzyme is orientated in the membrane so that the UDP-GlcNAc binding site is towards the interior of the cell since polyoxin A (K_i 5 x 10^{-7}M) had no effect upon spheroplast chitin synthesis (Keller and Cabib, 1971). The enzyme in filamentous fungi also appears to be present in the plasma membrane (Jan, 1974).

In the yeasts Saccharomyces cerevisiae, Saccharomyces carlsbergensis and Mucor rouxii, chitin synthetase existed mainly in the inactive or latent form which could be activated by protease treatment (Cabib and Farkas, 1971; Cabib, 1972; Ruiz-Herrera and Bartnicki-Garcia, 1974). The latent form

can be activated *in vitro* by an "activating factor" isolated from the vacuole fraction (Cabib *et al.*, 1973). The "activating factor" has been identified as proteinase B of yeast Cabib and Ulane, 1973; Hasilik, 1974; Ulane and Cabib, 1976) which can be inhibited by a soluble endogenous protein inhibitor (Cabib and Farkas, 1971; Ulane and Cabib, 1974; Cabib and Keller, 1971). On the basis of these results, Cabib (1975) proposed a theory to explain the restriction of chitin synthesis to specific areas (bud scar) and times (budding) during yeast growth. According to this contention, the latent chitin synthetase exists in the entire membrane and is activated at specific times by a protease transported to the correct site by vacuoles.

Chitin synthetase from filamentous fungi also exists in a latent form which can be activated by protease digestion (Ryder and Peberdy, 1977; Lopez-Romero and Ruiz-Herrera, 1976; Archer, 1977). The identities of the endogenous proteases and possible inhibitors have not been pursued, but in *Neurospora crassa*, a protease-inhibitor system has been found (Yu *et al.*, 1973; Lampkin IV *et al.*, 1976). Whether this protease system affects chitin synthetase remains to be demonstrated.

Chitin synthetase has been found to be difficult to purify, presumably due to its integral position in the membrane. The enzyme from *Coprinus cinereus* has been solubilized using the detergent digitonin (Gooday and de Rousett-Hall, 1975). It had a large molecular weight (several million) which could

be reduced to 150,000 when treated with 200 mM NaCl; possibly the enzyme is a multimolecular aggregate (Gooday, 1975, 1977). Incubation of crude enzyme preparations from Mucor rouxii with UDP-GlcNAc resulted in a small degree of solubilization of chitin synthetase (Ruiz-Herrera and Bartnicki-Garcia, 1974). Also, high speed centrifugation of crude extracts left ten percent of the total activity in the supernatant (Ruiz-Herrera et al., 1975). Both "soluble" preparations from Mucor rouxii actually consisted of large granules (M.W. 7×10^6) of chitin synthetase referred to as "chitosomes" (Bracker et al., 1976; Ruiz-Herrera et al., 1977).

In general, all chitin synthetases transfer GlcNAc from UDP-GlcNAc to an endogenous acceptor. All enzymes are activated by or require Mg^{++}, are activated by GlcNAc and inhibited by polyoxin D. The pH optima range from pH 6 to pH 8 and the Km's for UDP-GlcNAc vary between 5×10^{-4}M and 3×10^{-3}M (Glaser and Brown, 1957; Jaworski et al., 1965; Porter and Jaworski, 1966; Camarago et al., 1967; Endo et al., 1970a; McMurrough et al., 1971; Keller and Cabib, 1971; Jan, 1974; Peberdy and Moore, 1975; Gooday and deRousset-Hall, 1975; Moore and Peberdy, 1976; Lopez-Romero and Ruiz-Herrera, 1976; Ryder and Peberdy, 1977). At low substrate concentrations, the enzymes gave sigmoidal kinetics but in the presence of GlcNAc (Ka 6×10^{-4}M - 1×10^{-2}M) the sigmoidal nature of the velocity-substrate concentration plots disappeared.

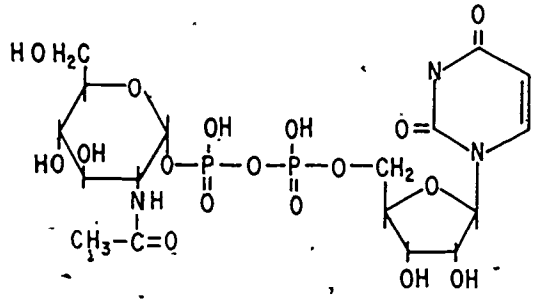
The nature of the endogenous acceptor is unknown. Enzyme

activities from different sources vary in their response to the addition of "primer" chitodextrins. Effects ranging from an absolute requirement for activity (Glaser and Brown, 1957) to inhibition have been described (Peberdy and Moore, 1975). One could understand a lack of effect in crude preparations due to saturating endogenous levels of acceptor but even purified C. cinereus preparations (Gooday and deRousset-Hall, 1975) have no "primer requirements".

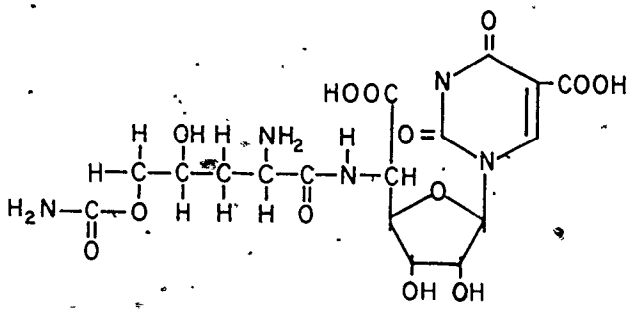
(a) inhibitors of chitin synthesis

Polyoxins are a family of nucleoside antibiotics produced by Streptomyces cacaoi (Isono et al., 1965, 1967, 1968) which inhibit fungal growth. The degree of inhibition of growth of Colchliobolus miyabeanus by several polyoxins correlated well with the inhibition of the incorporation of [^{3}H] glucosamine into chitin (Ohto et al., 1970). Polyoxin D (Figure 4) had no effect on respiration, protein or nucleic acid biosynthesis in C. miyabeanus or Neurospora crassa but was an effective competitive inhibitor of the enzyme chitin synthetase from either organism (Ki 10^{-5}M - 10^{-6}M) (Sasaki et al., 1968; Hori et al., 1971; Endo et al., 1970a). Polyoxin D was also found to be a competitive inhibitor of chitin synthetase from Mucor rouxii (Bartnicki-Garcia and Lippman, 1972) and Coprinus cinereus (Gooday et al., 1976).

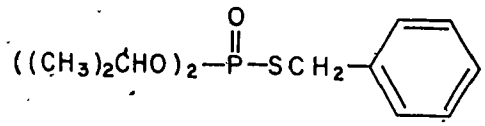
Nikkomycin is another nucleoside antibiotic isolated from the fermentation broth of S. tendae similar in structure to the polyoxin family and also an inhibitor of chitin synthetase in M. hiemalis. This compound however, also inhibits



UDP-GlcNAc



Polyoxin D



Kitazin P

Figure 4. Structure of UDP-GlcNAc and some chitin synthetase inhibitors.

RNA synthesis in this organism (Dahn et al., 1976).

Kitazin P, a synthetic organophosphorus compound (Figure 4) inhibited chitin synthetase in Pyricularia oryzae (Misato and Kakiki, 1977) and caused the accumulation of UDP-GlcNAc in vivo (Maeda et al., 1970). It had no effect on respiration, protein or nucleic acid synthesis (Kakiki et al., 1969).

1.3 Glucan biosynthesis

Very little is known about this area in fungi. β-1,3-glucan synthetases have been found in particulate preparations from Neurospora crassa (Mishra and Tatum, 1972) and Phytophthora cinnamomi (Wang and Bartnicki-Garcia, 1966) which apparently use UDP-glucose as substrate. As with chitin synthetase, the enzyme from P. cinnamomi was stimulated by trypsin digestion (Wang and Bartnicki-Garcia, 1976).

1.4 Insertion of polymers

It has long been known that filamentous fungi grow apically (Grove et al., 1970; Butler, 1961; Marchant and Smith, 1968) but the location of polymer insertion into the wall was in doubt. Burnett (1968) interpreted early work with Phycomycetes to indicate that these elements were inserted at a region behind the tip. However, autoradiographic studies of various polymers in different fungi have indicated that the wall polymers are inserted at the extreme tip (Bartnicki-Garcia and Lippman, 1969; Katz and Rosenberger, 1970, 1971; Gooday, 1971).

During the microscopic examination of hyphal tips, a

dark staining apical body referred to as "Spitzenkorper" (Brunswik, 1974) can be seen clearly. It was associated only with actively growing hyphae and disappeared when growth was arrested (Gibardt, 1955, 1957; McClure et al., 1968). Electron micrographs identified this apical body as a large concentration of cytoplasmic vesicles (Grove et al., 1970). The vesicles fuse with the hyphal tip during growth, thus expanding the plasma membrane. The vesicles give a positive periodic acid reaction for carbohydrate (Heath et al., 1971), but whether or not they contain material for cell wall biosynthesis must await further characterization.

The hyphal tip is very sensitive to various external factors. In the presence of water, hyphae of most species burst at the tip (Robertson, 1958, 1959; Bartnicki-Garcia and Lippman, 1972b). The bursting is not just an osmotic phenomenon, as a large number of other agents also trigger bursting. Lysozyme, as well as the antibiotic polyoxin D cause bursting of the apical tips of M. rouxii (Bartnicki-Garcia and Lippman, 1972b). It has been suggested that apical growth was a result of a balance between cell wall degradation and cell wall synthesis (Bartnicki-Garcia, 1973). Little evidence for this model has been given but lytic enzymes are found to be associated with the fungal cell wall (Mahadevan and Mahadkar, 1970; Ploacheck and Rosenberger, 1975).

As in the growing hyphal tip, budding yeast show a large number of vesicles concentrated in the areas of the bud (Bowers et al., 1974; Cortat et al., 1972; Moor, 1967;

Sentandreu and Northcote, 1969) which are thought to be involved in the synthesis of the cell wall (Cabib, 1975). Cortat et al. (1972) found that the vesicles appeared to contain glucanase activity which could be involved in the initial softening of the wall. The mechanism for determining the bud site and initiating bud formation remains unknown.

1.5 Protoplasts

(a) general properties

The term "protoplast" refers to the spherical cell derived from a walled vegetative cell by removal of the entire cell wall. Originally, the term was used in reference to bacterial systems (Weibull, 1953), but this has been extended to encompass a variety of walled organisms, including fungi, (McQuillen, 1960). Brenner et al. (1958) put forth a series of criteria for bacterial protoplasts which have also been applied to other systems. The term spheroplast was used when residues of the cell wall remained attached to the plasma membrane. In practice, criteria for protoplasts include osmotic fragility and the loss of rigidity resulting in a spherical form (Villanueva, 1966). Both protoplasts and spheroplasts satisfy these criteria and the question has to be approached in more specific terms.

In fungi there is no identifying marker for cell walls, unlike some bacteria where diaminopimelic acid provides a convenient chemical marker. Electron microscopy (Necas, 1971; Peberdy, 1972) and fluorescent dye studies (Peberdy and Gibson, 1971; Gull et al., 1972) of fungal protoplasts have shown that

most protoplasts lack cell wall residues. The mode of protoplast release (extrusion through a pore, leaving the undigested wall) (Peberdy, 1972) also suggests that protoplasts lack cell wall material. Although this evidence eliminates ninety-five percent of the wall material from the protoplast, one must make the differentiation between the terms protoplast and spheroplast on strictly relative grounds.

Protoplasts are normally obtained through the action of specific enzyme preparations (Villanueva and Garcia-Acha, 1971). Metabolic disturbance methods (Hamilton and Calvet, 1964; Cohen et al., 1969; Berliner and Reca, 1970) and mechanical fractionation methods (Necas, 1956) have been tried with limited success but the enzymatic procedures yield more protoplasts. The ability of the digestive juice of the snail Helix pomatia to attack fungal cell walls was first noted by Giaja (1914). Since this time it has been successfully used to obtain protoplasts from yeast (Eddy and Williamson, 1957; Svilla et al., 1961; Longley et al., 1968; Lebeault et al., 1969) and filamentous fungi (Emerson and Emerson, 1958; Bachmann and Bonner, 1959; Strunk, 1969). Other enzyme preparations derived from bacterial strains such as Streptomyces (Garcia-Mendoza and Villanueva, 1962; Rodriguez-Aguirre et al., 1964; Bartnicki-Garcia and Lippman, 1966; Sietsma et al., 1967) or fungi such as Trichoderma (de Vries and Wessels, 1972, 1973, 1975) have been used successfully. The enzymatic digestion methods have also been applied to conidia (Bachmann and Bonner, 1959; Weiss, 1965; Garcia-Acha et al., 1966; 1968)

with limited success.

Aquatic Phycomycetes produce flagellated zoospores which lack a cell wall (Cantino et al., 1963) and have been regarded as "natural" protoplasts (Bartnicki-Garcia and Lippman, 1966). They are much more stable to changes in osmotic pressure than protoplasts artifically derived from mycelium. Another "natural" protoplast system is the slime mutant of Neurospora crassa which apparently grows as a single wall-less cell (Emerson, 1963).

Protoplasts have numerous advantages over normal mycelia in examining various features of fungal metabolism. Protoplasts are easily broken and have been used for the preparation of cell membranes (Boulton, 1965; Garcia-Mendoza and Villanueva, 1967; Longley et al., 1968) and various other cell organelles including nuclei (Eddy, 1959), vacuoles (Svilla and Schlenk, 1960; Matile and Wiemken, 1967) and mitochondria (Duell et al., 1964). Recently, protoplast fusion studies have become important, particularly in relation to plant systems (Cocking, 1972), but this aspect has also been investigated in fungal systems (Anne and Peberdy, 1975, 1976). An important use has been in the investigation of cell wall biosynthesis since they have the ability to revert to normal growing cells. Most investigations in the past have concentrated on reverting yeast protoplast systems (Necas, 1971) but now investigators are turning to protoplast reversion systems of filamentous fungi (Garcia-Acha et al., 1966; Moore and Peberdy, 1976; Peberdy and Gibson, 1971).

(b) protoplast reversion and cell wall biosynthesis

In the field of protoplasts, the term regeneration is used in reference to the ability of the protoplast to synthesize new cell wall material; the term reversion is used in reference to the ability of the protoplast to return to the form from which it was derived (i.e. hyphal growth). Svoboda et al. (1969) have pointed out that the study of protoplast reversion enables one to trace a gradual construction of single wall components, trace these components in their arrangement in the overall cell wall, trace regulatory mechanisms, and relate the cell wall to other structures. Progress has been made in all of these areas, particularly with protoplasts of the yeast S. cerevisiae. Protoplasts of S. cerevisiae produced aberrant cell walls, fibrillar in nature when placed in liquid nutrient media (Necas, 1965; Kopecka et al., 1967). In contrast, protoplasts placed in agar or gelatin media reverted quantitatively to normal cells (Necas, 1961; Svoboda, 1966; Svoboda and Necas, 1966) where the wall consisted of both a fibrillar and amorphous matrix components. This phenomena allowed the study of each component separately. Chemical analysis of the fibrillar material originally showed mannose, glucose and large amounts of glucosamine (18-20%) (Eddy and Williamson, 1959; Garcia-Mendoza and Novaes-Ledieu, 1968), the latter being derived from chitin (Necas, 1971). Bacon et al. (1969) subsequently demonstrated contamination of the protoplast preparations with bud scars which are mainly chitin. Further examination

(Kreger and Kopecka, 1975) revealed that in fact chitin was part of the fibrillar structure. The percentage of chitin in this fraction was considerably larger than the one percent by weight in normal cells, indicating that a considerable portion of the wall was missing.

The glucan portion of this fibrillar element lacked β-1,6 branch points found in normal cell wall glucan (Manners et al., 1974), possibly accounting for the longer chain length of the glucan (Kreger and Kopecka, 1975). The formation of glucan was not affected by the proteolytic enzymes trypsin or pronase and hence was not synthesized on the surface of the plasma membrane (Kopecka et al., 1970). Also, cycloheximide had no effect on fibrillar β-1,3 glucan biosynthesis (Necas et al., 1968; Soskova et al., 1968), therefore, glucan synthesis was independent of protein synthesis. Inhibitors of polysaccharide synthesis like 2-deoxyglucose (Farkas et al., 1976; Johnson, 1968) did inhibit the synthesis of the fibrillar material (Farkas et al., 1970; Svoboda et al., 1969).

The amorphous matrix consisted of polysaccharides (mannan and amorphous glucan), protein and lipid (Necas, 1971). Its formation was a function of the physical properties of the surrounding medium (Necas, 1961, 1962; Necas and Svoboda, 1967). In liquid medium protoplasts continued to produce mannans (Lampen, 1968); yet, normal walls were not formed, indicating that some of the matrix materials were lost into the medium. In contrast to glucan synthesis, matrix formation was inhibited by cycloheximide (Necas et al., 1969) and

hence required protein synthesis.

The requirement of solid media in the reversion process occurs in all the budding yeasts examined; S. carlsbergensis (Svobodă and Masa, 1970); S. fragilis (Rost and Venner, 1965) and S. utilis (Svoboda, 1967). Non-budding yeasts were able to undergo complete reversion in liquid media. In Schizosaccharomyces pombe protoplasts (Svoboda, 1967), a dense fibrillar network was synthesized before the appearance of the matrix components, and perhaps it prevented the loss of the matrix material.

The description of protoplast reversion in mycelium-forming fungi has not progressed as far as the yeast systems. In contrast to the budding yeast, all filamentous fungal protoplasts were able to revert to normal mycelium in liquid media (Bachmann and Bonner, 1959; Garcia-Acha et al., 1966; Sietsma and DeBoer, 1973; Peberdy and Buckley, 1973; de Vries and Wessels, 1975). Reversion has in all cases followed various combinations of three patterns described by Garcia-Acha et al.,(1966): 1) formation of a chain of yeast-like forms, the last of which produces the germ tube, 2) direct formation of the germ tube from the protoplast and 3) formation of a bud or globular form from which the germ tube arises. The significance or cause for the three patterns of reversion is unknown. As in the case of yeast, regeneration starts with the production of a microfibrillar network surrounding the protoplast (Sietsma et al., 1975; Benitez et al., 1975). After a time lag (variable in different systems, the germ tube forms.

Biochemical studies on the reversion process had been lacking until the recent examination of wall polymers in the Schizophyllum commune reversion sequence (de Vries and Wessels, 1975). Cycloheximide inhibited protein synthesis by ninety-eight percent but had no effect on chitin synthesis or α-1,3 glucan (S glucan) synthesis although β-1,3 glucan (R glucan) formation and reversion to hyphal growth was inhibited. Polyoxin D inhibited chitin and R glucan synthesis as well as reversion to hyphal growth. These results seemed to implicate R glucan synthesis as a requirement for the initiation of hyphal growth.

The regeneration of Aspergillus nidulans and A. flavus protoplasts was stimulated by the presence of GlcNAc (Peberdy and Buckley, 1973; Moore and Peberdy, 1976). The levels of chitin synthetase activity gradually increased during reversion; the addition of GlcNAc enhanced this increase in the enzyme. These results suggested a close relationship between chitin synthesis and protoplast reversion. Conidial protoplasts of A. flavus which had very low initial levels of chitin synthetase compared to the mycelial protoplasts can also revert but the degree of reversion has not been documented.

Zoospores undergo a similar sequence of events between the zoospore and the formation of a germ tube. Phytophthora palmivora zoospores first lose their flagella then undergo a rapid encystment stage followed by germination and formation of the germ tube. During encystment, the naked zoospores syn-

thesize microfibrils (Tokunaga and Bartnicki-Garcia, 1971b) and then produce a germ tube which consists of microfibrils covered with an amorphous material. The amorphous material in Phytophthora was β-1,3 glucan while the microfibrillar elements were mixtures of β-glucans (1-3, 1-4 and 1-6 linkages). The encystment process could be synchronized by agitation and was complete within fifteen minutes (Tokunaga and Bartnicki-Garcia, 1971a). The cyst wall glucan apparently originated from internal water-soluble glucans. Similar sequences occurred in other zoospore systems (Kroh et al., 1976). During early stages of encystment, the Phytophthora palmivora zoospores showed a strong adhesive phase (Sing and Bartnicki-Garcia, 1972). This involved the secretion of a glycoprotein material from a peripheral vesicle (Sing and Bartnicki-Garcia, 1975a, 1975b).

1.6 Polyprenoid intermediates and polysaccharide biosynthesis

The first report of an alcohol soluble intermediate in polysaccharide biosynthesis came from investigations of cellulose synthesis in Acetobacter xylinum (Colvin, 1959). Several years passed before this observation was followed with the discovery of similar intermediates in cell wall peptidoglycan synthesis in Staphylococcus aureus and Micrococcus lysodeikticus (Anderson et al., 1965). Intermediates were found in the synthesis of a large number of bacterial polysaccharides; the O-antigen portion of lipopolysaccharide cell envelopes (Weiner et al., 1965; Wright et al., 1965, 1967), the teichoic acid polymers (Baddiley, 1972) and the mannan

polymers (Scher and Lennarz, 1969). The intermediates were identified as undecaprenol (C_{55} isoprenoid alcohol) attached via a pyrophosphate bridge to the appropriate sugar moiety (Higashi et al., 1967, 1970).

Although most of the early work concentrated on bacterial systems, recently, more emphasis has been placed on the role of isoprenoid intermediates in mammalian glycoprotein systems (Waechter and Lennarz, 1976). In these systems, the isoprenoid family dolichol (I-dihydro-XV, XVI-ditrans-polycis-prenols C_{120}) (Evans and Hemming, 1973) accepted sugar residues from guanosine diphosphate mannose or uridine diphosphate N-acetyl glucosamine and subsequently passed the sugars on to appropriate acceptors.

(a) "lipid intermediates" in fungal polysaccharide biosynthesis

The first report of a lipid intermediate in fungal systems came from an investigation of mannan biosynthesis in Saccharomyces cerevisiae (Tanner, 1969). The lipid which appeared to be dolichol phosphate (Tanner et al., 1971, 1972; Jung and Tanner, 1973) accepted mannose from guanosine diphosphate mannose and subsequently transferred the mannose to threonine or serine on the protein backbone. The remaining mannose units were added via guanosine diphosphate mannose directly (Babczinski and Tanner, 1973; Sharma et al., 1974; Lehle and Tanner, 1974). The other mannose chains which are attached via a GlcNAc-GlcNAc-Asparagine linkage are attached directly from GDP-mannose (Lehle and Tanner, 1975). The

various linkages of mannan, α-1,6, α-1,2 and α-1,3, involve different specific mannosyl transferases (Schutzbach and Ankel, 1971; Schutzbach et al., 1974; Nakajima and Ballou, 1975; Farkas et al., 1976). A similar mechanism has been described in the yeast Hansenula holstii (Bretthauer and Wu, 1975; Bretthauer et al., 1973; Bretthauer and Chen Tacky, 1974). The synthesis of the chitobiose unit apparently also involves a dolichol intermediate. Lehle and Tanner (1975) identified dolichol-PP-GlcNAc and dolichol-P-P-$(GlcNAc)_2$ in reactions involving UDP-GlcNAc and S. cerevisiae membrane preparations.

In Aspergillus niger, mannan biosynthesis also involved a polyprenol type intermediate (Barr and Hemming, 1972a; Letoublon et al., 1973). The polyprenols were identified as an exo-methylene hexahydropolyprenol family (C_{70}-C_{90}) (Barr and Hemming, 1972b). Neurospora crassa membrane fractions (Gold and Hahn, 1976) catalyzed similar reactions.

Various investigations have indicated that polyprenols appear to have no role in the formation of glucan or chitin polymers. Saccharomyces cerevisiae was not able to form lipid intermediates from UDP-glucose, neither in whole cells treated with toluene (Sentandreu et al., 1975) nor in crude membrane preparations which were capable of forming polyprenoid sugars from the substrate GDP-mannose (Lehle and Schwarz, 1976). These types of intermediates have also been looked for in relation to chitin biosynthesis, yet, they have not been detected (Endo et al., 1970a; McMurrough and

Bartnicki-Garcia, 1971). The best evidence against lipid intermediates participating in chitin biosynthesis comes from investigations of the mode of action of the antibiotic tunicamycin, produced by Streptomyces lysosuperificus (Takatsuki et al., 1971). It specifically inhibits the formation of polyprenol sugars from uridine diphosphate-N-acetylglucosamine in calf liver microsomes (Tkacz and Lampen, 1975), B. subtilis membranes (Brettinger and Young, 1975), and S. cerevisiae membranes (Lehle and Tanner, 1976). It had no effect on chitin biosynthesis in yeast (Kuo and Lampen, 1974, 1976).

CHAPTER II MATERIALS AND METHODS

MATERIALS

Calcofluor White M 2R New was a kind gift from Dr. J. R. Trevithick (Department of Biochemistry, University of Western Ontario) and Cyanamid of Canada Ltd.

Tinopal BOPT was a gift from CIBA-GEIGY (U.K.) Limited.

Polyoxin D was a gift from Dr. K. Isono (Institute of Physical and Chemical Research, Wako-shi, Saitama, Japan).

Kitazin P was a gift from Dr. Y. Uesugi (National Institute of Agricultural Chemicals, Nishigahara, Kita-Ku, Tokyo, Japan).

Suc D'Helix pomatia was obtained from L'Industrie Biologique Francaise, 35, Quai du Moulin de Cage, 92 Gennevilliers, France.

chemicals	supplier
N-acetyl D-glucosamine	Sigma Chem. Co.
α-amylase (pancreatic, type 1-A)	Sigma Chem. Co.
Amylo-α-1,4-α-1,6-glucosidase	Boehringer Mannheim
Bovine serum albumin	Sigma Chem. Co.
Chitinase (Streptomyces griseus)	ICN Pharmaceuticals Inc.
Chitin (practical grade)	Eastman Kodak Co.
Cysteine-HCl, monohydrate	Mann Research Labs. Inc.
Deoxyribonucleic acid (calf thymus)	Worthington Biochemicals
N,N'-Diacetylchitobiose	Sigma Chem. Co.
o-dianisidine hydrochloride	Sigma Chem. Co.

EDTA (dipotassium salt)	British Drug House Ltd.
Fructose 6-PO_4, disodium salt (grade I)	Sigma Chem. Co.
Galactosamine-HCl	Sigma Chem. Co.
Glucosamine-HCl	Aldrich Chem. Co.
Glucosamine-6-PO_4, sodium salt (grade II)	Sigma Chem. Co.
Glucosamine-6-$^3H(N)$-HCl (10.13 Ci/mmole, 20.7 Ci/mmole)	New England Nuclear Corp.
L-glutamine	Nutritional Biochem. Co.
Glucose oxidase (type V)	Sigma Chem. Co.
Glycogen (type II)	Sigma Chem. Co.
Peroxidase (type I)	Sigma Chem. Co.
Phenylmethylsulfonyl fluoride	Sigma Chem. Co.
Trypsin (type I)	Sigma Chem. Co.
Trypsin inhibitor (soybean type I-s)	Sigma Chem. Co.
UDP-GlcNAc, sodium salt (grade I)	Sigma Chem. Co.
UDP-N-acetyl[U-^{14}C]$GlcNH_2$ ammonium salt (300 mCi/mmole)	Amersham/Searle Co.
UDP-N-acetyl[6-^3H]$GlcNH_2$ (6.6 Ci/mmole)	New England Nuclear Corp.

Other chemicals and solvents were obtained from either the Fisher Chem. Co. or Canadian Scientific Products Ltd.

METHODS

2.1 Organisms and culture conditions

Neurospora crassa wild type (F.G.S.C. no. 987) (obtained from the University of Western Ontario, Botany Department Culture Collection) was maintained on Fries minimal medium

(no. 3) slants supplemented with 1.5% sucrose and 2% agar (Beadle and Tatum, 1945). Large quantities of conidia were obtained from 1-2 week(s) old Roux bottle cultures initially seeded with conidia from slant cultures. Roux bottle cultures were grown in the light at 22°C. Conidia (1×10^9) were washed from the Roux bottle cultures with water, filtered through glass wool, concentrated by centrifugation and introduced into 200 ml flasks containing 50 ml of liquid medium. For enzyme assays, young hyphae were harvested by filtration after incubation on a gyrotory shaker (New Brunswick, 200 rpm) at 30°C for 24 hours. For protoplast formation, young hyphae were grown for 11.5 hours and harvested by centrifugation (Sorvall RC-2, 10 minutes, 27,000 x g, SS-34 rotor).

A growth curve for the wild type strain was constructed by filtering the mycelia at the specified times, drying the resulting mat for 24 hours *in vacuo* over P_2O_5 and finally weighing the dried mat (Figure 5).

The *Neurospora crassa* slime mutant (F.G.S.C. no. 1118) [fz (no number), sg (no number), and os-1 (B135A)] was a kind gift from Dr. P. Galsworthy (Department of Biochemistry, University of Western Ontario). Cells were grown at 30°C for 72 hours on agar plates of Vogels medium N (Vogel, 1956) supplemented with 2% sucrose. Ten agar disks (1.0 cm diameter) were used to innoculate 250 ml flasks containing 50 ml liquid medium supplemented with 2% sucrose, 2% mannitol, 0.75% nutrient broth and 0.75% yeast extract. Cultures were grown at

30°C for 44 hours on a gyrotory shaker (120 rpm). A growth curve was constructed by reading the optical density of an aliquot of cells at 600 mμ (Figure 6). Cells were routinely isolated by centrifugation (700 x g, 10 minutes).

All culture transfers were conducted under sterile conditions using a laminar air flow chamber (Environmental Air Control Inc.).

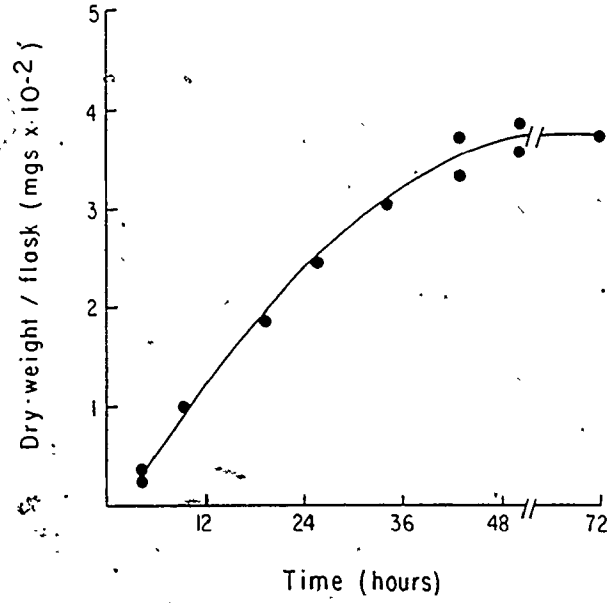
2.2 Germination of ethylene glycol treated conidia

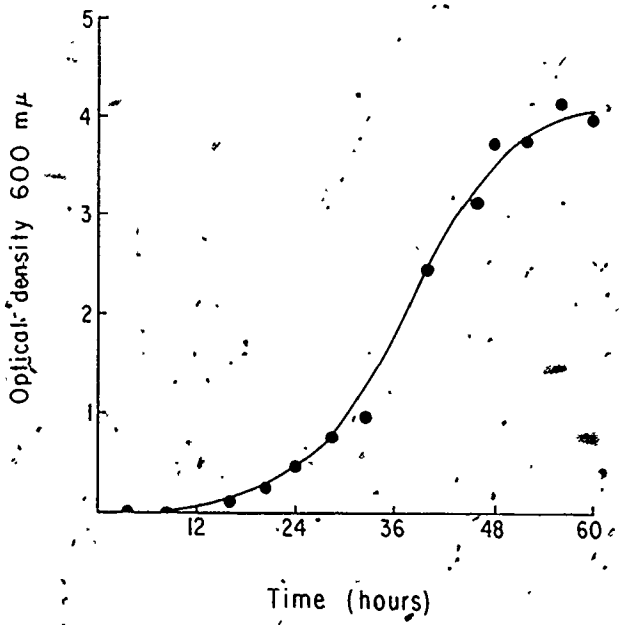
Conidia were isolated from Roux bottles as described with the exception that they were allowed to remain in water after filtration for at least one hour prior to isolation by centrifugation. Conidia (1×10^9) were innoculated into 50 ml of Vogel's liquid medium containing 3.22 M ethylene glycol (250 ml flasks) (Bates and Wilson, 1974). The cultures were incubated on a gyrotory shaker (150 rpm) at 25°C for the stated times.

At specified times, 10 ml aliquots of the growing cells were pipetted into a 200 ml Erlenmeyer flask and incubated at 25°C in a slowly shaking water bath (40 spm). Growth medium without the ethylene glycol was added using a peristalic pump at the following rates: 1 ml/min for 10 minutes, 2 ml/min for 10 minutes and 4 ml/min for 15 minutes (Bates and Wilson, 1974). Samples were removed at various time intervals, examined by phase contrast microscopy and counted using a haemocytometer.

Figure 5. Growth curve of wild type Neurospora crassa.

Figure 6. Growth curve of the Neurospora crassa slime mutant.


Dry weight / flask (mgs x 10-2)
5
4
3
2
1
12
24
36
48
72
Time (hours)


Optical density 600 mμ
5
4
3
2
1
12
24
36
48
60
Time (hours)

2.3 Protoplasts

(a) preparation

Protoplasts were prepared by a modification of the method of Bachmann and Bonner (1959). The harvested mycelia (from 50 ml growth medium) were washed twice with 0.1 M PO_4 buffer, pH 5.8 containing 20% sucrose. The material was taken up in 9.5 ml of the same buffer and transferred to a sterile 25 ml Erlenmeyer flask. "Snail gut" juice (0.5 ml) was added after treatment with 0.025 ml of a 1% cysteine-HCl solution in the same buffer which had been sterilized by passage through a Gelman Metricel filter-type GA-8, pore size 0.20 μ. The resulting suspension was incubated at 30°C in an Amino-Dubnoff reciprocal shaker (40 spm) for 15 hours. All the above procedures were carried out under sterile conditions. For large scale protoplast production, the procedure was scaled up ten fold.

(b) isolation

The "snail gut" incubation mixture was passed through glass wool to remove hyphal fragments. The resulting solution was centrifuged at 200 x g for 10 minutes and the supernatant was further centrifuged at 2000 x g for 10 minutes. The protoplast pellet was washed with reversion medium (normal growth medium supplemented with 20% sucrose) and then dispersed very gently in 5.0 ml of reversion medium. All procedures were conducted under sterile conditions.

(c) reversion

Reversion was carried out in 25 ml Erlenmeyer flasks containing 5 ml reversion medium and a total of 5×10^8 proto-

plasts. The flasks were incubated in a reciprocal shaking water bath (40 spm) at 30°C for the specified times. The degree of reversion was determined by counting the number of protoplasts which had formed germ tubes after brief sonication (MSE ultrasonic power unit - 1.5 amps) to separate the clumped cells.

2.4 Microscopy

(a) light microscopy

The protoplasts were counted with a haemocytometer using phase contrast microscopy (400 x magnification - Reichert Zetopan microscope). Photographs were taken with a 35 mm Nikon Microflex EFM attachment.

(b) fluorescence microscopy

Samples of mycelial and conidial suspensions in water were mixed (1/1 by volume) with a 1% Calcofluor solution. Protoplast suspensions were mixed with a 1% Calcofluor solution in either reversion medium supplemented with 20% sucrose or 0.1 M PO_4 buffer, pH 5.8 supplemented with 20% sucrose depending upon the stage of isolation. Slime cell suspensions were mixed with a 1% Calcofluor solution in normal growth medium. After five minutes, samples were examined under a Reichert Zetopan microscope (mercury vapor lamp) with an E2 exciter filter, Sp 2 absorption filter and a Kodak 18 A filter.

(c) electron microscopy

Mycelia were fixed in 5.6% glutaraldehyde in 0.05 M PO_4 buffer, pH 7.0 for two hours at 22°C. Protoplasts or slime cells were combined with one volume of normal growth medium supplemented with four percent agar to reduce lysis

during fixation (personal communication, Dr. P. C. Fitz-James). After cooling, pieces of agar were fixed as stated above for four hours at 22°C. The fixed material was washed several times with buffer. The agar pieces were post-fixed with 1% osmium tetroxide in the same buffer for two hours at 22°C, washed several times with Kellenberger buffer (Ryter and Kellenberger, 1958) and stained with 0.1% uranyl acetate (45 minutes) in Kellenberger buffer. The material was dehydrated through an ethanol series to propylene oxide and embedded in an Araldite-Epon mixture (Mollenbauer, 1964). Reynold's lead citrate (Reynolds, 1963) was used to stain sections on the grid. The sections were examined with an R.C.A.-E.M.U. 3 electron microscope.

2.5 Tinopal BOPT uptake by reverting protoplasts

Protoplasts (1×10^8) were incubated in 1.0 ml of reversion medium for varying times (Corex test tubes, 15 ml) at 30°C in a shaking water bath. Tinopal BOPT (20 µg/ml - in reversion medium) (4 ml) was added to the reverting protoplasts and the mixture was left standing for 5 minutes prior to centrifugation at top speed in a clinical centrifuge for 10 minutes. The optical density of the supernatant was read at 350 mµ. A zero time sample of protoplasts was used as a control. The difference between the O.D. at 350 mµ of the original dye solution (4.0 ml diluted with 1.0 ml reversion medium) and the protoplast supernatant was taken to be the amount of dye absorbed by the protoplasts. All procedures involving

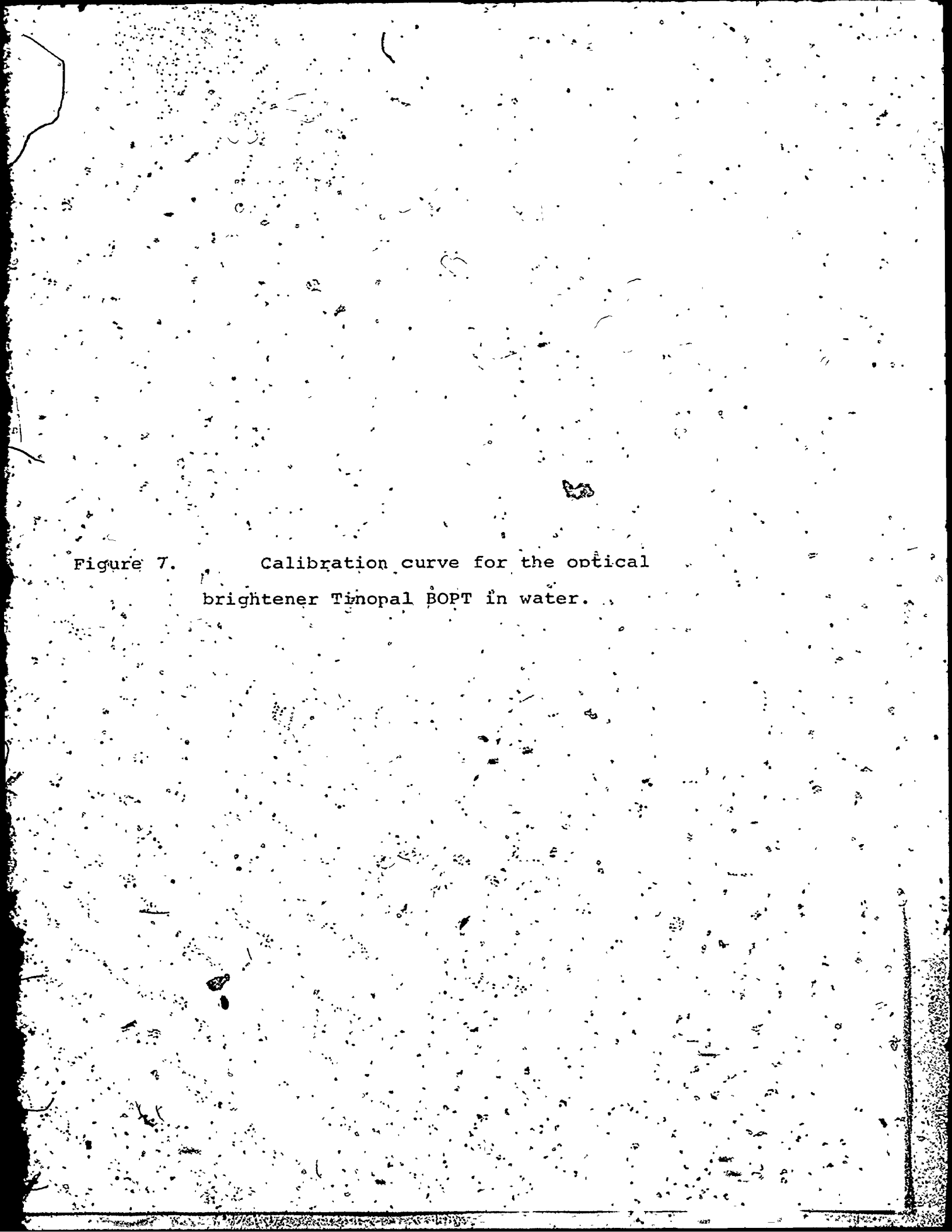
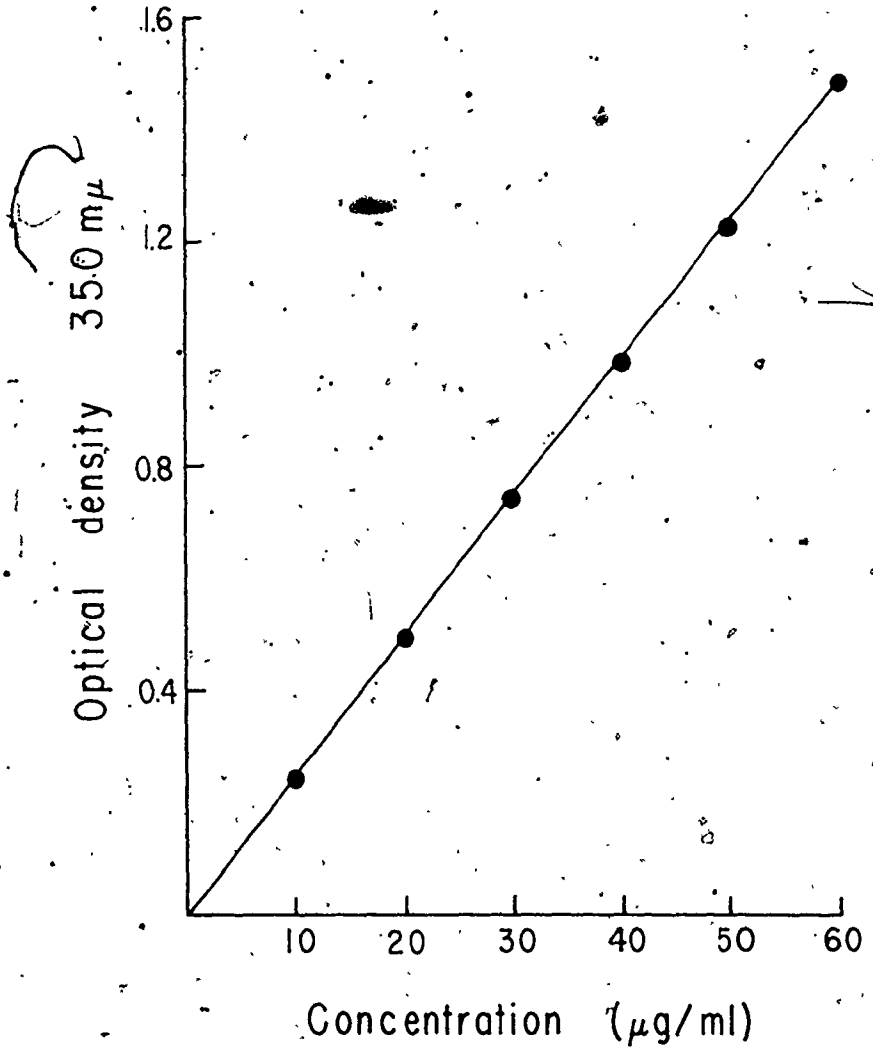


Figure 7. Calibration curve for the optical brightener Tinopal BOPT in water.


Optical density 350 mμ
1.6
1.2
0.8
0.4
10
20
30
40
50
60
Concentration (μg/ml)

the dye were conducted in the dark since the dve is light sensitive (Peberdy and Buckley, 1973).

2.6 Radioactive precursor incorporation

(a) protoplasts

UDP-N-acetyl[6-^{3}H]GlcNH$_2$ (5 µCi) (50 µl) was added directly to 10 ml of reversion medium in a 50 ml Erlenmeyer flask containing 1 x 10^9 protoplasts. At specified times, 1.0 ml samples were taken, diluted with 5 ml of reversion medium and centrifuged at 2000 x g for 10 minutes. The washing procedure was repeated twice and an aliquot of the pellet was oxidized (Oxymat) and the incorporated radioactivity was counted as ^3H_2O. A time zero protoplast sample was used as a control.

Glucosamine-6-^{3}H-HCl (10.13 Ci/mmole) (75 µCi) (75 µl) was added to 10 ml of reversion medium in a 50 ml Erlenmeyer flask containing 1 x 10^9 protoplasts. Aliquots (0.5 ml) were treated as described above. A particulate fraction was obtained by sonicating the cell pellet in water (1.5 amps - 5 minutes) and centrifuging (Beckmann L2-65 ultracentrifuge) the resulting homogenate at 101,000 x g for 60 minutes. The high speed pellet was washed twice and an aliquot was oxidized and counted.

The non-reverted protoplasts were isolated at 48 hours by passage of the cells through glass wool which removed the new mycelia. The cells were isolated and treated as described above. A particulate fraction was obtained by homogenization with a motor-driven Potter-Elvehjem type glass homogenizer followed by centrifugation at 40,000 x g for 30 minutes.

(b) wild type mycelia and the slime mutant

Glucosamine-6-^{3}H-HCl (10.13 Ci/mmole)(50 µCi) (50 µl) was added at time zero to 50 ml of growth medium containing conidia (1×10^{9}) or slime cells. After 24 hours, the mycelia were removed by filtration and thoroughly washed with water. After 44 hours, the slime cells were isolated by centrifugation and washed 3 times with growth medium. The growth medium was subjected to exhaustive dialysis against water at 4°C. The mycelium was ground with sand and taken up in water while the slime cells were lyzed in water. The resulting homogenates were centrifuged at 101,000 x g for 60 minutes. The pellets were washed and re-centrifuged twice, the three high speed supernatants were combined and an aliquot of each fraction was oxidized and counted.

(c) conidia

Glucosamine-6-^{3}H-HCl (20.7 Ci/mmole) (50 µCi) (50 µl) was added at time zero to 50 ml growth medium containing 1×10^{9} conidia. At specific times, 5.0 ml aliquots were removed and centrifuged in a clinical centrifuge at top speed for 5 minutes. The supernatant was discarded and the pellet was washed twice with 5 ml water. Particulate fractions were obtained by grinding the conidia with sand and centrifuging the resulting homogenates at 40,000 x g for 30 minutes. The pellets were washed twice with water. The same procedure was used for ethylene glycol treated conidia with the exception that the conidia were washed with normal growth medium.

2.7 Analysis of high speed pellet fractions

(a) acid hydrolysis

Aliquots of pellet fractions (5 x 10^7 protoplasts) were treated with 6-N-HCl in sealed tubes at 100°C for 6 hours. Samples were cooled, diluted with water and centrifuged at 101,000 x g for 60 minutes. The pellets were washed twice with water and the combined supernatants were taken to dryness under reduced pressure and stored *in vacuo* over KOH.

(b) alkaline hydrolysis

Pellet aliquots (5 x 10^7 protoplasts) were treated with 1N KOH at 100°C (boiling water bath) for 1 hour. The samples were cooled and centrifuged at 101,000 x g for 60 minutes for protoplast and slime material and 40,000 x g for 30 minutes for conidial material. The pellets were washed once with 5 ml 1N KOH, and washed three times with 5 ml water.

(c) chitinase hydrolysis

The protoplast pellet aliquots after alkaline hydrolysis were incubated with 5 mg chitinase in a total volume of 1 ml at 37°C for 24 hours. After incubation, 1 ml of ethanol was added and the resulting suspension was centrifuged at 12,000 x g for 15 minutes. The supernatant was taken to dryness under reduced pressure and the residue taken up in a small volume of water for chromatography.

"Chitin" pellets from mycelia (0.25 ml growth medium), conidia (1.67 x 10^8), slime cells (5 ml growth medium), as well as from *in vitro* chitin synthetase assays were incubated with 2.5 mg chitinase in a total volume of 0.5 ml water at

37°C for 24 hours. The incubation mixture was subjected to ultrafiltration through an Amicon-UM 2 membrane to obtain a small molecular weight fraction (less than 1000 M.W.). In some digestions, pellet material was incubated without the enzyme to act as a control. The control samples gave less than 1% of the total starting radioactivity in the small molecular weight fraction.

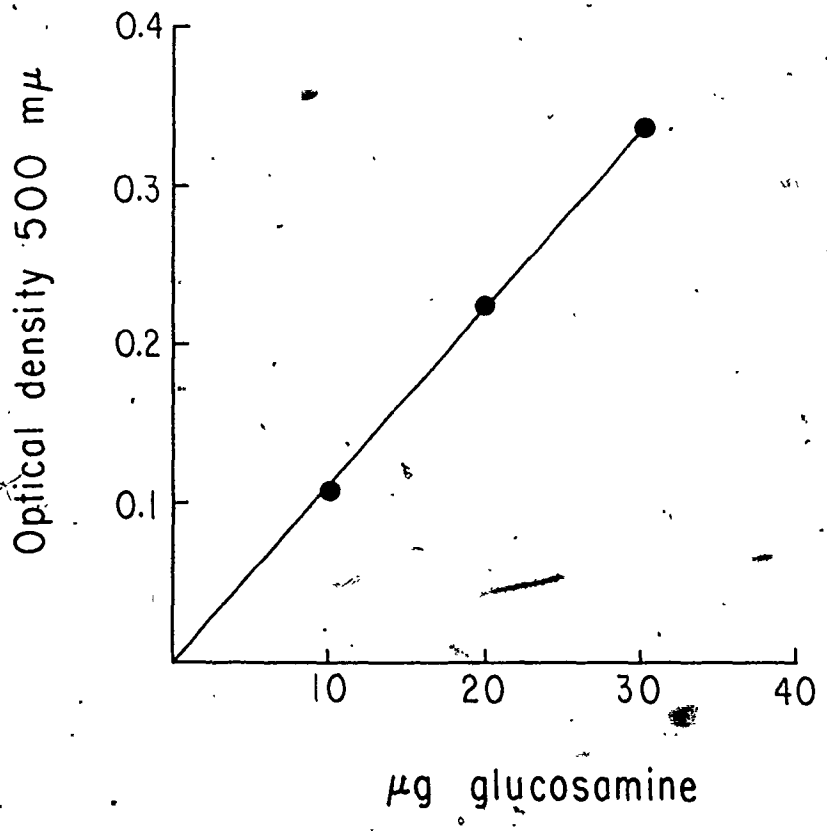
2.8 Determination of glucosamine levels in particulate fractions of wild type mycelia and the slime mutant

Slime cells were lyzed in 3 ml of water while mycelia were put through a French Pressure Cell Press (Aminco, 16,000 psi) with five volumes of water. Particulate fractions were isolated by centrifugation at 101,000 x g for 60 minutes. The pellets were digested with 1N KOH followed by hydrolysis with 6N HCl. The acid hydrolysates were taken up in water and aliquots were spotted on Whatmann 3MM paper with glucosamine standards. After electrophoresis, the quantity of glucosamine was determined by the cadmium-ninhydrin procedure of Atfield and Morris (1961). This procedure involves dipping the dried paper in cadmium-ninhydrin reagent, heating at 100°C for 10 minutes, eluting the developed color with 3 ml methanol for 2 hours and reading the optical density at 500 mμ (Figure 8).

2.9 Electrophoresis

Electrophoresis was carried out using Whatmann 3MM paper (24 x 112 cm) for thirty minutes at 3000 volts (Savant Instruments Inc.) in pyridine-acetic acid-water (1:10:69) buffer, pH 3.5 (Endo et al., 1970a).

Figure 8. Calibration curve for the determination of glucosamine by the cadmium-ninhydrin method of Atfield and Morris (1961).


Optical density 500 mμ
0.4
0.3
0.2
0.1
10
20
30
40
μg glucosamine

2.10 Chromatography

(a) paper chromatography

Chromatography was carried out using Whatmann 3MM paper in paper lined tanks at 22°C in the following solvent systems: A. ascending, 1N NH_4OH/isobutyric acid (3/5); B. ascending, pyridine/1-propanol/acetic acid/water (40/40/15/20); C. descending, pyridine/ethyl acetate/n-butanol/butyric acid/water (10/10/5/1/5).

(b) column chromatography

Dowex I formate was prepared from Dowex I chloride by sequential washing with 0.5 M NaOH, water, 0.5 M formic acid and water. After loading with the slime cell radioactive high speed supernatant (1.0 ml), the column (0.9 x 30 cm) was eluted with 50 ml water followed by a linear gradient of 1N ammonium formate (200 ml water - 200 ml 1N ammonium formate). 5 ml fractions were collected. The salt concentration was estimated using an osmometer (Advanced Instruments Inc.). Standards were prepared by dissolving 5 mg UDP-GlcNAc in 0.5 ml MeOH and adding UDP-N-acetyl[U-^{14}C]$GlcNH_2$ (20 µl). 0.5 ml of 0.1 N HCl was added and the mixture was heated in a boiling water bath for 45 seconds. After cooling, the mixture was loaded onto the column and eluted as described.

The isolated fractions were desalted using a Sephadex G-10 column (1.5 x 90 cm); 2 ml fractions were collected.

The Amberlite MB-3 columns (1 cm x 10 cm) were washed with 100 ml of water prior to use. Samples (1.0 ml) of chitinase digested material were loaded onto the column and eluted

with 100 ml of water to obtain a neutral fraction.

2.11 Detection of radioactivity

A Nuclear Chicago Mark I scintillation counter was used in conjunction with a liquid scintillation system consisting of 10 ml toluene containing 0.4% Omnifluor. For water containing samples, 10 ml of toluene-ethylene glycol monoethylether (10:8) fluor containing 0.4% omnifluor was used. Tritium samples were oxidized to ^3H_2O using an Intertechnique sample oxidizer (Oxymat) and counted with the fluor 700 ml dioxane, 300 ml toluene, 20 gms naphthalene and 7 gms butyl-PBD. These samples were corrected for quenching using the external standard ratios method.

A Panax thin layer radiochromatogram scanning system (RTLS-1A) with a RCMS-3 paper chromatogram scanning attachment was used to detect radioactivity on chromatograms.

2.12 Preparation of colloidal chitin

Colloidal chitin was prepared by the method of Berger and Reynolds (1958). Chitin (10 gms) was made into a paste by grinding in a mortar with acetone. Concentrated HCl (80 ml) was added with more grinding and after 10 minutes, the material was filtered through glass wool into a rapidly stirred solution of 50% ethanol. The precipitate was collected by centrifugation, washed several times with water, dialyzed against tap water over night and dried *in vacuo* over KOH.

2.13 Protein determination

The assay was carried out using the method of Lowry *et al.*

(1951). Five ml of a freshly prepared reagent consisting of 1 ml of 1% $CuSO_4 \cdot 5H_2O$, 1 ml of 2% sodium tartrate and 100 ml of 2% Na_2CO_3 in 0.1 N NaOH was added to 1.0 ml of sample and mixed. After 10 minutes, 0.5 ml of freshly prepared 1N Folin-Phenol reagent was added with mixing, the developed color was measured at an optical density of 750 mμ after 30 minutes. Bovine serum albumin was used as a standard.

2.14 Preparation of extracts for enzyme assays

(a) L-glutamine D-fructose-6-PO_4 transamidase

Isolated slime cells (from 50 ml growth medium) were lyzed in three volumes of 0.05 M Tris buffer, pH 7.5 while wild type mycelia (from 50 ml growth medium) were ground with sand and taken up in three volumes of the same buffer. High speed supernatant fractions were obtained by centrifugation at 101,000 x g for 60 minutes. The homogenate from mycelia was centrifuged at 2000 x g prior to high speed centrifugation. All procedures were carried out at 0°-4°C.

(b) chitin synthetase

Isolated slime cells (from 50 ml growth medium) were lyzed in 3 ml of 0.05 Tris buffer, pH 7.5 containing 10 mM $MgCl_2$ and 1 mM EDTA while protoplasts were lyzed in 0.3-1.0 ml of the same buffer. Wild type mycelia (from 50 ml growth medium) were ground with sand and taken up in 10 ml of the same buffer. Conidia were taken up in 3 ml of buffer and put through the French Press 3 times. Reverted protoplasts (48 hours) were ground with sand and taken up in 2 ml of buffer. Non-reverted protoplasts (48 hours) were homogenized in 1 ml of buffer using a motor-driven Potter-Elvehjem type glass homogenizer. All procedures were carried out at 0°-4°C.

2.15 Enzyme assays

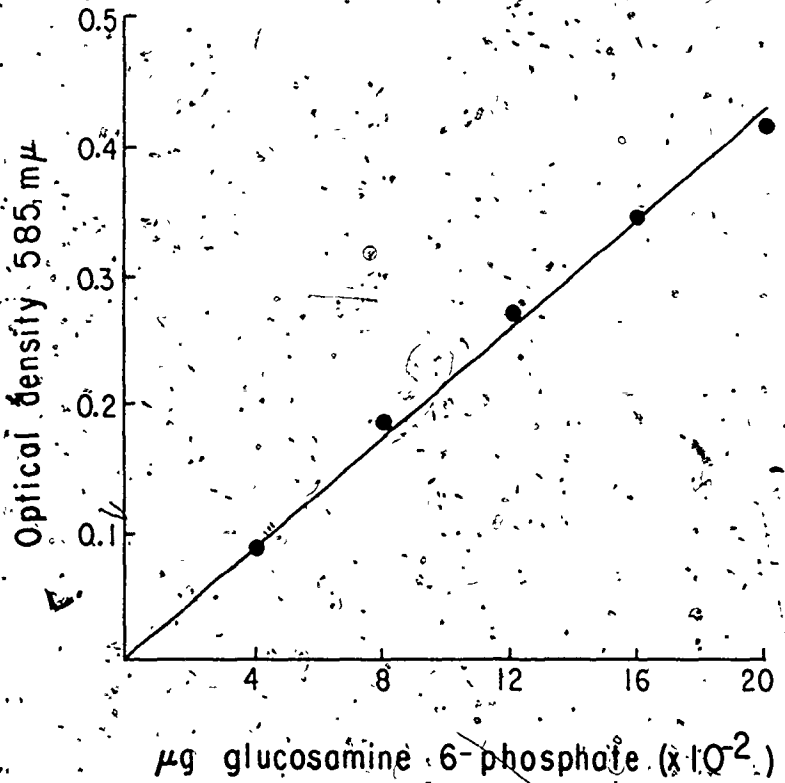
(a) L-glutamine D-fructose-6-PO_4 transamidase

The assay was carried out as described by Endo et al. (1970b). The incubation mixture contained 6 mM fructose-6-PO_4, 9 mM L-glutamine, 2 mM EDTA, 30 mM PO_4 buffer, pH 6.6 and enzyme in a total volume of 0.5 ml. The mixture was incubated at 30°C for 30 minutes, the reaction was stopped by placing the tubes in a boiling water bath for 1 minute.

Glucosamine-6-PO_4 was assayed colorimetrically by the Morgan-Elson method described by Ghosh et al. (1960). 50 µl of saturated $NaHCO_3$ solution was added to all tubes at room temperature. 50 µl of cold 5% aqueous acetic anhydride was added (this reagent was made up just prior to use in the cold) the tubes vortexed and left at room temperature for 3 minutes. The tubes were then placed in a boiling water bath for 3 minutes; after cooling, 100 µl of 0.8 M Na_2BO_3 (pH 9.0) was added and the tubes placed in a boiling water bath for 3 minutes. After cooling to room temperature, 3 ml of Ehrlich's reagent (1 gm diaminobenzaldehyde, 1.25 ml of 10 N HCl and acetic acid to a total volume of 100 ml) was added; the tubes were vortexed and incubated at 37°C for 20 minutes. After cooling to room temperature, the optical density was read at 585 mµ. Glucosamine-6-PO_4 was used to prepare the standard curve (Figure 9).

One unit of activity is defined as the amount of enzyme catalyzing the formation of one µ mole of glucosamine-6-PO_4 in one minute under the above conditions.

Figure 9. Calibration curve for the determination of glucosamine-6-PO_4 by the Morgan-Elson method described by Ghosh et al., (1960).


0.5
0.4
0.3
0.2
0.1
Optical density 585 mμ
4
8
12
16
20
μg glucosamine 6-phosphate (x 10⁻²)

(b) chitin synthetase

The reaction mixture contained 50 mM Tris buffer, pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, 0.1 mM UDP-N-acetyl[U-^{14}C] $GlcNH_2$ (95,000 cpm) and enzyme in a total volume of 75 µl. After incubation at 25°C for a specified time, the reaction was stopped by dilution with 10 ml of cold water and the mixture filtered through Whatmann 3MM paper (2 cm diameter) supported by a 25 mm plastic filter. The filter was washed with 150 ml water and 50 ml acetone and dried under a heat lamp for 30 minutes before counting.

One unit of activity is defined as the amount of enzyme catalyzing the incorporation of one pmole of GlcNAc into the particulate fraction in one minute under the above conditions.

2.16 Trypsin activation

The homogenates (25 µl) were incubated at 25°C for 15 minutes in the presence of small amounts of trypsin (0-5 µg) in a total volume of 50 µl of 50 mM Tris buffer, pH 7.5, 10 mM $MgCl_2$ and 1 mM EDTA. After incubation, the reaction was stopped by adding double the theoretical quantity of soybean trypsin inhibitor in the same buffer.

2.17 Glycogen determination

Glycogen was estimated using the method described by Hufjing (1970). Protoplast samples were diluted 10-100 fold and boiled for three minutes to inactivate endogenous invertase activity (since excess sucrose was present) prior to assay. 0.2 ml of material was mixed with 0.2 ml of a solution containing α-glucosidase (50 µg/ml) and pancreatic

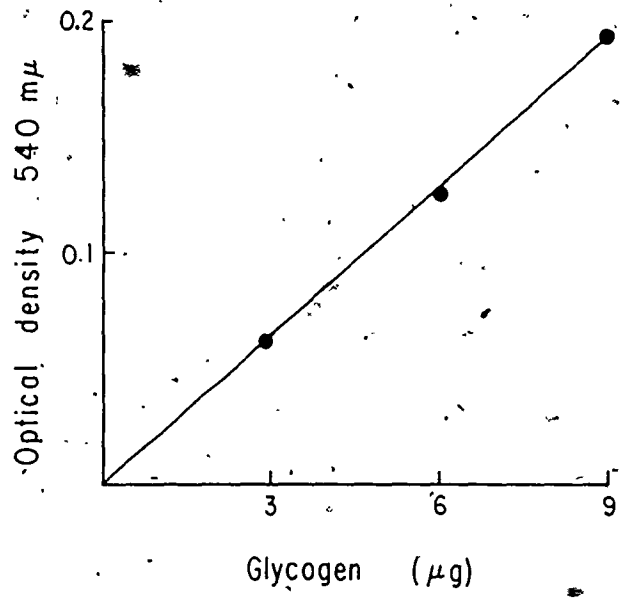
α-amylase (80 µg/ml) in 0.1 M sodium acetate buffer, pH 4.8. A control tube contained 0.2 ml sample and 0.2 ml buffer in place of the enzyme solution. The mixtures were incubated at 30°C for one hour. Released glucose was determined by the addition of 0.4 ml of a solution containing glucose oxidase (0.5 ml), peroxidase (3 mg) and o-dianisidine hydrochloride (10 mg) in 100 ml of Tris-phosphate-glycerol buffer (36.3 g Tris, 50 g $NaH_2PO_4.H_2O$ and 400 ml glycerol made up to one liter of H_2O, pH adjusted to pH 7.0 with solid $NaH_2PO_4.H_2O$). The mixture was incubated at 30°C for one hour before the addition of 5N HCl (0.8 ml). The optical density was measured at 540 mµ. Shell fish glycogen was used to prepare the standard curve (Figure 10).

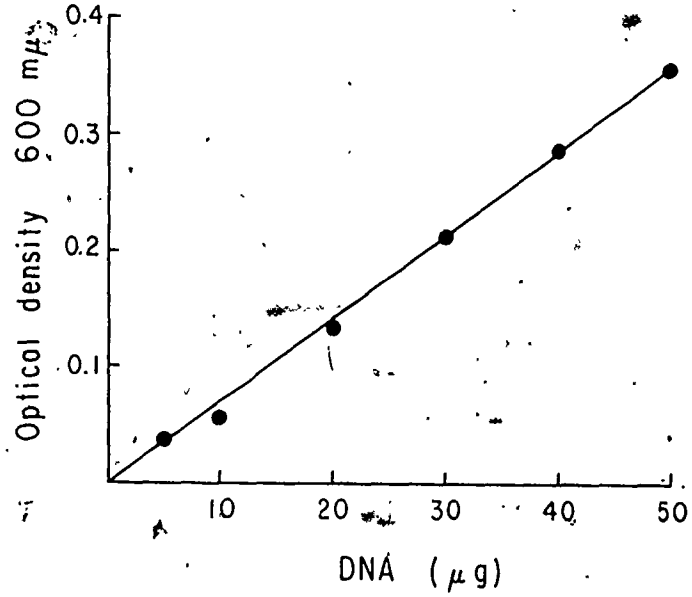
2.18 DNA determination

The DNA was isolated by the method described by Schneider (1957). One ml of the lyzed protoplast preparation was mixed with 2.5 ml of cold 10% TCA and centrifuged (clinical centrifuge). The precipate was washed once with 2.5 ml of cold 10% TCA and extracted twice with 5 ml 95% ethanol. RNA was removed by incubation of the precipitate with 2 ml 1N KOH at 37°C for 20 hours. The DNA was precipitated by the addition of 0.4 ml 6N HCl and 2 ml of 5% TCA. After centrifugation, the DNA was brought into solution by the addition of 0.5 ml H_2O and 0.5 ml 10% TCA and heating at 90°C for 15 minutes. The remaining precipitate was removed by centrifugation and washed with 1.0 ml 5% TCA. The resulting supernatants were

Figure 10. Calibration curve for the determination of glycogen using the procedure of Huijing (1970).

Figure 11. Calibration curve for the determination of DNA using the procedure of Burton (1956).


0.2
0.1
Optical density 540 mμ
3
6
9
Glycogen (μg)


0.4
0.3
0.2
0.1
Optical density 600 mμ
10
20
30
40
50
DNA (μg)

combined for the DNA assay as described by Burton (1956). Two ml of diphenylamine reagent was added to 1 ml of supernatant and incubated at 30°C for 20 hours. The optical density was taken at 600 mμ. The diphenylamine reagent consisted of diphenylamine (1.5 g) and concentrated H_2SO_4 (1.5 ml) in a total volume of 100 ml of glacial acetic acid. Aqueous acetaldehyde (0.1 ml) was added to 20 ml of reagent prior to use. Calf thymus DNA was used to prepare a standard curve (Figure 11).

2.19 Statistical analysis

In most cases data was presented as the mean of at least three separate determinations with the standard error of the mean.

CHAPTER III FORMATION AND REVERSION OF NEUROSPORA CRASSA PROTOPLASTS

The ability of enzyme preparation suc d'Helix pomatia, commonly known as "snail gut enzyme", to release protoplasts from Neurospora crassa mycelia or conidia has been known for some time (Bachmann and Bonner, 1959; Kinsky, 1962; Trevithick and Metzenberg, 1964; Manocha, 1968). Protoplasts have also been obtained by growing an osmotic strain of the same fungus in the presence of ten percent sorbose (Emerson and Emerson, 1958; Hamilton and Calvet, 1964). The enzyme β-glucuronidase (Helix pomatia) releases spherical cells from Neurospora crassa mycelia but since the wall remaining on these cells has not been examined, the term spheroplast has been used to describe them (Scarborough and Schulte, 1974). Since the "snail gut" procedure appeared to be the superior method, it was chosen in this study. A number of variables were examined in the procedure in an effort to optimize the yield of protoplasts obtained from mycelia.

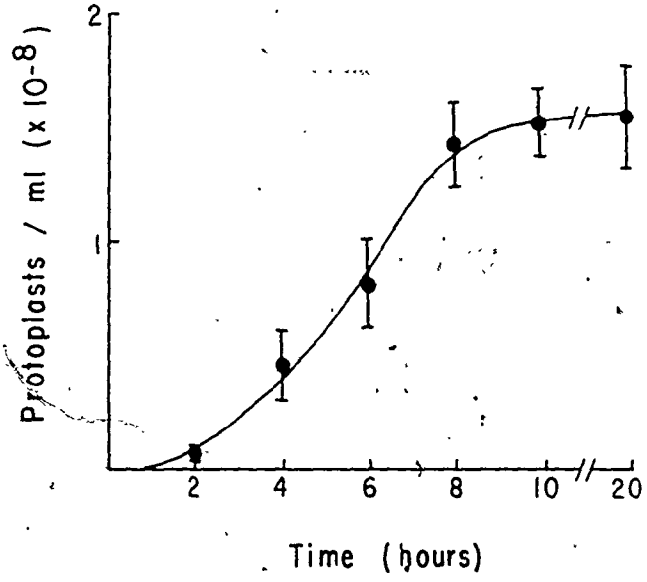
RESULTS

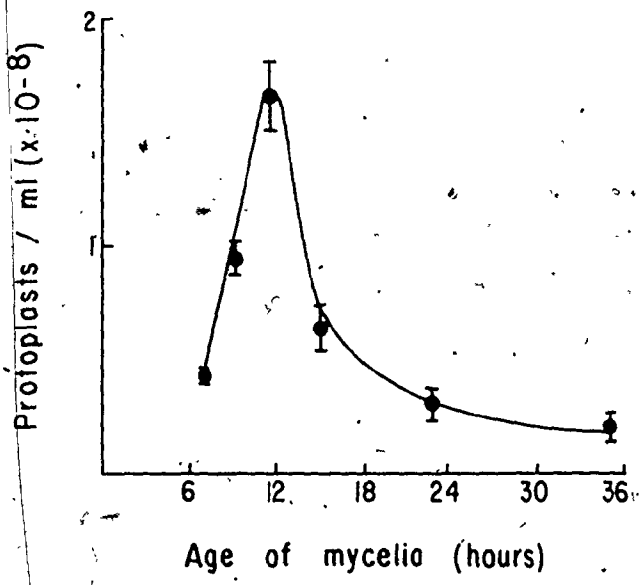
3.1 Formation of protoplasts

Figure 12 shows that an increasing number of protoplasts were released during the first ten hours of incubation. After that time, protoplast numbers remained constant for up to twenty hours. The age of the starting mycelia was found to be the critical factor in terms of yield. As shown in Figure 13, a sharp optimum occured with mycelia which were twelve hours old.

Figure 12. Time course of the release of protoplasts from Neurospora crassa mycelia (12 hours old) by "snail gut" enzyme.

Figure 13. Effect of the age of Neurospora crassa mycelia on protoplast yield. The yield was determined after a 15 hour digestion with "snail gut" enzyme.


Protoplasts / ml (x 10-8)
2
1
2
4
6
8
10
20
Time (hours)


Protoplasts / ml (x·10-8)
2
1
6
12
18
24
30
36
Age of mycelia (hours)

Examination of the treated hyphae using phase contrast microscopy indicated that protoplast release left behind "empty" compartments outlined by cell wall material (Figure 14). Using these "empty" compartments as markers for protoplast release, it was found that after two hours of digestion, 77.02 ± 8.18% of the protoplasts originated from the apical regions of the mycelia. As digestion proceeded, the protoplasts were released from the older regions of the hyphae.

Under the optimal conditions described, the yield was one hundred and sixty percent of the number of starting conidia. This procedure resulted in the production of a sufficient number of protoplasts for study.

3.2 Properties of the protoplasts

The protoplasts released were spherical in form, some with one large vacuole or numerous small vacuoles; others with no vacuoles (Figure 15a). All protoplasts were osmotically sensitive (Figure 15b). Electron micrographs of these forms indicated that cell wall was absent on most cells except in a small number of cases where small areas of cell wall residue remained (Figure 16).

A more convenient method of determining the presence of cell wall material utilizes the fluorescent brightener Calcofluor White M 2R New as described by Gull et al. (1972). This material binds only to β linked polysccharides (Meada and Ishida, 1967) and is an extremely sensitive technique for detecting cell wall material. Figures 18 and 19 illustrate

Figure 14. Phase contrast micrograph of a Neurospora crassa mycelium after a two hour digestion with "snail gut" enzyme showing the "empty" apical region. (magnification 2600 x)

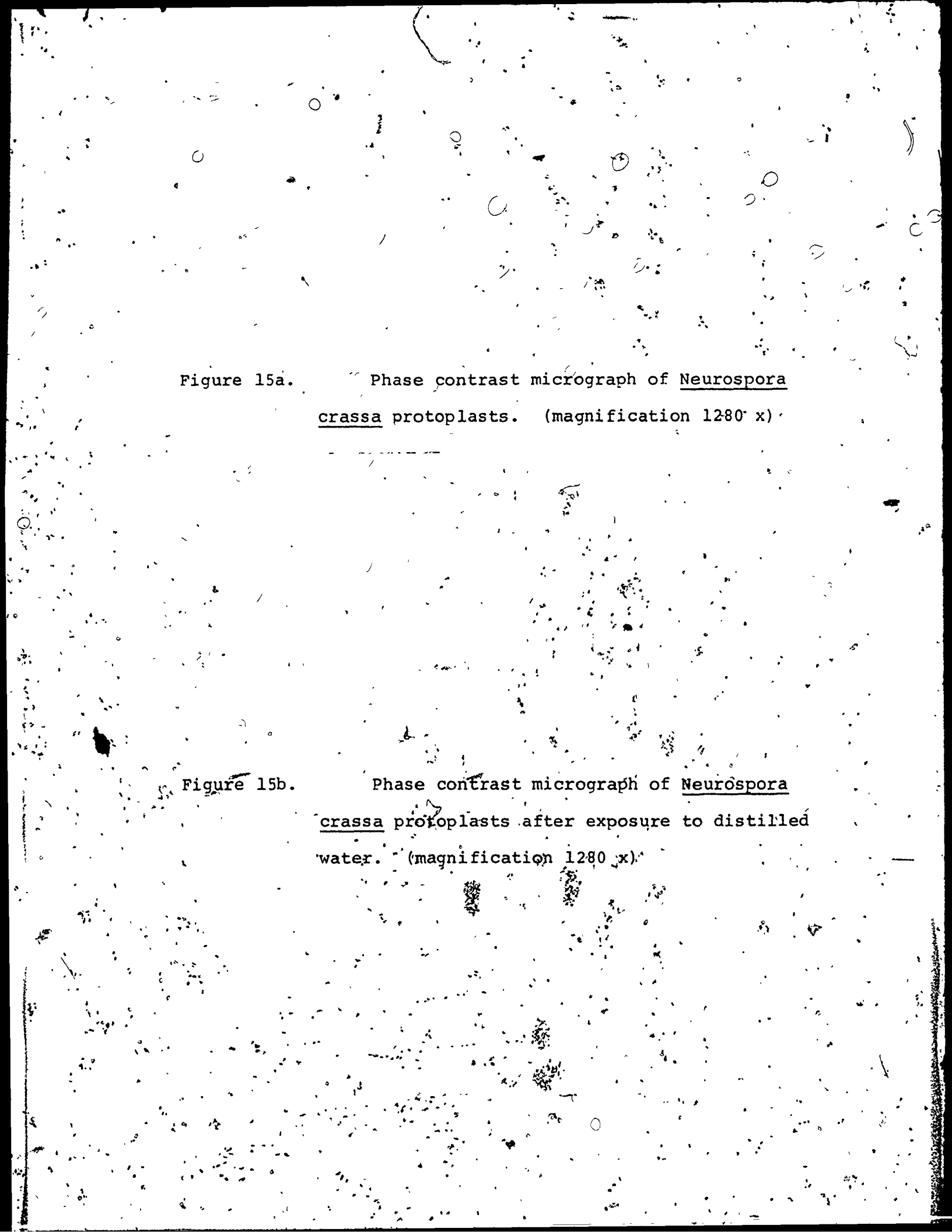
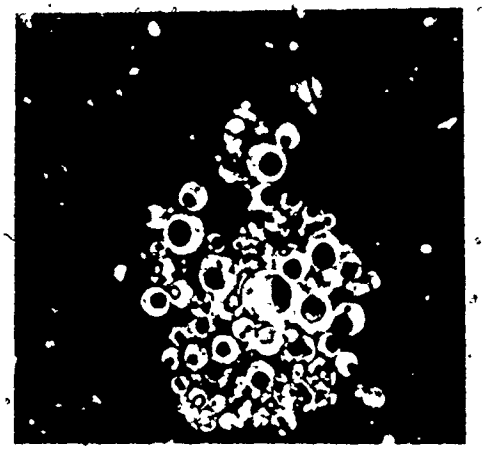


Figure 15a. Phase contrast micrograph of Neurospora crassa protoplasts. (magnification 1280 x)

Figure 15b. Phase contrast micrograph of Neurospora crassa protoplasts after exposure to distilled water. (magnification 1280 x)



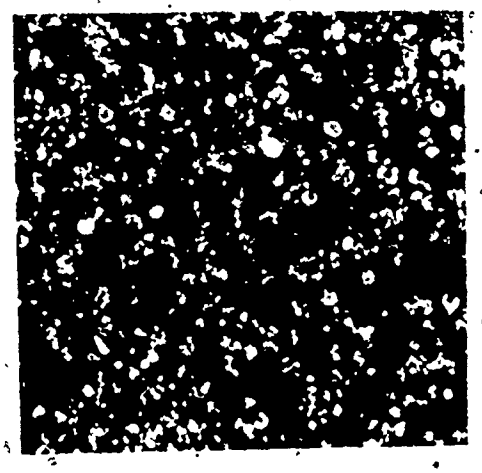
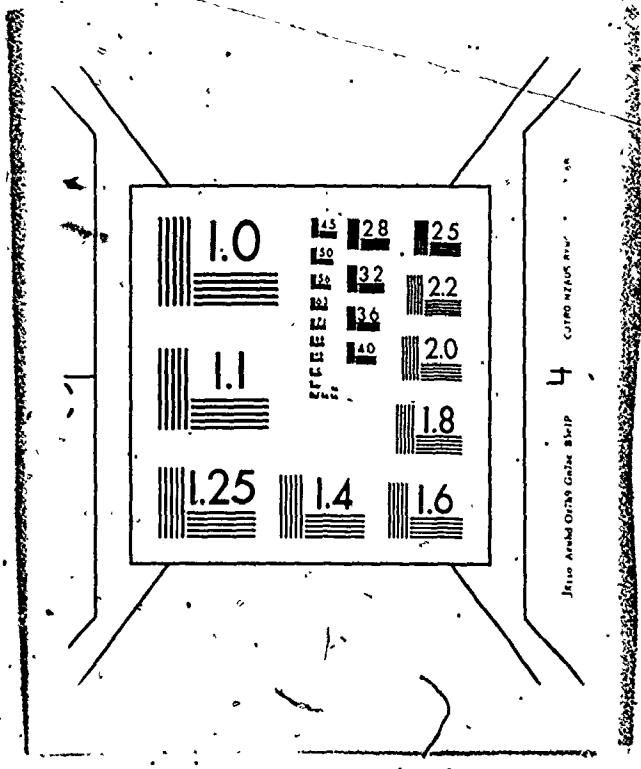


Figure 16. Electron micrograph of a Neurospora crassa protoplast. The arrow points to an area where two small "spots" of wall material remain. (magnification 28,000 x)

Figure 17. Electron micrograph of an eight hour reverted Neurospora crassa protoplast. (magnification 28,000 x)

2



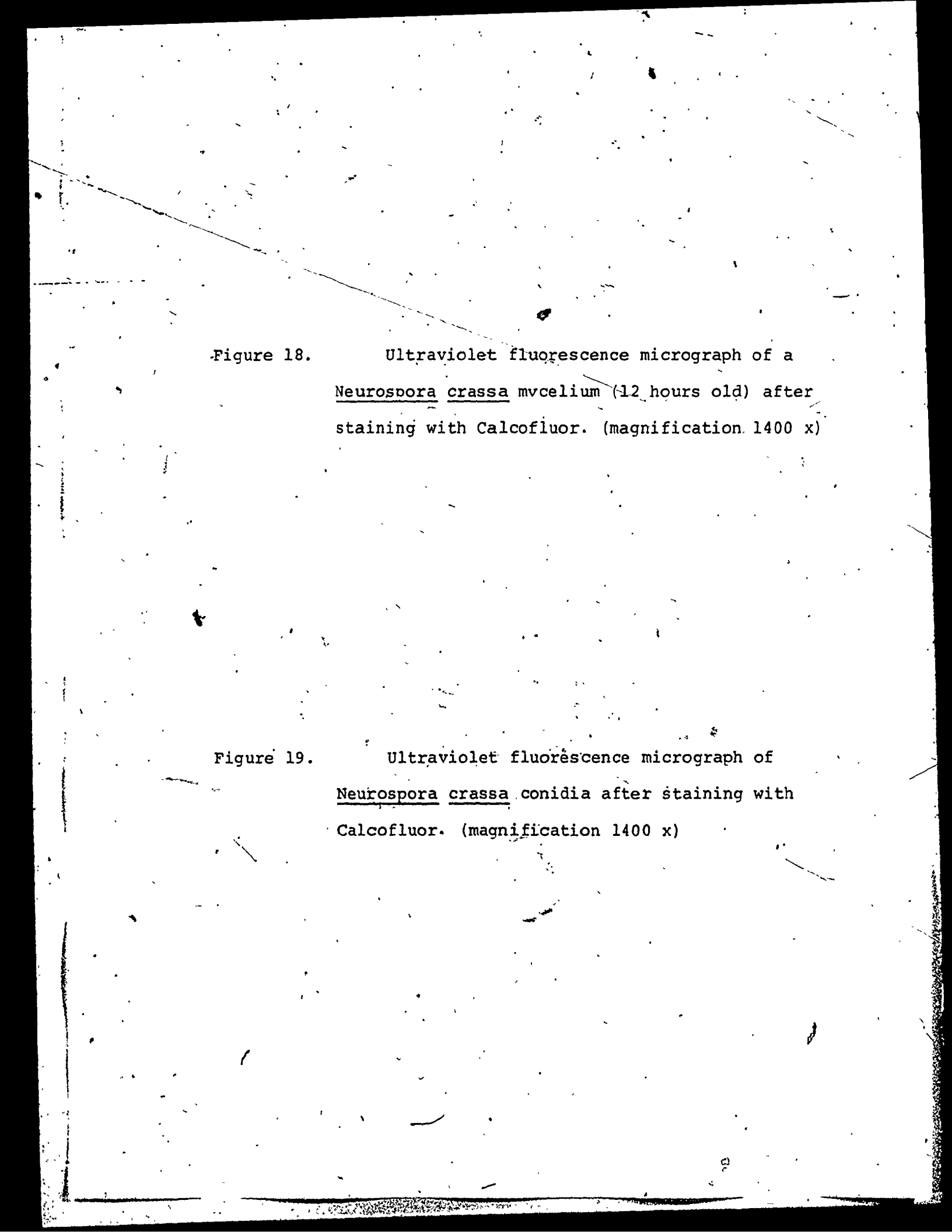
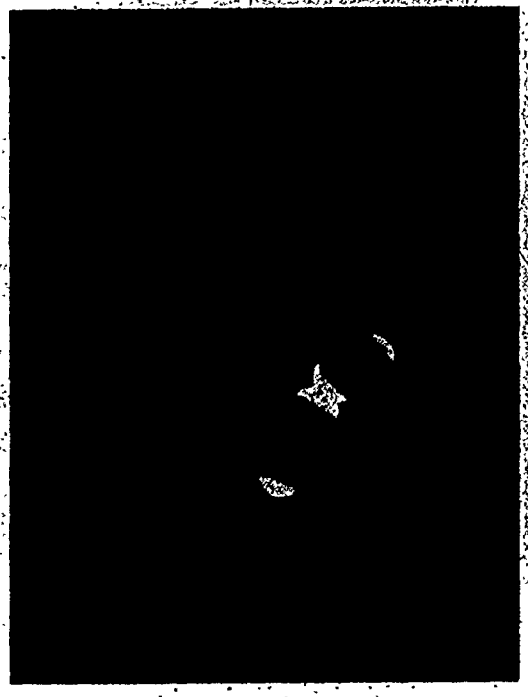
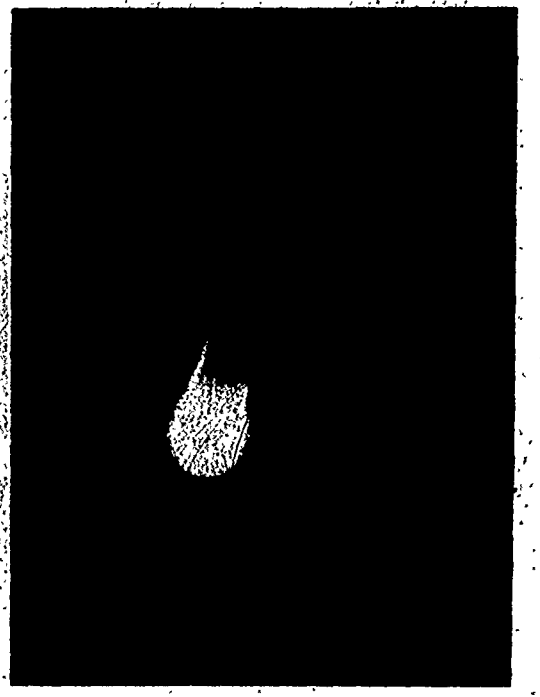


Figure 18. Ultraviolet fluorescence micrograph of a Neurospora crassa mycelium (12 hours old) after staining with Calcofluor. (magnification 1400 x)

Figure 19. Ultraviolet fluorescence micrograph of Neurospora crassa conidia after staining with Calcofluor. (magnification 1400 x)





the sensitivity of this method using growing mycelia and conidia. The strongest fluorescence was located at the apical and septal regions; non-apical regions showed a much weaker fluorescence. The majority of the conidia gave a bipolar fluorescence.

Using this technique approximately eighty percent of the protoplasts did not show fluorescence, six percent showed "spot" fluorescence and thirteen percent showed internal fluorescence; possibly an indication of membrane damage (Table 2). The "spot" fluorescence probably corresponds to small areas of cell wall remaining on the cell surface as shown in the electron micrographs.

3.3 Regeneration and reversion of protoplasts

The centrifugation procedure used to isolate the protoplasts gave low yields (twenty percent). Attempts to significantly increase the recovery by gradient centrifugation (sucrose, ficoll) failed, probably due to the heterogeneous size and density of the protoplast population. When the isolated protoplasts were stained with Calcofluor, a larger percentage (28%) showed "spot" fluorescence as compared to the starting suspension of protoplasts (7%) (Table 2).

Within thirty minutes of incubation in reversion medium, the protoplasts underwent aggregation to form "clumps" visible to the naked eye. Germ tubes started to appear at approximately six hours; the number increasing gradually until twenty-four hours where a synchronous emergence of germ tubes was

Table 2. Calcofluor White M 2R New staining properties of Neurospora crassa protoplasts.

Protoplast sample	Fluorescent* (%)	Nonfluorescent (%)	Internal fluorescence (%)
total	6.5 ± 1.4	79.9 ± 11.0	13.5 ± 10.1
isolated (2000 x g)	27.7 ± 8.8	62.5 ± 13.3	9.7 ± 7.3

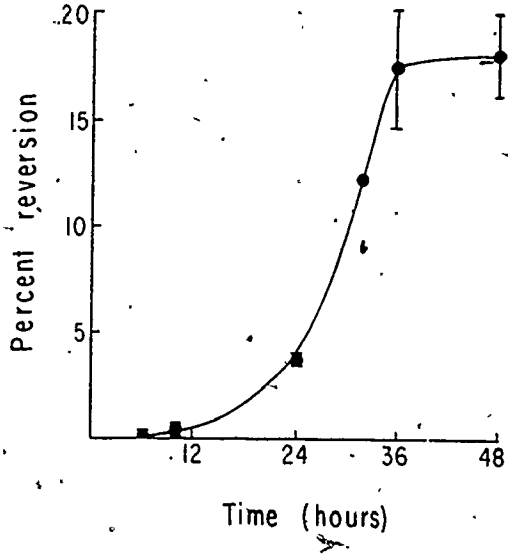
* refers to "spot" fluorescence

evident (Figure 20). Approximately twenty percent of the protoplasts produced germ tubes; they arose directly from the spherical cells but occasionally the germ tube arose from a bud-like structure formed by the protoplast (Figure 22a-c).

Prior to the formation of the germ tube, the protoplasts encysted by forming a wall-like polysaccharide at the cell surface. The encysted cells were osmotically stable. The encystment process could be conveniently followed by staining with Calcofluor. The protoplasts underwent synchronous encystment after one to two hours incubation in reversion medium. Sixty percent of the protoplasts formed this wall-like polysaccharide (Figure 21). The fluorescence at time zero and at one hour was "spot" fluorescence whereas at two hours the protoplasts showed a complete ring of fluorescence around the entire periphery. The protoplasts exhibiting internal fluorescence did not encyst (Figure 21). Electron micrographs of eight-hour regenerated protoplasts confirmed the presence of wall-like material (Figure 17). The synthesis of this polysaccharide could be followed directly by examining the amount of the fluorescent dye Tinopal BOPT which was taken up during regeneration (Peberdy and Buckley, 1973). This dye was taken up one hour after the start of the reversion sequence and continued to be taken up for at least eight hours. The amount of dye absorbed began to level out after six hours of regeneration as the amount of dye absorbed approached the levels of dye added to the system (Figure 23).

Figure 20. Time course of the reversion of Neurospora crassa protoplasts as determined by the percentage of protoplasts forming germ tubes.

Figure 21. Initiation of regeneration of Neurospora crassa protoplasts as determined by fluorescence after staining with Calcofluor.
protoplasts exhibiting internal fluorescence ⊙
protoplasts exhibiting external fluorescence ●


Percent reversion
20
15
10
5
12
24
36
48
Time (hours)

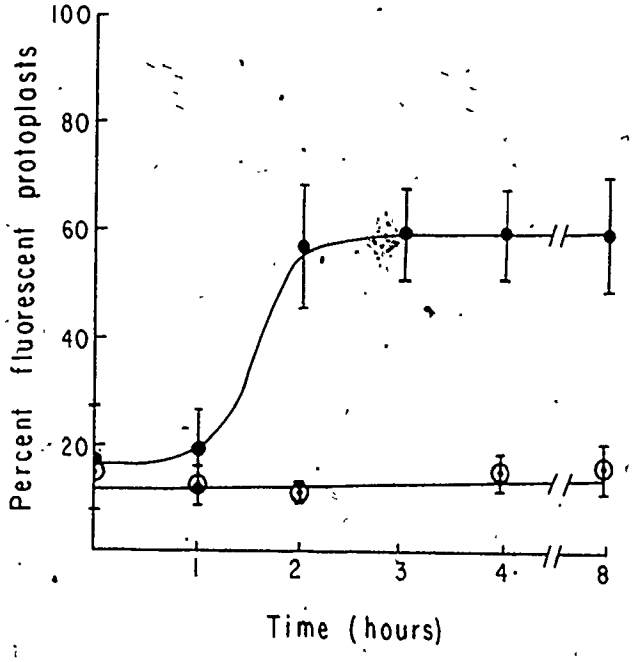

Percent fluorescent protoplasts
100
80
60
40
20
1
2
3
4
8
Time (hours)

Figure 22a,b,c. Ultraviolet fluorescence micrographs of eight hour reverted Neurospora crassa protoplasts (encysted) after staining with Calcofluor. (magnification 1400 x)

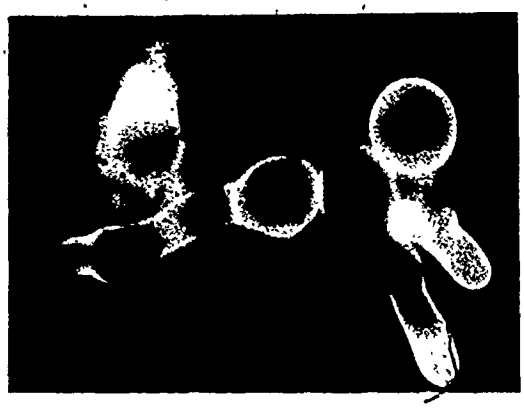
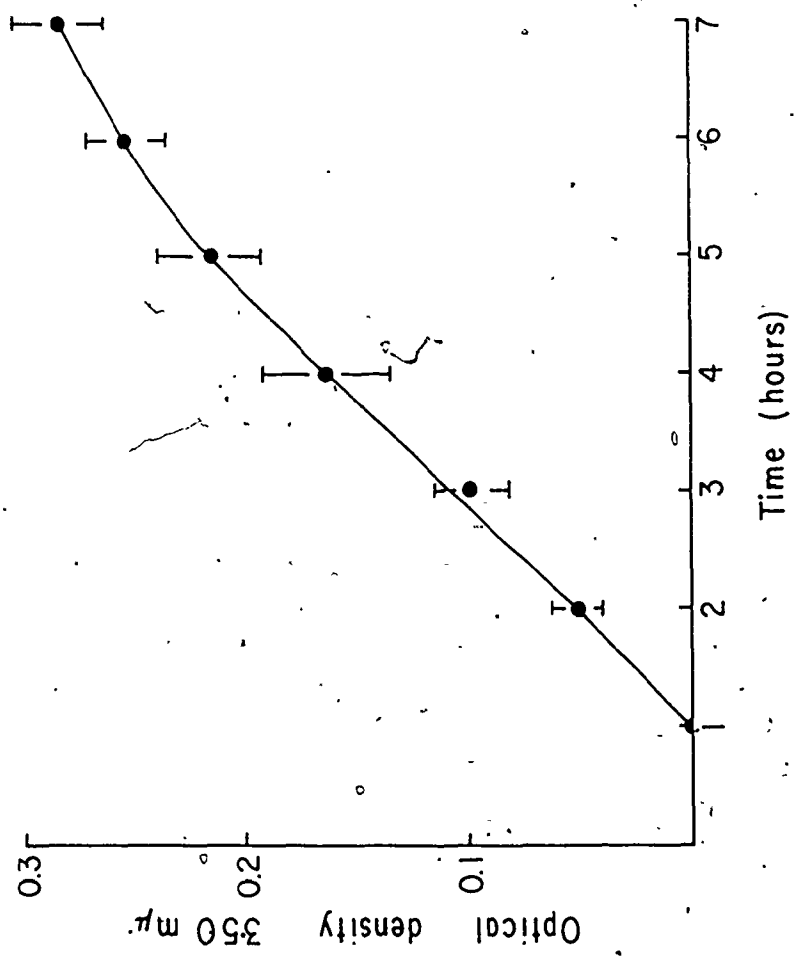


Figure 23. Uptake of Tinopal BOPT by regenerating *Neurospora crassa* protoplasts as a function of time.


Optical density 350 mμ
0.3
0.2
0.1
1
2
3
4
5
6
7
Time (hours)

DISCUSSION

The "snail gut" digestion procedure used in this study released much larger quantities of protoplasts than previously described for Neurospora crassa (Kinsky, 1962). The larger yields were a result of the use of young mycelia. The release of protoplasts from mycelia of different ages gave an extremely sharp optimum with twelve hour old mycelia. The increased resistance of growing mycelia to the enzymatic digestion suggests that "snail gut" resistant compounds were formed in older mycelia. Although components of the cell wall of Neurospora crassa have been defined (Mahadevan and Tatum, 1965), very little is known about their synthesis as related to mycelial age. The galactosamine polymers in the wall are produced ten hours after germination (Schmit et al., 1975) and would be one of the likely candidates whose resistance to hydrolysis by the mixture of enzymes present in "snail gut" juice might explain the increased resistance of older mycelia. The age of the mycelia has also been found to be a factor influencing protoplast release in Geotrichum candidum (Dooijewaard-Kloostezeil et al., 1973), Schizophyllum commune (de Vries and Wessels, 1972) and Aspergillus flavus (Peberdy et al., 1976).

The spherical forms released can be regarded as protoplasts since they satisfy the criteria for protoplasts put forth by Brenner et al. (1958). They are sensitive to osmotic shock and devoid of cell wall residues as determined

by electron microscopy and fluorescent staining techniques. A very small portion of the protoplasts did show isolated "spots" of wall residue as determined by these two techniques. The protoplasts consisted of a heterogeneous population in terms of size and occurrence of vacuoles. Peberdy (1972) has suggested that the large vacuolar protoplasts arise from the mature areas of the mycelia where large vacuoles are normally found.

The fluorescent brightener (Calcofluor) procedure was found to be a sensitive technique in detecting cell wall polymers in mycelia, conidia and protoplasts. The apex and septal regions of mycelia showed much stronger fluorescence than other areas of the mycelia. The wall polymer chitin forms the greatest proportion of the wall in both these areas (Hunsley and Kay, 1976; Hunsley and Gooday, 1974). Similar fluorescent characteristics of mycelia have been observed in Geotrichum lactis (Gull et al., 1972) and Trichoderma viride (Benitez et al., 1976). The conidia showed strong fluorescence at both poles suggesting a structural difference in the wall at these positions. The pole regions have been found to have "collars" which represent interconidial attachment sites (Searle, 1973) which probably correspond to the fluorescent areas. Some of the protoplasts showed an apparent internal fluorescence after treatment with Calcofluor. Gull et al. (1972) suggested that Calcofluor did not stain intracellular carbohydrate due to the fact that it cannot permeate the plasma membrane. Possibly, the internal fluorescence was a

result of damage to the plasma membrane. This was supported by the fact that these forms were unable to regenerate a wall.

The protoplasts underwent two distinct morphological events prior to the appearance of the germ tube; these were aggregation and encystment. The polymers formed at the plasma membrane during the encystment stage could have been the cause of aggregation. In order to investigate this possibility, the appearance of the encystment polysaccharides were followed with time using the fluorescent dyes Calcofluor and Tinopal. These polysaccharides began to appear at least one hour after incubation of the protoplasts in reversion medium while aggregation of the protoplasts was apparent in the first thirty minutes of incubation. Therefore, subject to the sensitivity of the fluorescent dye methods, it was concluded that the encystment polysaccharides were not responsible for aggregation. In a related study with a "natural" protoplast system, Phytophthora palmivora zoospore encystment, the zoospores became adhesive prior to the synthesis of glucan encystment material (Sing and Bartnicki-Garcia, 1972). Sing and Bartnicki-Garcia (1975a, 1975b) showed that the adhesive phase was the result of secreted glycoprotein.

The protoplasts in the present study reverted to hyphal growth by directly forming germ tubes from the encysted protoplast. Occasionally, the protoplasts formed a bud-like structure from which the germ tube developed. In contrast, Bachmann and Bonner (1959) found that a large proportion of

Neurospora crassa protoplasts reverted on solid media to form a chain of yeast-like cells. Reversion by the formation of a chain of yeast-like cells was the major form of development in other fungal protoplast systems (Garcia Acha et al., 1966; Peberdy and Gibson, 1971; Benitez et al., 1975).

A large proportion of the protoplasts (40%) were unable to form new wall polymers; a larger proportion (80%) were unable to revert to the hyphal form of growth. Since germ tubes were formed only from encysted protoplasts, it seemed that encystment was a prerequisite of germ tube formation. If the initial formation of a bud or germ tube depends on the internal turgor pressure (Robertson, 1968), then, perhaps in physical terms, this phenomena requires a rigid form to allow the force to develop in one direction.

The presence of a rigid form is not the only requirement for reversion since only a small proportion of the encysted forms (33%) could revert to form hyphae. The inability of most protoplasts to revert is a common feature of all protoplast systems examined (Sietsma and DeBoer, 1973; Dooijewaard-Kloosterziel et al., 1973; de Vries and Wessels, 1975). Numerous reasons for the lack of protoplast regenerative ability have been expressed. In yeast systems (Necas, 1971), the importance of cell wall residues remaining on the protoplast to act as "primers" for new polymer synthesis has been argued for some time. In this study, the majority of the protoplasts (62%) had no gross wall components as determined by electron microscopy and fluorescent dye studies yet,

60% were capable of regenerating a new cell wall indicating that "primer" carbohydrate was not required for regeneration of the wall. However, Van der Valk and Wessels (1976) have found that S. commune protoplasts still have "plasma membrane carbohydrate" associated with them which could act as "primer" material.

The study of cell wall biosynthesis in protoplast systems should uncover important aspects of the role of cell wall polymers in fungal morphogenesis.

CHAPTER IV CHITIN SYNTHESIS DURING NEUROSPORA CRASSA PROTOPLAST REVERSION

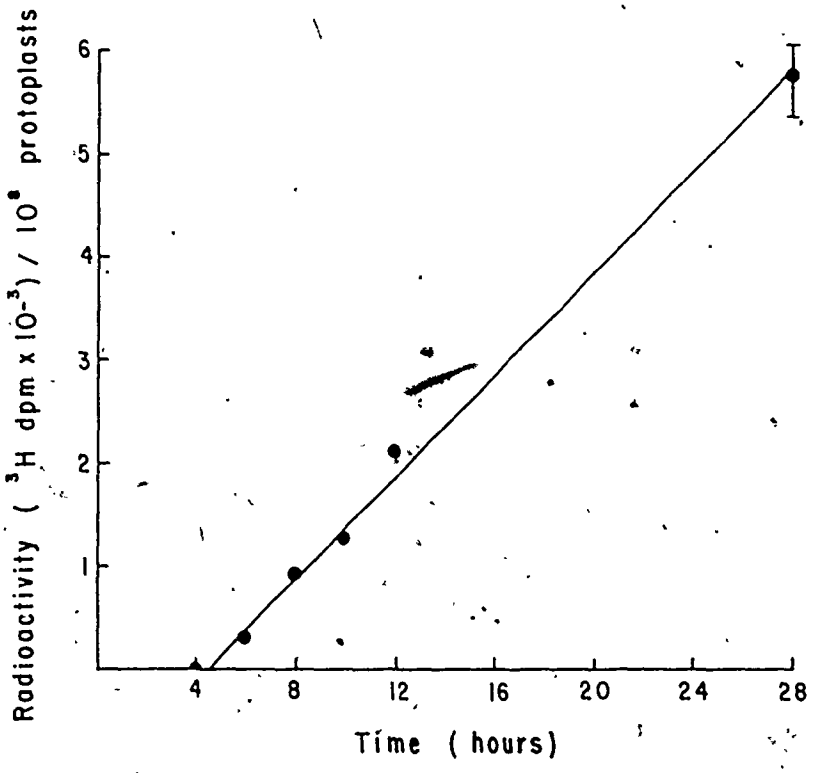
The reversion of Aspergillus nidulans protoplasts consisted of two phases: formation of a chain of yeast-like cells followed by the formation of a hypha from the last cell (Peberdy and Gibson, 1971). The transition time from the first phase to the second was considerably shortened by the presence of GlcNAc in the reversion medium (Peberdy and Buckley, 1973). Benitez et al. (1975) found that the regenerated wall formed in the first phase of Trichoderma viride protoplast reversion, had only relatively small quantities of chitin (approximately 10% of normal hyphal levels). These results indicated that chitin synthesis did not have any function in the first phase of reversion but apparently had some role in the formation of the hypha from protoplasts. Chitin synthesis in the Neurospora crassa protoplast system was examined to determine the role of this polymer in the reversion process.

RESULTS

4.1 Incorporation of UDP-N-acetyl[6-^{3}H]GlcNH$_2$ and [^{3}H] glucosamine by reverting protoplasts

In order to investigate chitin synthesis during protoplast reversion, known precursors of chitin were added to the reversion system. Figure 24 shows that the uptake of radioactivity from UDP-N-acetyl[6-^{3}H]GlcNH$_2$ had at least a five hour lag period. Examination of the reversion medium after twelve

Figure 24. Uptake of UDP-N-acetyl[6-^{3}H]GlcNH$_2$ as a function of time during Neurospora crassa protoplast reversion.


Radioactivity (^3H dpm x 10^{-3}) / 10^6 protoplasts
1
2
3
4
5
6
4
8
12
16
20
24
28
Time (hours)

hours by Sephadex G-10 chromatography indicated that the substrate had broken down into at least two smaller components. Therefore, the precursor was changed to [^{3}H]glucosamine. The major portion of the tritium taken up in this case became associated with the 101,000 x g pellet (Figure 25). Although the [^{3}H]glucosamine was taken up without a lag period, the radioactivity did not appear in the particulate fraction until six hours after addition of the precursor.

4.2 Examination of the high speed pellet after [^{3}H] glucosamine incorporation

The radioactivity in pellet fractions taken at 24 and 36 hours of the reversion sequence was resistant to 1N KOH digestion and sensitive to acid or chitinase hydrolysis (Table 3). The acid hydrolysates contained only one radioactive spot which migrated the same distance as standard glucosamine on electrophoresis (Figure 26). The products of chitinase hydrolysis migrated with the same R_f as GlcNAc in chromatography system A (Figure 27). On the basis of these properties, the largest proportion (80%) of the radioactive pellet was identified as chitin.

The specific activity, i.e. the radioactivity per µg glucosamine, of the high speed pellet remained constant over the first 48 hours of the reversion sequence (Table 4), hence the amount of radioactivity incorporated during reversion was directly proportional to the amount of chitin deposited. A plot of the degree of reversion (setting the amount of reversion at 36 hours equal to one) versus the relative amount

Figure 25. Incorporation of [^{3}H]glucosamine as a function of time during Neurospora crassa protoplast reversion.

total incorporation ● ___

101,000 x g pellet ⊙ ---

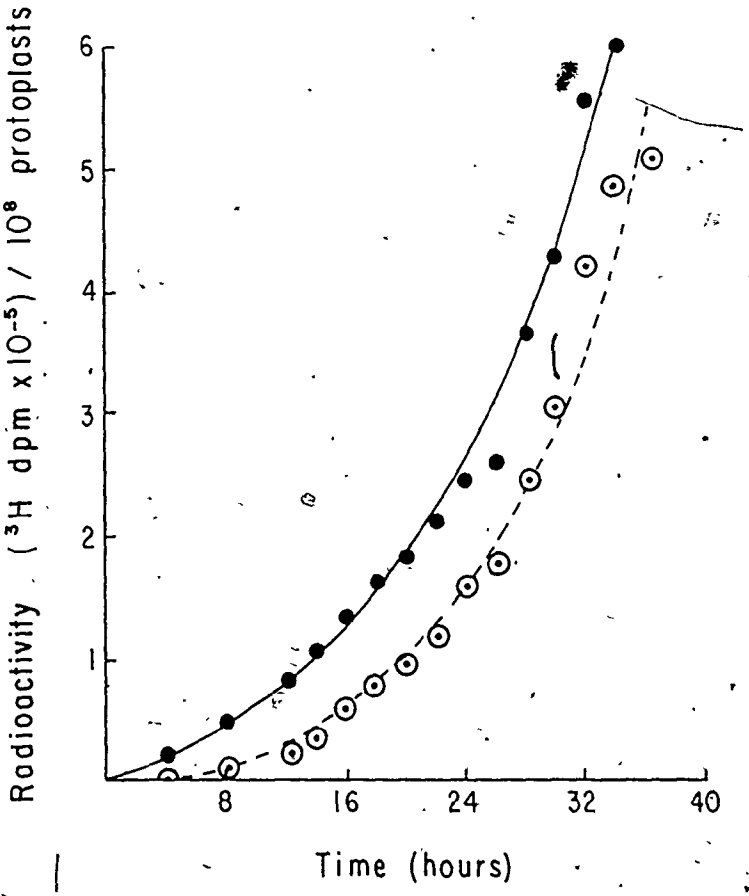

Radioactivity (^{3}H dpm x10^{-5}) / 10^8 protoplasts
6
5
4
3
2
1
8
16
24
32
40
Time (hours)

Table 3. Properties of the high speed pellet from reverting protoplasts after [^{3}H]glucosamine incorporation.

Time (hours)	% solubilization by		
	1N KOH	6N HCl	chitinase
24	5.9	98.4	83.0
36	5.3	98.9	76.4

Table 4. Specific activity of the high speed pellet from reverting protoplasts after [^{3}H] glucosamine incorporation.

Time (hours)	Specific activity (^{3}H dpm/µg glucosamine)
12	3504 ± 356
24	4804 ± 637
36	4918 ± 1009
48	3756 ± 358

Figure 26. Radiochromatograph scan of the reverted protoplast radioactive high speed pellet (36 hours) after acid hydrolysis and electrophoresis.

1. UDP-GlcNAc
2. $GlcNH_2.HCl$

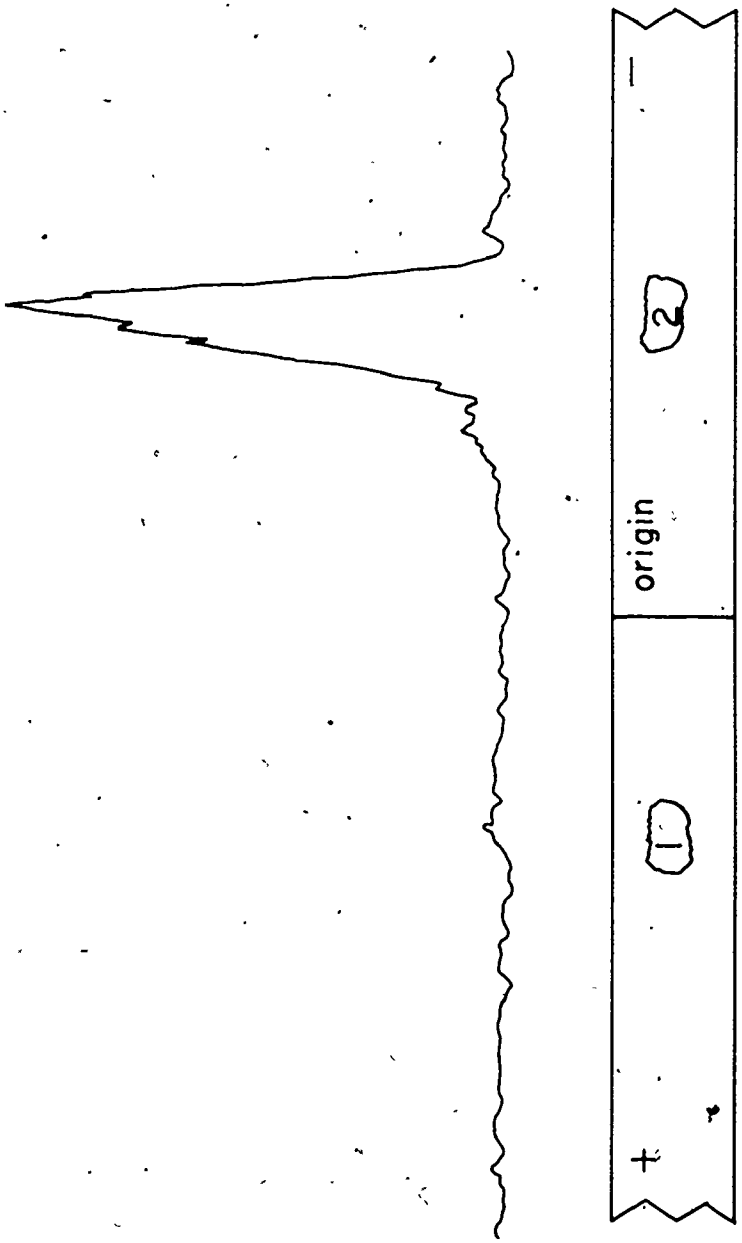
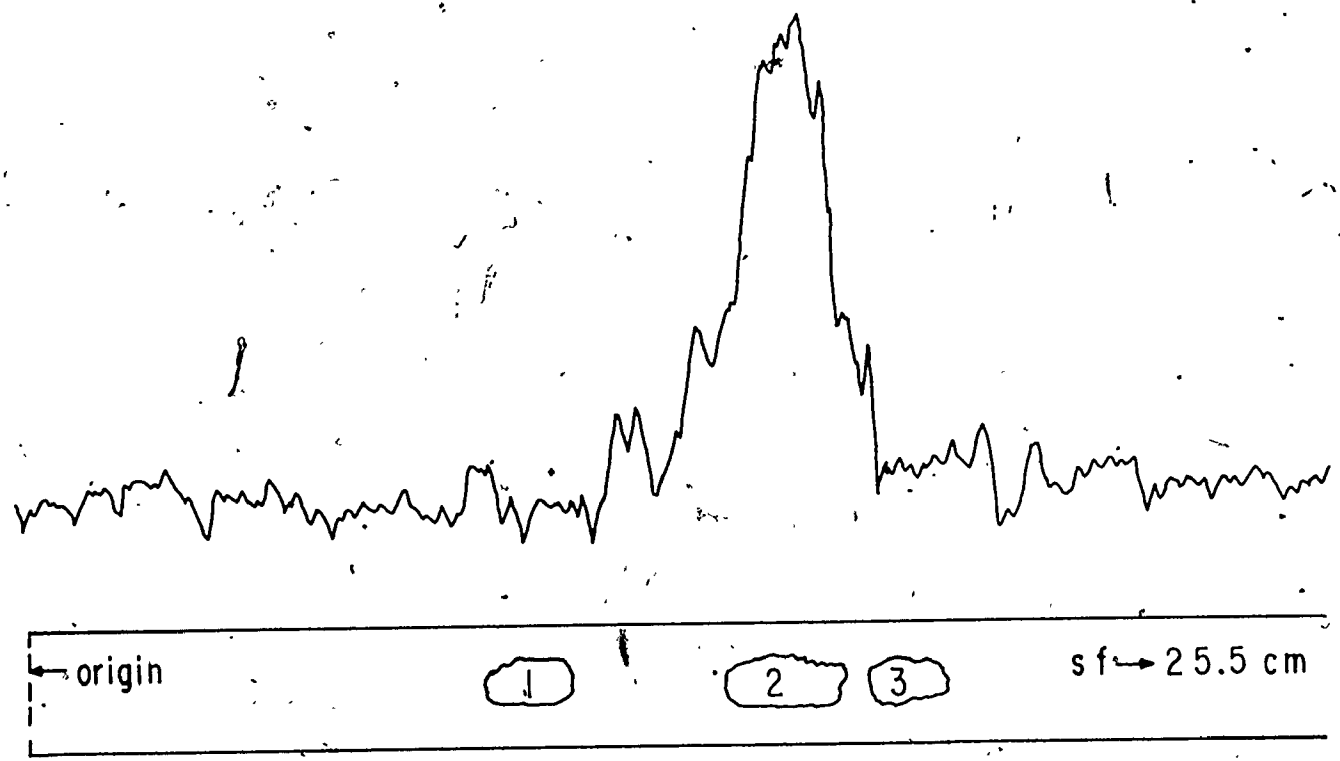

origin
1
2
+
−

Figure 27. Radiochromatograph scan of the reverted protoplast radioactive high speed pellet (36 hours) after chitinase hydrolysis.

1. UDP-GlcNAc
2. GlcNAc
3. $GlcNH_2$.HCl

Chromatography system A.


origin
1
2
3
sf→25.5 cm

of chitin synthesized (setting the amount of radioactivity incorporated at 36 hours equal to one) indicated a direct relationship between chitin synthesis and germ tube formation (Figure 28). In order to investigate whether chitin synthesis was required for or was a result of germ tube formation, the effect of polyoxin D (a specific chitin synthetase inhibitor) was examined.

4.3 Effect of polyoxin D on protoplast reversion

Polyoxin D (2×10^{-5}M) inhibited reversion by 68% regardless of the time at which it was added to the reverting protoplasts (0 or 24 hours). A ten-fold increase in the polyoxin D concentration to 2×10^{-4}M increased the inhibition of reversion to 86% (Table 5). [^{3}H]glucosamine incorporation into chitin in the presence of polyoxin D (2×10^{-4}M) was reduced to the same extent (88%) as the degree of inhibition of reversion (Table 7). Two distinct effects of polyoxin D were obtained. The total radioactivity taken up by the reverting protoplasts was less than that of the controls, probably a result of the reduction in growth. Also, the distribution of tritium between the soluble and particulate fractions was greatly altered; the label was largely in the soluble fraction in the presence of polyoxin D.

When polyoxin D (2×10^{-5}M) was added to the reverting protoplast system at 24 hours there was a four hour time lag before any effect on reversion was noted (Table 6). This probably resulted from penetration difficulties since no effect on the incorporation of tritium into the high speed

Figure 28. Relationship between germ tube formation and chitin synthesis during protoplast reversion.

relative reversion ●

relative chitin synthesis O

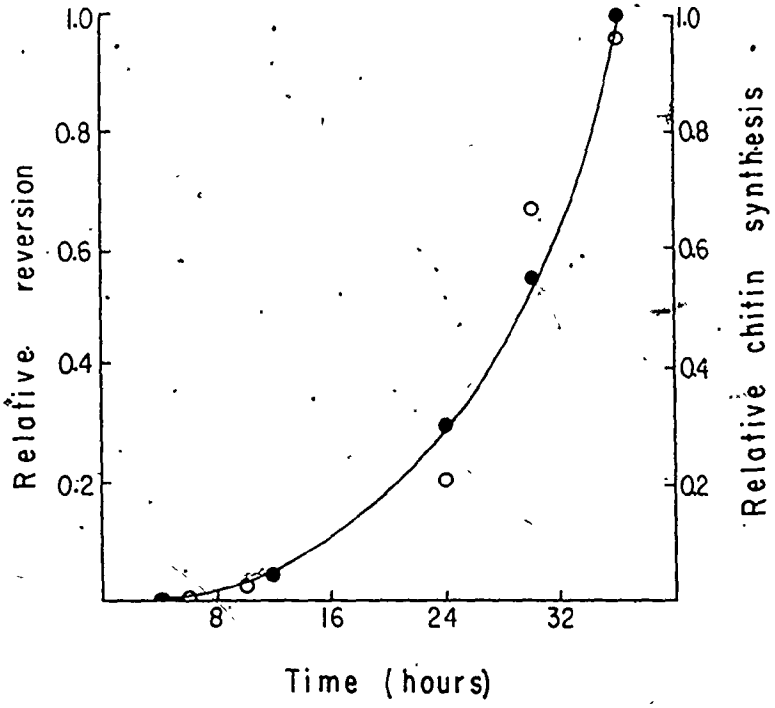

1.0
0.8
0.6
0.4
0.2
Relative reversion
1.0
0.8
0.6
0.4
0.2
Relative chitin synthesis
8
16
24
32
Time (hours)

Table 5. Effect of polyoxin D on protoplast reversion.

Polyoxin D concentration (molar)	Time of addition (hours)	% inhibition of reversion at 36 hours
2×10^{-5}	0	68.6
	24	68.2 ± 8.0
2×10^{-4}	0	86.3
	24	70.2

Table 6. Time course of the effect of polyoxin D on protoplast reversion.

Time (hours)	% reversion of total protoplasts	
	control	polyoxin D*
28	9.6	9.5 ± 1.6
32	12.5	8.9 ± 2.7
36	17.5	9.4 ± 1.2
48	17.8	9.5

* polyoxin D (2×10^{-5}M) added at 24 hours.

Table 7. Effect of polyoxin D on the incorporation of [^{3}H]glucosamine during protoplast reversion.

Sample	Time (hours)	Total radioactivity incorporated (dpm x 10^6)	Radioactivity in 40,000 x g pellet (dpm x 10^6)	% inhibition of [^{3}H] in 40,000 x g pellet caused by polyoxin D	% of total radioactivity in 40,000 x g pellet
control	24	2.44	1.97	----	80.7
polyoxin D (2×10^{-4}M)	24	0.84	0.20	89.9	23.8
control	36	6.83	5.36	----	78.5
polyoxin D (2×10^{-4}M)	36	1.32	0.62	88.4	47.0

pellet fraction was found during this four hour period.

The effects of polyoxin D on reversion suggested that chitin synthesis was a requirement for protoplast reversion. The majority of the protoplasts (80%) were unable to revert back to the hyphal mode of growth and perhaps this was a result of their inability to form chitin.

4.4 Incorporation of [^{3}H]glucosamine by non-reverting protoplasts

The non-reverting protoplasts were separated from the reverted protoplasts after the total protoplast population had been incubated in reversion medium for 48 hours in the presence of [^{3}H]glucosamine. These cells took up a small quantity of [^{3}H]glucosamine, some of which was chitinase sensitive radioactivity (Table 8). Although some chitin was associated with these cells, the data does not allow any comparison of the potential capacity of the two cell types to form chitin, due to the differences in [^{3}H]glucosamine uptake and the time factors involved.

4.5 Chitin synthetase of protoplasts, reverted protoplasts and non-reverting protoplasts

A more direct method to determine chitin synthesizing ability of the two forms of protoplasts was to examine the activity of the enzyme chitin synthetase in each protoplast type. Mycelial homogenates incorporated radioactivity from UDP-N-acetyl[U-^{14}C]GlcNH$_2$ (0.1mM) into chitin only in the presence of 20 mM GlcNAc, (see chapter V). Therefore, only the GlcNAc stimulated activity of protoplasts was regarded

Table 8. Incorporation of [^{3}H]glucosamine by non-reverting protoplasts*.

Experiment	Total [^{3}H] incorporated (dpm)	% of total radioactivity incorporated		% of total radioactivity solubilized by chitinase
		101,000 x g pellet	101,000 x g pellet after 1N KOH digestion	
1	71,005	82.5	63.6	43.6
2	58,470	91.5	76.0	33.8

* non-reverting protoplasts were isolated from the total protoplast population after 48 hours of reversion in the presence of [^{3}H]glucosamine.

as chitin synthetase. The different protoplast samples assayed gave linear incorporation of radioactivity from UDP-N-acetyl[U-^{14}C]GlcNH$_2$ into particulate material for at least sixty minutes (protein 38-108 µg) under all assay conditions.

Freshly isolated protoplasts had small amounts of chitin synthetase activity (Table 9). Since protoplasts have most of the cell wall removed, colloidal chitin was added to the assay to provide the enzyme with "primer". Although this addition did stimulate the incorporation of radioactivity in the control, it had little effect on the activity in the presence of GlcNAc. Activity assayed under all conditions was completely inhibited by polyoxin D (2×10^{-4}M). Reversion in protoplasts was reflected by a ten-fold increase in the specific activity of chitin synthetase. The non-reverting protoplast fraction exhibited highly variable activity under all the assay conditions employed; the GlcNAc stimulated activity varied between 1.08 and 5.24 units/mg.

The chitin synthetase activity of Neurospora crassa mycelia existed mainly in a latent form which could be activated by trypsin digestion (see chapter V). Protoplast homogenates also showed large stimulations in activity on treatment with trypsin (Figure 29). Over 95% of the total chitin synthetase activity of freshly prepared protoplasts existed in the latent form. Upon reversion, the activity of the latent form of the enzyme doubled; the proportion of the active form of the total activity increased to approximately 20%. The non-reverting protoplast fraction had very small

Table 9. Chitin synthetase activity of protoplasts.

Assay condition	Chitin synthetase (units/mg protein)	
	protoplasts	reverted protoplasts*
control	1.2 ± 0.7	16.8 ± 11.7
+ 20 mM GlcNAc	4.3 ± 0.4	44.4 ± 11.6
+ colloidal chitin (0.5 mg)	3.5 ± 0.9	28.7 ± 6.1
+ colloidal chitin (0.5 mg) and 20 mM GlcNAc	6.6 ± 1.7	61.3 ± 7.0
+ polyoxin D** (2×10^{-4}M)	0	0

* reverted protoplasts assayed after 48 hours of reversion.

** polyoxin D eliminated all activity under all assay conditions.

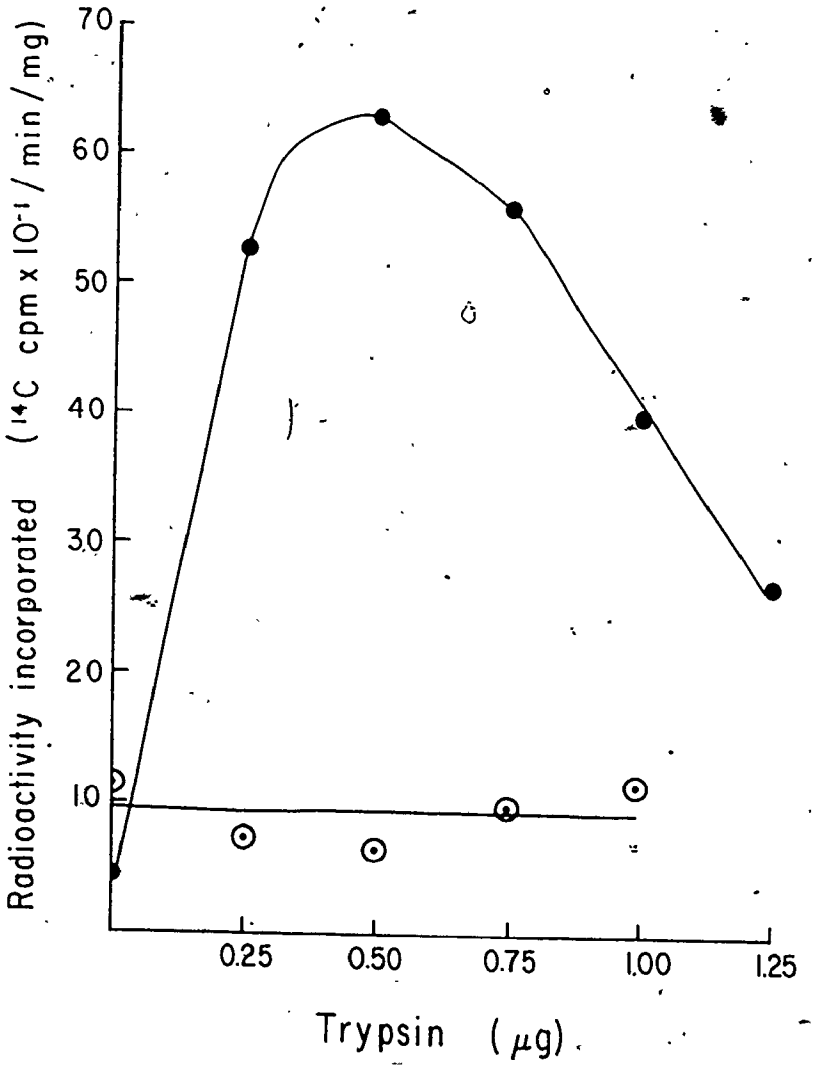
Table 10. Effect of trypsin digestion on protoplast "chitin synthetase" activity.

Sample	"Chitin synthetase" (units/mg protein)		
	before trypsin	after trypsin	
		control	+20 mM GlcNAc
protoplasts	1.2 ± 0.7	48.2 ± 12.8	97.5, 68.5
reverted protoplasts	16.8 ± 11.7	131.4 ± 22.2	204.3
non-reverting protoplasts	11.1	12.2	----
	10.4	7.1	17.0

Figure 29. Effect of trypsin digestion on the incorporation of radioactivity from UDP-N-acetyl[U-^{14}C]GlcNH$_2$ into particulate material in protoplast homogenates.

protoplast homogenate ●

non-reverting protoplast homogenate ⊙


Radioactivity incorporated (^{14}C cpm x 10^{-1} / min / mg)
70
60
50
40
30
20
10
0.25
0.50
0.75
1.00
1.25
Trypsin (μg)

amounts of the latent enzyme activity (Table 10).

These results indicate either that the initial protoplast suspension contained two populations of protoplasts in terms of total chitin synthetase activity or that the low chitin synthetase activity of the non-reverting protoplasts was a result of general deterioration of these cells.

4.6 Chitin synthetase of protoplasts released from Neurospora crassa mycelia at different times

Protoplasts were released from Neurospora crassa mycelia over a ten hour time period as a result of digestion with "snail gut" enzyme (see chapter III). The chitin synthetase activity of protoplasts released between zero and two hours had the highest levels of activity. As digestion proceeded, the specific activity of this enzyme progressively decreased until ten hours where protoplast release had ceased. After that time, the activity remained stable up to at least sixteen hours. The latent form of the enzyme was also highest in 0-2 hour protoplasts; it decreased to the same extent as the active form of the enzyme up to ten hours. Between ten and sixteen hours, the latent activity increased (Table 11).

Although these results seemed to suggest two populations of protoplasts, the specific activity of chitin synthetase of the 0-2 hour protoplasts (14.2 units/mg) was the same as the enzyme activity of mycelia (11.6 units/mg) from which the protoplasts were derived. Therefore, the decrease in activity was likely a result of prolonged contact with the "snail gut" preparation since the activity stabilized when protoplast

Table 11. Chitin synthetase activity of protoplasts released from Neurospora crassa mycelia during the "snail gut" digestion period.

Time of protoplast release (hours)	Chitin synthetase (units/mg protein)	
	control	after trypsin
0 - 1	12.7	----
0 - 2	14.2 ± 1.0	55.4 ± 2.1
0 - 5	9.5	35.7
0 - 10	4.2	18.4
0 - 16	4.3 ± 0.4	68.5 , 97.5

assays included 20 mM GlcNAc.

release ceased. The deterioration in the protoplasts was apparently specific since there was essentially no change in the glycogen-protein or DNA-protein ratios over the ten hour release period (Table 12). The failure of the majority of the protoplasts to revert was probably a result of general membrane damage caused by the "snail gut" preparation.

The protoplasts released between zero and two hours of digestion were mainly apical protoplasts (see chapter III). On examination of these protoplasts, it was found that only 13% underwent reversion. Although the specific activity of the chitin synthetase of these protoplasts was the same as the mycelial enzyme, suggesting limited or no membrane damage, the specific activity of the latent enzyme (55.2 units/mg) was already less than that of the mycelial enzyme (135 units/mg). Perhaps these protoplasts are already considerably damaged.

DISCUSSION

The study of *in vivo* chitin biosynthesis during protoplast reversion requires the incorporation of a radioactive precursor of chitin. The uptake of tritium from UDP-N-acetyl [6-^{3}H]GlcNH$_2$, the substrate of chitin synthetase, into reverting protoplasts proceeded with a five hour lag period. Since nucleotide sugars have generally been shown not to be transported across the plasma membrane (Keenan and Morre, 1975; Pratt and Grimes, 1976) and the substrate was found to hydrolyse during the reversion process, the incorporation of

Table 12. Glycogen and DNA levels of protoplasts released from Neurospora crassa mycelia during the "snail gut" digestion period.

Time of protoplast release (hours)	Glycogen (µg/mg protein)	DNA (µg/mg protein)
0 - 2	76.3, 74.6	3.9, 3.0
0 - 5	52.3	----
0 - 10	73.9	2.3, 3.6

tritium was likely a result of the transport of a hydrolysis product. The hydrolysis of UDP-N-acetyl[6-^{3}H]GlcNH$_2$ could be a result of contamination of the preparation with "snail gut" enzyme which readily hydrolyses UDP-GlcNAc. Also, the plasma membrane of various cells, for example liver hepatocytes, have nucleotide sugar degrading enzymes bound to the plasma membrane (Fleischer and Fleischer, 1969; Evans, 1964).

The radioactivity incorporated into the particulate fraction from [^{3}H]glucosamine was identified as chitin on the basis of the following properties. The material was insoluble in water and 1N KOH but could be hydrolyzed by strong acid and chitinase. The product from acid hydrolysis was identified by electrophoresis as glucosamine. This technique does not permit the separation of glucosamine from galactosamine. Trevithick and Metzenberg (1966) as well as Schmit et al., (1975) have shown that wild type Neurospora crassa mycelium have much lower levels of galactosamine polymers compared to the levels of chitin. The galactosamine polymers of Neurospora crassa are soluble in alkali (Mahadevan and Tatum, 1965) and since only 5% of the particulate fraction was alkali soluble in this case, only small quantities of galactosamine would be present in our hydrolysate. The product released by chitinase hydrolysis was identified as GlcNAc by paper chromatography. Chitinases normally release diacetylchitobiose from chitin which is subsequently hydrolyzed to GlcNAc by chitobiase (Jeuniaux, 1966). The

Streptomyces griseus preparation of chitinase used in this study has been shown to have both activities (Reynolds, 1954). Only 80% of the particulate fraction was sensitive to chitinase digestion and therefore this fraction contains glucosamine in other polymers.

The biosynthesis of chitin proceeded with at least a six hour lag period even though chitin synthetase activity was present in freshly prepared protoplasts. One can argue that chitin was produced immediately but was lost into the reversion medium due to the lack of supporting wall material as is the case in yeast protoplast cell wall biosynthesis (Necas, 1971). This possibility is unlikely, however, since dense wall-like material was formed as early as one hour after incubation in reversion medium (see chapter III).

Chitin was formed after the protoplasts had encysted and appeared at the same time as the germ tubes. The encysted non-reverting protoplasts did have a small amount of chitin associated with them after 48 hours of incubation in reversion medium. In contrast to our results, no apparent relationship between germ tube formation and chitin synthesis was found in the S. commune protoplast system by de Vries and Wessels, (1975). In their study, the encysted protoplasts formed large amounts of chitin prior to the appearance of the germ tube. Germ tube formation did not result in an increased rate of chitin synthesis. Regardless of this difference both reversion systems were inhibited by polyoxin D, a specific inhibitor of chitin synthetase. Polyoxin D, a specific competi-

tive inhibitor of chitin synthetase in Neurospora crassa, was able to inhibit reversion at the same concentrations used to inhibit mycelial growth. Polyoxin D inhibited protoplast reversion 68% and 86% at 2×10^{-5}M and 2×10^{-4}M respectively; Endo et al. (1970a) found that these concentrations inhibited mycelial growth 56% and 89%. The effective concentrations of polyoxin D were much higher than the Ki determined for the inhibition of the in vitro chitin synthetase activity by Endo et al. (1970a) but were in the concentration range which inhibited the incorporation of [^{3}H] glucosamine into chitin under in vivo conditions. Endo et al. (1970a) suggested that this was the result of a transport problem. In agreement with this suggestion, the addition of polyoxin D (2×10^{-5}M) to the protoplast reversion system at 24 hours did not effect reversion until after 28 hours; this lag was the result of poor transport since no effect on the incorporation of [^{3}H]glucosamine into the particulate fraction occurred during this four hour interval. The addition of polyoxin D to the reverting protoplast system after the regeneration phase did not change its effect; apparently polyoxin D affected a specific event associated with germ tube formation. Even though polyoxin D inhibited reversion, it was very difficult to show that this effect is due to the specific inhibition of chitin synthetase in this system. In this context, it should be kept in mind that polyoxin D has been found to have no effect on respiration and the synthesis of DNA, RNA, protein or phospholipid in Neurospora crassa (Endo et al.,

1970a). Also, polyoxin D did not have any effect on the growth of the "natural protoplast", the N. crassa slime mutant (see chapter V), which does not form chitin. Other chitin lacking organisms including bacteria, plants and animals (Isono et al., 1967) also are unaffected by polyoxin D. Based on this specificity, active chitin synthesis appears to be a requirement for protoplast reversion.

The mycelial chitin synthetase formed chitin from UDP-N-acetyl[U-^{14}C]GlcNH$_2$ (0.1 mM) only in the presence of 20 mM GlcNAc although smaller amounts of radioactivity were incorporated into non-chitinous material in the absence of GlcNAc (see chapter V). Protoplasts obtained from Neurospora crassa mycelia also incorporated small amounts of radioactivity from UDP-N-acetyl[U-^{14}C]GlcNH$_2$ (0.1 mM) into particulate material, a reaction which was greatly stimulated by 20 mM GlcNAc. The GlcNAc stimulated activity of protoplast preparations was, therefore, taken as chitin synthetase activity. Protoplasts had small amounts of chitin synthetase activity. Since protoplasts apparently lack chitin, colloidal chitin was added to the assay system to act as a possible "primer". Addition of colloidal chitin had no effect on activity (GlcNAc stimulated activity) but it did stimulate the incorporation of radioactivity from UDP-N-acetyl[U-^{14}C]GlcNH$_2$ into particulate material. Both of these activities increased during reversion of the protoplasts. These activities are likely due to two distinct enzymes since Glaser and Brown (1957) succeeded in

solubilizing the GlcNAc independent enzyme. The existence of these two enzymes might clarify the inconsistency of results reported by various investigators on the effects of addition of chitodextrins to crude chitin synthetase preparations (see chapter I). The significance of the GlcNAc independent enzyme in chitin synthesis is unknown; possibly it represents a nonspecific transferase activity.

Chitin synthetase activity in Neurospora crassa mycelium was found to exist mainly (90%) in a latent form which could be activated by trypsin digestion. Protoplast chitin synthetase also existed primarily as a latent form. On reversion, the activity of the latent form of the enzyme increased 2-3 fold, whereas the active form of the enzyme increased some 10 fold, hence a decreasing proportion of the total activity was latent. Both forms of the enzyme also increase in specific activity during mycelial growth. The relationship between the active and latent activities is unknown but they have similar properties; stimulation by GlcNAc and inhibition by polyoxin D.

The non-reverting protoplasts had low total chitin synthetase levels (lack of the latent form) compared to freshly isolated protoplasts. This condition could have resulted from either a general deterioration of the non-reverting protoplasts or the existence in the original protoplast suspension of two different protoplast populations which differed in their chitin synthetase activity. The original protoplast

suspension did consist of at least two populations of protoplasts in terms of their origin, apical and non-apical protoplasts (see chapter III). Autoradiographic studies of chitin synthesis in Neurospora crassa mycelium have indicated that most of the chitin was deposited at the apex (Gooday, 1971). Therefore, one might expect apical protoplasts to have higher levels of chitin synthetase than protoplasts originating behind the apex. Indeed, examination of the chitin synthetase activity of protoplasts released from mycelia at different times showed a progressive decrease in activity. The apical protoplasts which were released first (see chapter III) had the highest levels of activity. If the apical protoplasts did have much higher levels of chitin synthetase activity as compared to non-apical protoplasts, then their specific activity should have been greater than the mycelia from which the total protoplast suspension was derived. This was not found to be the case. Archer (1977) found that the apical protoplasts of Aspergillus fumigatus had high levels of chitin synthetase compared to mycelia. However, the protoplasts were derived from mycelia which were grown under different conditions (agar cultures) than the mycelia used for comparison (shake culture). Since specific activity of fungal chitin synthetase varies tremendously with age (Peberdy and Moore, 1975; McMurrough et al., 1971), then the above comparison might not be valid. The autoradiographic results on Neurospora crassa cannot be explained on the basis of large concentration of chitin synthetase at the apex. Possibly, the large chitin synthesizing

capacity of the apex is a result of high localized levels of the activator GlcNAc.

The progressively lower levels of chitin synthetase activity found during the protoplast release was probably the result of contact with the "snail gut" enzyme since the decrease in activity stopped at the same time as protoplast release ceased (10 hours). The overall decrease in activity was not due to a general deterioration of these cells since no changes in DNA/protein or glycogen/protein ratios occurred during this time period. The specific location of chitin synthetase in Neurospora crassa is unknown but in other fungal systems, it has been found to be localized in the plasma membrane (Duran et al., 1975; Jan, 1974). This location would be consistent with the effects of the "snail gut" preparation on chitin synthetase since this preparation must work at the level of the plasma membrane. Since the 0-2 hour protoplasts had the same levels of activity of active chitin synthetase as the mycelia from which they were derived, then all of the active form must be located within the plasma membrane rather than in the cell wall which was removed by the "snail gut" enzyme. The latent activity of the 0-2 hour protoplast preparation was only 50% of the mycelial activity, perhaps a good part of the latent activity is in the cell wall. Alternatively, the latent activity might be more sensitive to the snail enzyme than the active form but this is unlikely since both activities decreased at the same rate during the digestion period.

The relationship between reversion ability, protoplast origin and chitin synthetase levels was not clear. The apical protoplasts (0-2 hours) had little contact with the "snail gut" preparation and apparently were undamaged as the chitin synthetase levels were "normal", yet, they still had a low ability to revert. Apparently, the reverting protoplasts arise from the non-apical portions of the mycelia. The reverting protoplasts are most likely those released last from the mycelia and only had very short contact time with an active "snail gut" enzyme. The protoplasts may be damaged by the "snail gut" preparation to varying degrees depending on the contact time. This is indicated by the numerous protoplast types found in the total population, the "leaky" protoplasts which have internal Calcofluor staining, the protoplasts which were not "leaky" but could not regenerate a wall, the protoplasts which regenerated a wall but could not revert and finally those protoplasts which regenerated and did revert.

CHAPTER V CHITIN BIOSYNTHESIS IN THE SLIME MUTANT OF NEUROSPORA CRASSA

The slime mutant of Neurospora crassa grows mainly as a single cell which is apparently devoid of a cell wall (Bigger et al., 1972). It can therefore be regarded as a "natural protoplast" which does not possess the ability to revert back to the mycelial state, presumably due to genetic defects. Genetic analysis has shown that the slime mutant involves at least three distinct morphological locii referred to as spontaneous germination (sg), fuzzy (fz) and osmotic (os) (Emerson, 1963).

In view of the apparent relationship between chitin biosynthesis and germ tube formation in reverting protoplasts or germ tube extension in wild type Neurospora crassa, the chitin biosynthesis of this "natural protoplast" was examined.

RESULTS

5.1 Chitin content of slime mutant and wild type mycelia of Neurospora crassa

Examination of the slime mutant by electron microscopy (Figure 30) confirmed the absence of the bulk of cell wall material. Closer examination of the plasma membrane indicated the presence of fuzzy regions of material at the cell surface. The slime mutant did not stain with Calcofluor White M 2R New (see chapter III).

The amount of chitin present in the slime mutant was compared to that present in wild type mycelia by quantitating the amount of glucosamine present in the 101,000 x g pellets ob-

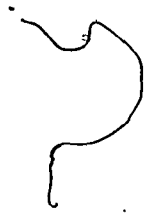
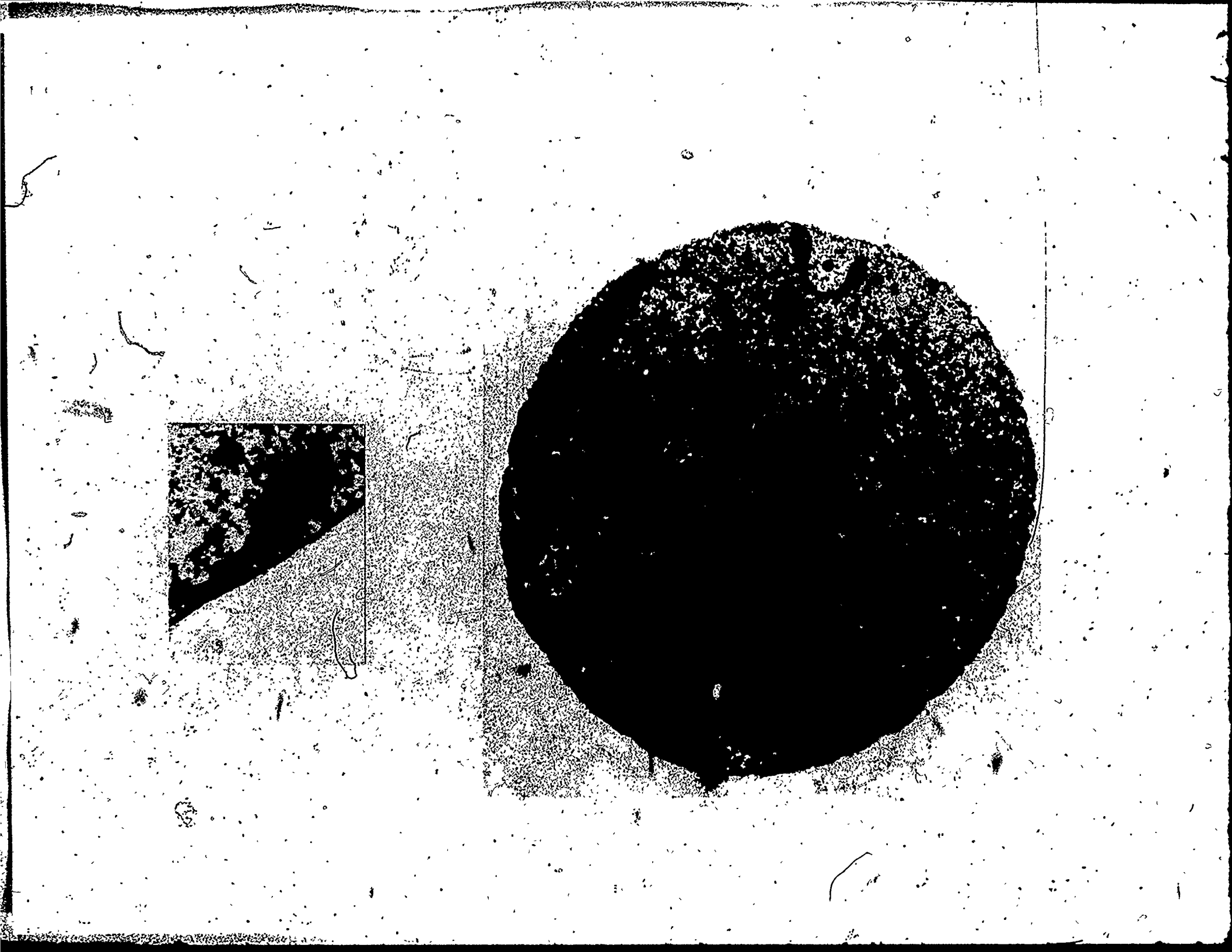


Figure 30a. Electron micrograph of the Neurospora crassa slime mutant.
(magnification - 25,600 x)

Figure 30b. Electron micrograph of the Neurospora crassa slime mutant showing the plasma membrane.
(magnification - 53,600 x)



tained from crude homogenates after acid hydrolysis. As shown in Table 13, the mutant possessed only 7.1% of the glucosamine levels of the wild type mycelia expressed in terms of µg glucosamine per total cellular protein. A high speed pellet from the growth medium of the slime mutant also contained small quantities of glucosamine.

5.2 Incorporation of [^{3}H]glucosamine by the slime mutant and wild type mycelia of *Neurospora crassa*

Although these results indicated that the slime mutant of *Neurospora crassa* did not produce much chitin, they did not tell us whether the small amount of glucosamine found was derived from chitin. In order to determine the source of this material, the following experiments were carried out. The slime mutant was grown in the presence of [^{3}H]glucosamine to mid-log phase and then harvested. The cells incorporated a large amount of tritium, the largest proportion of which was found in the high speed supernatant (Table 14). The radioactivity in the high speed pellet was largely (80%) soluble in 1N KOH. Acid hydrolysis of this fraction indicated that the radioactivity was present as galactosamine, not glucosamine (Table 15). However, the 1N KOH insoluble fraction gave glucosamine upon acid hydrolysis. The alkali insoluble fraction was solubilized to the extent of 66% by chitinase digestion. Further examination of the chitinase hydrolysate by ion exchange (Table 16) and paper chromatography (Table 17) indicated that the products released by chitinase hydrolysis were

Table 13. "Chitin" content of the slime mutant and wild type mycelia of Neurospora crassa.

Organism	µg glucosamine per total cellular protein (mg)
wild type	10.9 ± 2.8
slime	0.8 ± 0.2
slime*	2.1 ± 0.8
slime**	0.6

Note: The 101,000 x g pellet from crude homogenates was subjected to digestion with 1N KOH prior to acid hydrolysis.

* slime mutant was grown in a medium containing 2.5% GlcNAc.

** 101,000 x g pellet obtained from the growth medium of the slime mutant.

Table 14. Metabolism of [^{3}H]glucosamine by the slime mutant and wild type mycelia of Neurospora crassa.

Organism	Total radio-activity incorporated (^{3}H dpm x 10^7)	% of the total radioactivity incorporated					
		pellet	supernatant	growth media	pellet after 1N KOH digestion	pellet after chitinase digestion	growth media after chitinase digestion
slime mutant	3.28 ± 0.26	45.0 ± 0.7	54.1 ± 0.7	13.2 ± 3.1	9.2 ± 3.3	3.2	12.5
wild type	2.64 ± 0.15	83.4 ± 1.1	11.7 ± 1.1	7.1 ± 3.4	59.8 ± 5.2	5.7	----

Note: The pellet and supernatant fractions were obtained by centrifugation at 101,000 x g for 60 minutes. The radioactivity in the growth media was determined after exhaustive dialysis against water.

The chitinase digested growth media was examined for small molecular weight components by subjecting the hydrolysate to ultrafiltration (UM-2 membrane).

Table 15. Chromatography of the acid hydrolysate of the 101,000 x g pellet of the slime mutant of Neurospora crassa after incorporation of [^{3}H]glucosamine.

Fraction	R_f*
101,000 x g pellet	0.47 (80)** 0.56 (20)
101,000 x g pellet after 1N KOH digestion	0.57
glucosamine	0.57
galactosamine	0.46

* chromatography system C.

** values in parenthesis represent percentages of total radioactivity in each peak.

Table 16. Ion exchange chromatography (Amberlite MB-3) of the chitinase solubilized radioactivity from the 101,000 x g pellet of the slime mutant of Neurospora crassa after incorporation of [^{3}H]glucosamine.

Experiment	[^{3}H] loaded onto column (dpm)	[^{3}H] recovered in neutral fraction (dpm)	% of [^{3}H] recovered in neutral fraction
1	76,473	3,231	4.2
2	148,632	17,100	11.5

Table 17. Paper chromatography of the neutral fraction from the Amberlite MB-3 column.

Sample	R_f*
neutral fraction	0.00 (33)**
	0.34 (67)
GlcNAc	0.53
N,N-diacetylchitobiose	0.43

* chromatography system B.

** values in parenthesis represent percentages of total radioactivity in neutral fraction.

not N,N-diacetylchitobiose nor GlcNAc, the two products normally formed by chitinase digestion of chitin. Therefore, the conclusion was reached that chitin was completely absent from the slime mutant of Neurospora crassa.

In order to examine the possibility that chitin was synthesized but excreted by the slime mutant, the growth medium from the above experiments was examined for chitin. The growth medium after dialysis did contain some radioactivity in high molecular weight compounds (MW >5000) but it was found to be resistant to chitinase digestion (Table 14).

For comparative purposes, the metabolism of [^{3}H]glucosamine by wild type mycelia was also examined. In contrast to the slime mutant, over 80% of the radioactivity incorporated was found in the high speed pellet. This fraction was largely insoluble in 1N KOH but was completely sensitive to chitinase digestion. A small proportion (7%) of the total radioactivity incorporated was also excreted in the form of high molecular weight (MW >5000) components (Table 14).

5.3 Effect of polyoxin D on the growth of the slime mutant of Neurospora crassa

Since the slime mutant was totally devoid of chitin, polyoxin D should have no effect on its growth. Indeed, a concentration of 2×10^{-4}M polyoxin D had no effect on the growth of the slime mutant (Table 18). It also had no effect on the incorporation of [^{3}H]glucosamine into the 1N KOH resistant high speed pellet (Table 19).

Table 18. Effect of polyoxin D on growth of the slime mutant of Neurospora crassa.

Experiment	Sample time (hours)	Optical density (600 mμ)	
		control	polyoxin D (2×10^{-4}M)
1	0	0.10	0.11
	24	1.21	1.28
2	0	0.16	0.12
	24	1.06	1.11

Table 19. Effect of polyoxin D on the incorporation of [^{3}H]glucosamine by the slime mutant of Neurospora crassa*.

Sample	Total [^{3}H] incorporated (dpm)	% of total radioactivity incorporated	
		101,000 x g pellet	101,000 x g pellet after 1N KOH - digestion
control	338,443	29.1	13.2
polyoxin D (2×10^{-4}M)	463,854	24.2	13.2

* [^{3}H]glucosamine was added at time 0 and the cells were harvested after 24 hours.

5.4 Identification of soluble metabolites of [^{3}H]glucosamine in the slime mutant of Neurospora crassa

Since the slime mutant was unable to produce chitin, an effort was made to locate the defect in the chitin biosynthetic pathway. The high speed supernatants from the [^{3}H] glucosamine incorporation experiments were examined for known intermediates of the chitin pathway by ion exchange chromatography. Figure 31 shows the elution profile of radioactivity from a Dowex I formate column eluted with water followed by a linear gradient of 1M ammonium formate. Three peaks were eluted which consisted of twenty-nine (I), seventeen (II) and fifty-four (III) percent of the radioactivity loaded onto the column (Figure 31). The column was standardized with GlcNAc, GlcNAc-1-PO_4 and UDP-GlcNAc (Figure 32). The three unknowns eluted in the same positions as the standards.

The two salt-eluted fractions (II and III) were loaded onto a Sephadex G-10 column and eluted with water (Figure 33). Although this step was carried out to desalt the fractions, fraction II was resolved into a large (II-1) and a small (II-2) molecular weight component. Fraction III gave a single peak at the void volume. The fractions were further examined by paper chromatography and electrophoresis (summarized in Table 20). Fraction III migrated with the same R_f (0.1) as standard UDP-GlcNAc in chromatography system B and migrated the same distance as UDP-GlcNAc upon electrophoresis (7.0 cm). After acid hydrolysis, fraction III gave a single peak with

Figure 31. Dowex I column chromatography of the 101,000 x g supernatant of the Neurospora crassa slime mutant after [^{3}H]glucosamine incorporation.

- the NH_4COOH gradient was started at tube 10

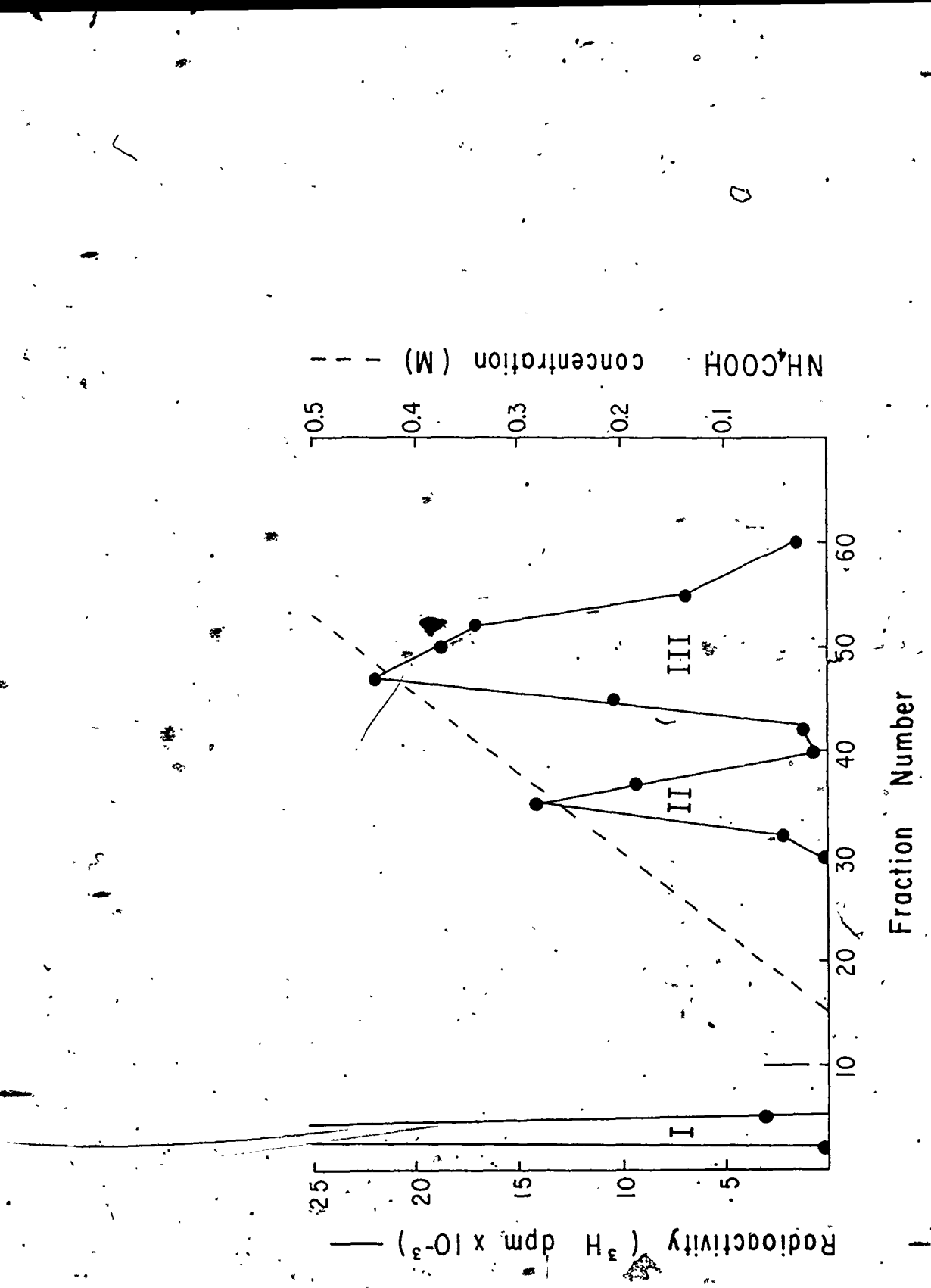

NH4COOH concentration (M) ---
0.5
0.4
0.3
0.2
0.1
I
II
III
10
20
30
40
50
60
Fraction Number
Radioactivity (3H dpm x 10-3) ——
25
20
15
10
5

Figure 32. Dowex I column chromatography of UDP-GlcNAc, GlcNAc-1-PO_4 and GlcNAc obtained by mild acid hydrolysis of UDP-N-Acetyl[U-^{14}C]$GlcNH_2$ (Molnar et al., 1971).

I GlcNAc

II GlcNAc-1-PO_4

III UDP-GlcNAc

- the two unmarked peaks absorbing at an optical density of 260 mμ correspond to UMP and UDP respectively.

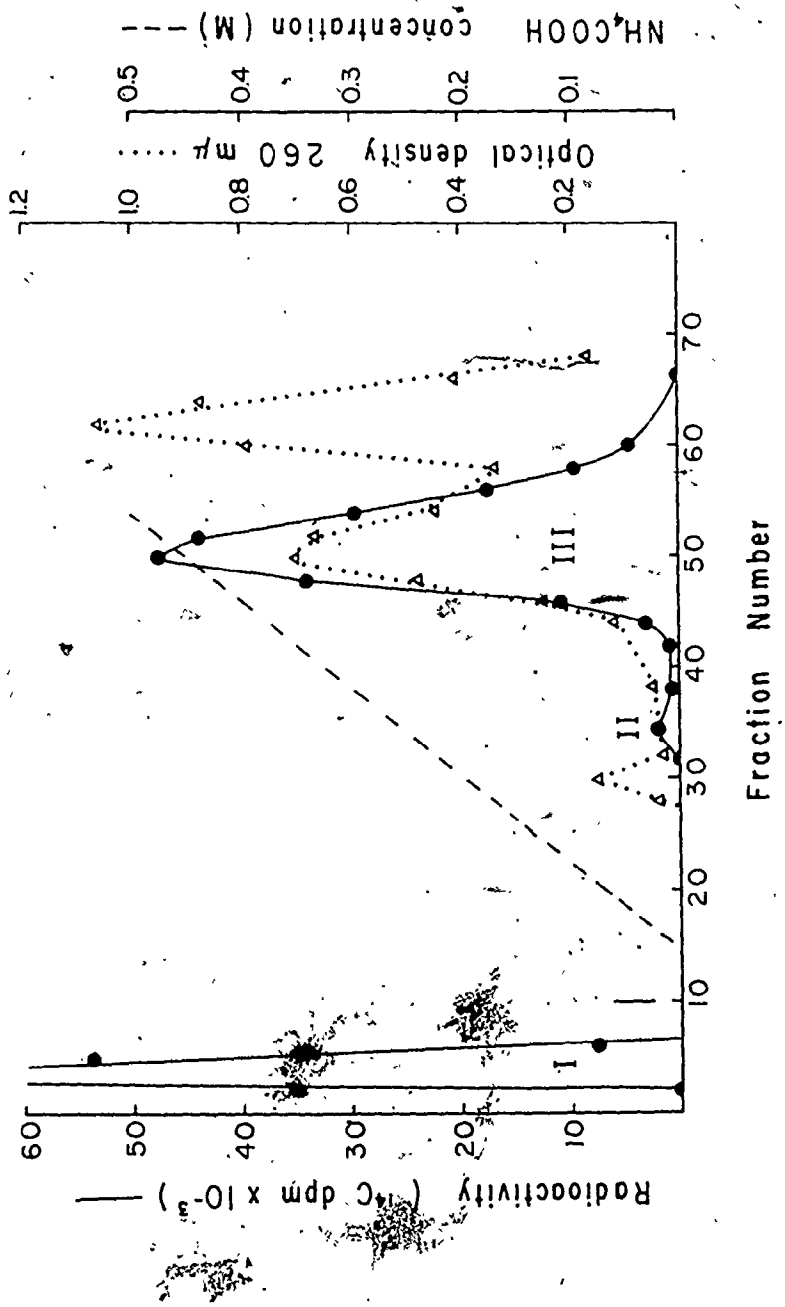

Radioactivity (^{14}C dpm x 10^{-3}) ——
Optical density 260 mμ ····
NH_4COOH concentration (M) ---
Fraction Number
I
II
III

Figure 33. Sephadex G-10 column chromatography of the fractions obtained from the Dowex I column.

Fraction III ● ——

Fraction II △ - - -

- the larger molecular weight species of fraction II was labelled II-1, the smaller species II-2.

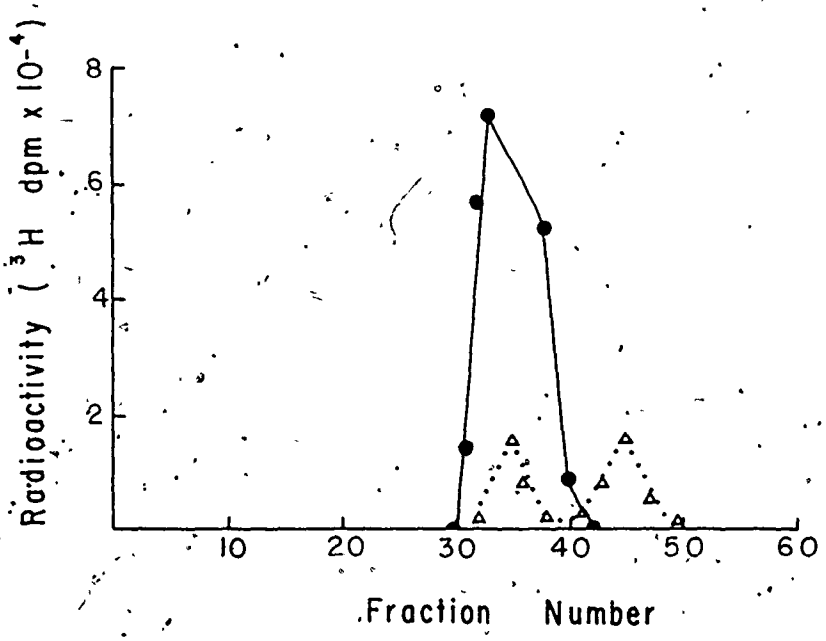

Radioactivity (^{3}H dpm x 10^{-4})
8
6
4
2
10
20
30
40
50
60
Fraction Number

Table 20. Properties of soluble metabolites of the slime mutant of Neurospora crassa after [^{3}H]glucosamine incorporation.

system	R_f or distance migrated from origin (cm)							
	III	II-2	II-1	I	UDP-GlcNAc	$GlcNH_2$	$GalNH_2$	GlcNAc
chromatography B	0.10	0.53	0.00	0.00(50)*	0.10	0.37	-	0.53
	-	-		0.53(50)	-	-	-	-
chromatography B**	0.37	-		0.37	-	-	-	-
chromatography C**	0.57(75)	-	-	-	-	0.57	0.46	-
	0.46(25)	-	-	-	-	-	-	-
electrophoresis	7.0 (+)***	0	-	-	7.0 (+)	10.0 (-)	-	0

* in the case of multiple chromatographic peaks, the percentage of each peak is in parenthesis.

** after hydrolysis with 3 N HCl (100°C) for 3 hours

*** the + or - in parenthesis represents the direction of migration from the origin during electrophoresis.

the same R_f (0.37) as glucosamine in chromatography system B. Upon chromatography of the acid hydrolysate in chromatography system C, a small peak with the same R_f as galactosamine (0.46) also appeared. Therefore, fraction III consisted of UDP-GlcNAc with smaller quantities (25%) of UDP-GalNAc.

Fraction I gave two peaks in chromatography system B, one of which migrated with the same R_f (0.53) as GlcNAc; the other remained at the origin. After acid hydrolysis, it gave a single peak which migrated with the same R_f (0.37) as $GlcNH_2$. Therefore, fraction I was a mixture of 50% GlcNAc and 50% of an unidentified compound or polymer. Fraction II-1 remained at the origin in chromatography system B and was unidentified. Fraction II-2 migrated with the same R_f as GlcNAc (0.53) in chromatography system B and remained at the origin during electrophoresis. Since the original components of fraction II were compounds possessing a net charge, then the GlcNAc must have resulted as a breakdown product of the original compound. Fraction II-2 was tentatively identified as GlcNAc-$1PO_4$.

5.5. Examination of the turnover of the radioactive soluble fraction from the slime mutant of Neurospora crassa

Since the major soluble metabolite of [^{3}H]glucosamine metabolism in the slime mutant was identified as UDP-GlcNAc, it was of interest to see if it was the precursor of the particulate material. Indeed, most of the soluble fraction (72%) turned over within the first 4 hours. The radioactivity appeared mainly in the high speed pellet (60%) but a significant portion (40%) was found in the growth medium. The in-

crease in radioactivity in the pellet fraction was divided between the alkali resistant (60%) and alkali sensitive (40%) fractions (Table 21).

Since UPD-GlcNAc was the major soluble metabolite of [^{3}H]glucosamine in the slime mutant, the *in vivo* defect in chitin synthesis was apparently at the level of the enzyme chitin synthetase.

In order to examine the possibility of an additional defect existing prior to glucosamine-6-PO_4 in the chitin pathway since the first step of the chitin pathway is by-passed in the [^{3}H]glucosamine experiments, the slime mutant was grown in the presence of GlcNAc as an extra carbon source. Growth in GlcNAc containing medium did not significantly increase the levels of glucosamine in the particulate fraction of the slime mutant (see Table 13), and, therefore, it was unlikely that a defect prior to glucosamine-6-PO_4 in the chitin pathway was responsible for the lack of chitin.

5.6 L-glutamine D-fructose-6-PO_4 transamidase

Although it was unlikely that a defect occurred in the chitin pathway prior to the formation of glucosamine-6-PO_4, the specific activity of the first enzyme in the chitin pathway L-glutamine D-fructose-6-PO_4 transamidase was determined in the slime mutant and wild type mycelia since it is an important control point in this pathway. High speed supernatants from both mycelial and slime mutant homogenates formed glucosamine-6-PO_4 from L-glutamine and fructose-6-PO_4

Table 21. Examination of the "flow" of radioactivity in the slime mutant of Neurospora crassa* after [^{3}H]glucosamine incorporation.

Time (hours)	Radioactivity in 101,000 x g supernatant (dpm)	Radioactivity in 101,000 x g pellet (dpm)		Radioactivity in growth medium (dpm)
		alkali soluble	alkali insoluble	
0	28,140	10,120	3,345	10,088
4	10,178	15,570	6,549	15,675
6	8,100	15,054	5,389	16,151
8	7,112	15,147	7,208	17,520
change	-20,328	+ 5,450	+3,863	+ 7,432

* slime cells were previously grown for 24 hours in presence of [^{3}H]glucosamine prior to isolation and suspension in non-radioactive media.

in direct proportion to the incubation time and the amount of protein in the assay mixture (Figures 34a and 34b). Using 30 minute incubation times and protein concentrations in the linear range for the slime mutant (protein 0.2-0.3 mg) and the mycelial enzyme (protein 0.25-0.45 mg), the specific activity of slime mutant enzyme was found to be three times that of the mycelia (Table 22).

5.7 Incorporation of radioactivity from UDP-N-acetyl[U-^{14}C] $GlcNH_2$ into particulate material by homogenates of the slime mutant and wild type mycelia of Neurospora crassa

The in vivo results indicated that the defect in chitin synthesis of the slime mutant was at the level of the enzyme chitin synthetase. In order to confirm this the level of this enzyme in the slime mutant was compared to that found in wild type mycelia.

Crude slime mutant homogenates gave linear incorporation of radioactivity from UDP-N-acetyl[U-^{14}C]GlcNAc into particulate material over sixty minutes (protein - 425 µg). The incorporation of radioactivity was also linear with respect to protein levels between 100-1000 µg (Figure 35a and 35b). This activity varied between different batches of culture material but the values were constant within a single batch (Table 23). Cells disrupted by sonication lost all activity.

For comparison, the incorporation of radioactivity from UDP-N-acetyl[U-^{14}C]$GlcNH_2$ into the particulate fraction catalyzed by conidial and mycelial homogenates was also investi-

Figure 34a. L-glutamine D-fructose-6-PO_4 transamidase activity as a function of time.

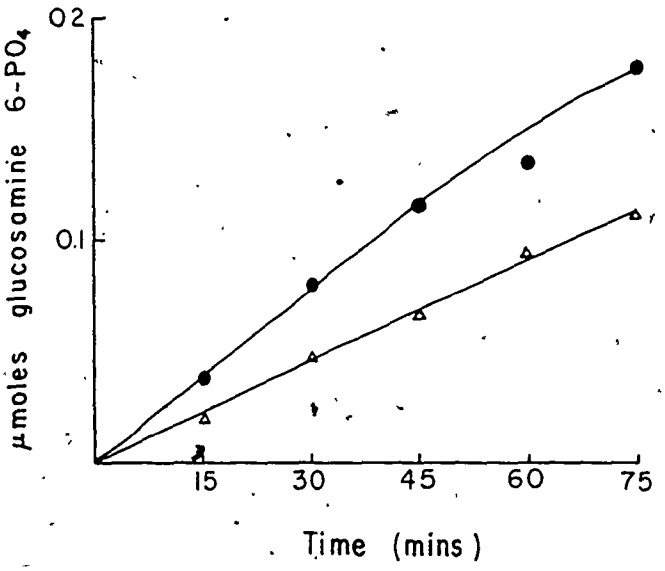
wild type (413 µg protein) Δ

slime mutant (290 µg protein) ●

Figure 34b. L-glutamine D-fructose-6-PO_4 transamidase activity as a function of protein concentration.

wild type mycelia Δ

slime mutant ●


0.2
0.1
µmoles glucosamine 6-PO4
15
30
45
60
75
Time (mins)

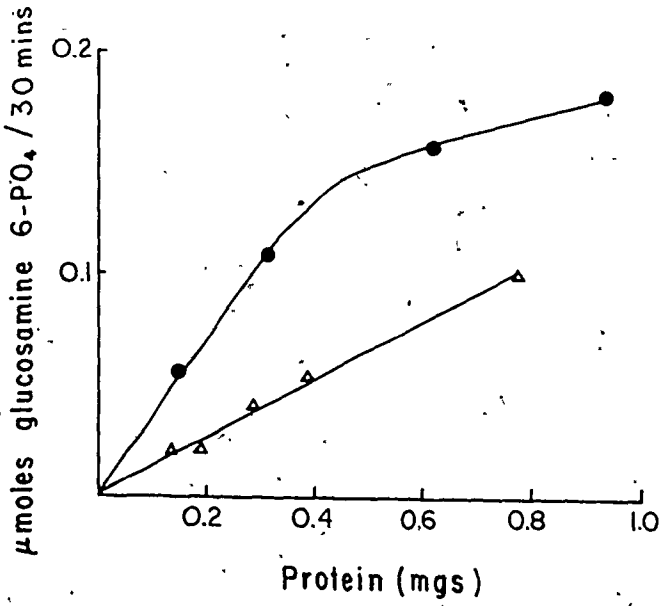
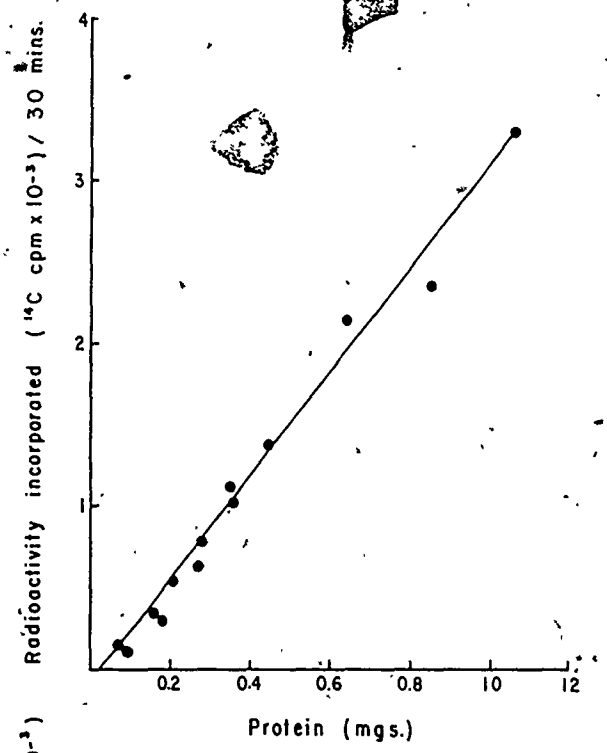

0.2
0.1
µmoles glucosamine 6-PO4 / 30 mins
0.2
0.4
0.6
0.8
1.0
Protein (mgs)

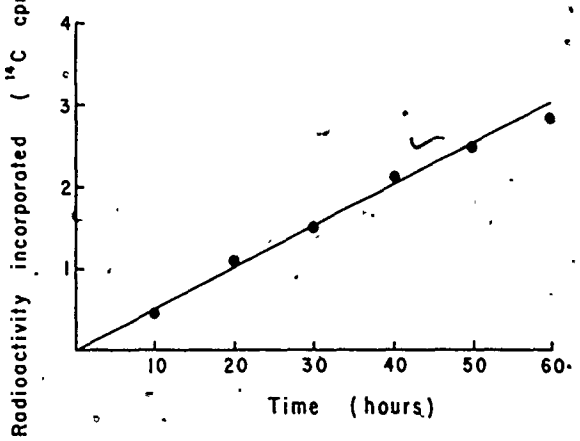
Table 22. L-glutamine-D-fructose-6-phosphate-transamidase activity in the slime mutant and wild type mycelia of Neurospora crassa.

Organism	L-glutamine-D-fructose-6-PO_4 transamidase (units/mg protein)
wild type mycelia	0.004 ± 0.001
slime mutant	0.011 ± 0.003

Figure 35a. Incorporation of radioactivity from UDP-N-acetyl[U-^{14}C]GlcNH$_2$ into particulate material by a homogenate of the slime mutant of Neurospora crassa as a function of protein concentration.

Figure 35b. Incorporation of radioactivity from UDP-N-acetyl[U-^{14}C]GlcNH$_2$ into particulate material by a homogenate of the slime mutant of Neurospora crassa as a function of time (protein - 425 µg).


Radioactivity incorporated (^{14}C cpm x 10^{-3}) / 30 mins.
4
3
2
1
0.2
0.4
0.6
0.8
10
12
Protein (mgs.)


Radioactivity incorporated (^{14}C cpm x 10^{-3})
4
3
2
1
10
20
30
40
50
60
Time (hours)

gated. The conidial homogenates gave linear incorporation of radioactivity up to sixty minutes with protein levels between 500 µg and 800 µg. High protein concentrations and long incubation times were required due to the low activity. Mycelial homogenates gave linear incorporation of radioactivity for thirty minutes with protein levels between 100 µg. and 1000 µg. Both the slime and mycelial homogenates gave approximately the same incorporation rates (Table 23).

5.8 Effect of GlcNAc and polyoxin D on the incorporation of radioactivity from UDP-N-acetyl[U-^{14}C]GlcNH$_2$ into particulate material by homogenates of the slime mutant and wild type mycelia of Neurospora crassa

The mycelial enzyme preparation was greatly stimulated by the addition of GlcNAc to the assay system; concentrations of up to 20 mM failed to saturate the system (Figure 36). A concentration of 20 mM GlcNAc was used in the standard assay procedures. While the mycelial preparation was stimulated over six fold, the activity of the slime mutant homogenate was only stimulated 2.6 fold by 20 mM GlcNAc.

Polyoxin D inhibited the incorporation of radioactivity from UDP-N-acetyl[U-^{14}C]GlcNH$_2$ into particulate material of a mycelial homogenate in the presence of 20 mM of GlcNAc. Fifty percent inhibition was obtained at a concentration of 10 µM polyoxin D; concentrations of over 100 µM were required for complete inhibition (Figure 37). The incorporation of radioactivity in mycelial and slime mutant homogenates was completely inhibited by polyoxin D (2×10^{-4}M) (Table 24).

Table 23. Incorporation of radioactivity from UDP-N-acetyl [U-^{14}C]GlcNH$_2$ into particulate material by homogenates of conidia, mycelia and the slime mutant of Neurospora crassa.

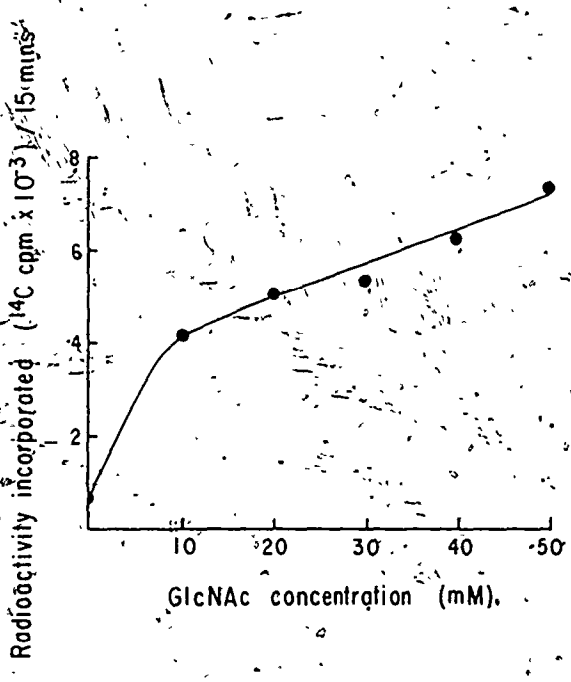
Organism	Radioactivity incorporated (cpm/min/mg protein)
conidia	6.1 ± 0.9
mycelia	101.0 ± 33.2
slime mutant	80.8 ± 39.5

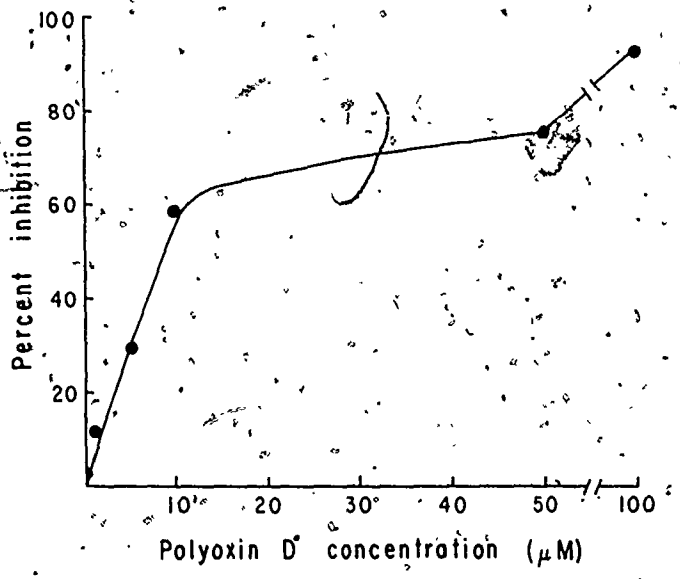
Table 24. Effect of GlcNAc and polyoxin D on the incorporation of radioactivity from UDP-N-acetyl [U-^{14}C]GlcNH$_2$ into particulate material by homogenates of the slime mutant and wild type mycelia of Neurospora crassa.

Assay condition	Radioactivity incorporated (cpm/min/mg protein)	
	slime	mycelia
control	125.4 ± 8.7	119.0 ± 8.7
+20 mM GlcNAc	324.4 ± 27.9	738.8 ± 133.2
+polyoxin D (2×10^{-4}M)	2.4 ± 0.1	8.5 ± 3.0

Figure 36. Effect of GlcNAc concentration on the incorporation of radioactivity from UDP-N-acetyl[U-^{14}C]$GlcNH_2$ into particulate material by a mycelial homogenate of Neurospora crassa.

Figure 37. Effect of polyoxin D on the incorporation of radioactivity from UDP-N-acetyl[U-^{14}C]$GlcNH_2$ into particulate material by a mycelial homogenate of Neurospora crassa. The assay was carried out in the presence of 20 mM GlcNAc.


Radioactivity incorporated (^{14}C cpm $\times 10^{-3}$) / 15 mins
2
4
6
8
10
20
30
40
50
GlcNAc concentration (mM).


Percent inhibition
20
40
60
80
100
10
20
30
40
50
100
Polyoxin D concentration (μM)

5.9 Characterization of the radioactive particulate material formed from UDP-N-acetyl[U-^{14}C]GlcNH$_2$ in the in vitro incorporation experiments

The particulate material from large scale in vitro incorporation experiments from the slime mutant and mycelial homogenates was isolated by high speed centrifugation (40,000 x g). All the radioactivity incorporated into particulate material as determined by the filter paper method was recovered in this fraction. The particulate material formed from UDP-N-acetyl [U-^{14}C]GlcNH$_2$ in the presence and absence of 20 mM GlcNAc in both the slime mutant and mycelial homogenates was alkaline resistant (Table 25). In the absence of GlcNAc, very little of the particulate material was chitinase sensitive in either the slime mutant or mycelial homogenates. The slime mutant particulate material formed from UDP-N-acetyl[U-^{14}C]GlcNH$_2$ in the presence of 20 mM GlcNAc showed an increased sensitivity to chitinase. Most of this chitinase solubilized material was neutral (passed through an Amberlite MB-3 column). The neutral fraction had the same R_f as GlcNAc (0.53) in chromatography system B. Therefore, chitin made up only 30% of the particulate material formed from UDP-N-acetyl[U-^{14}C]GlcNH$_2$ in the presence of 20 mM GlcNAc in the slime mutant homogenate. In contrast, chitin made up 84% of the particulate material in the mycelial homogenates in the presence of 20 mM GlcNAc. On this basis, the chitin synthetase activities (GlcNAc stimulated) of the slime

Table 25. Characterization of the particulate material formed from UDP-N-acetyl [U-^{14}C]GlcNH$_2$ by homogenates of the slime mutant and wild type mycelia of Neurospora crassa.

Organism	Assay condition	Radioactivity in* 40,000 x g pellet (cpm)		Soluble radio-** activity after chitinase digestion and ultrafiltration (cpm)	Neutral fraction from MB-3 column after chitinase digestion and ultrafiltration (cpm)	% chitin of original 40,000 x g pellet
		original	after 1N KOH digestion			
slime	control	260,000	202,400	36,205	8,996	3.5
	+20 mM GlcNAc	270,000	210,096	97,448	81,870	30.3
wild type mycelium	control	227,408	204,542	38,219	----	<16.8
	+20 mM GlcNAc	229,248	----	214,332	192,568	84.0

* the radioactivity in the 40,000 x g pellet does not represent the total radioactivity incorporated in the assay but represents the aliquot taken for this experiment.

** chitinase digested material was ultrafiltered through an UM-2 membrane to obtain a soluble fraction.

mutant and mycelial homogenates could be estimated at 7.6 units/mg protein and 51.2 units/mg protein respectively.

5.10 Effect of colloidal chitin on the incorporation of radioactivity from UDP-N-acetyl[U-^{14}C]GlcNH$_2$ into particulate material by homogenates of the slime mutant and wild type mycelia of Neurospora crassa

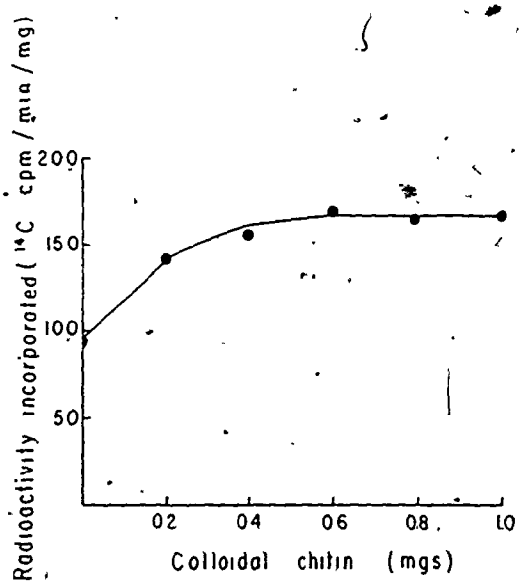
Since the slime mutant does not have any chitin associated with it, colloidal chitin was added to the assay system to act as a possible primer. Colloidal chitin stimulated the incorporation of radioactivity from UDP-N-acetyl[U-^{14}C]GlcNH$_2$ into particulate material in the slime mutant homogenate with saturation at 0.5 mg colloidal chitin (Figure 38). This contration also stimulated incorporation by the mycelial homogenate (Table 26). The GlcNAc stimulated activity of the slime mutant was not effected by colloidal chitin but the wild type mycelial incorporation was doubled. The presence of colloidal chitin in the incorporation experiments increased the amount of chitinase sensitive particulate material present in the slime mutant homogenate (Table 27). However, even in the presence of colloidal chitin, the slime mutant GlcNAc stimulated incorporation did not increase to the levels of the GlcNAc stimulated activity of the mycelial homogenate.

5.11 Effect of trypsin digestion on the incorporation of radioactivity from UDP-N-acetyl[U-^{14}C]GlcNH$_2$ into particulate material by homogenates of the slime mutant and wild type mycelia of Neurospora crassa

Trypsin digestion of the crude homogenate from the slime

Figure 38. Effect of colloidal chitin on the incorporation of radioactivity from UDP-N-acetyl[U-^{14}C] $GlcNH_2$ into particulate material by a homogenate of the slime mutant of Neurospora crassa.

Figure 39. Effect of trypsin digestion on the incorporation of radioactivity from UDP-N-acetyl[U^{14}C] $GlcNH_2$ into particulate material by a homogenate of the slime mutant of Neurospora crassa.


Radioactivity incorporated (^{14}C cpm / min / mg)
200
150
100
50
02
04
06
08
1.0
Colloidal chitin (mgs)

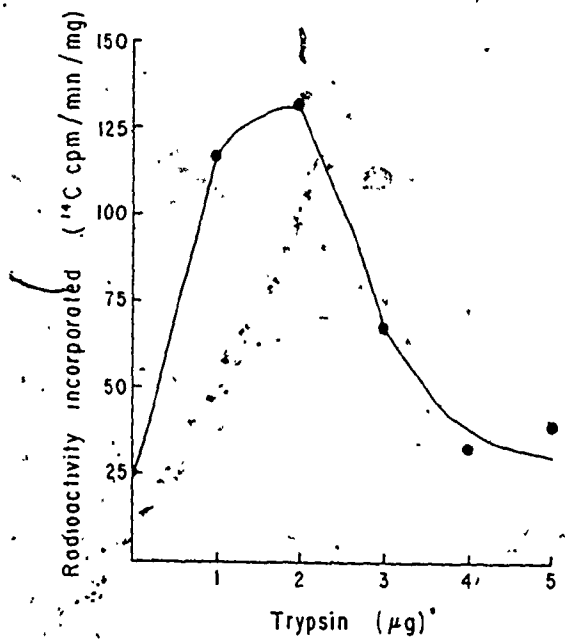

Radioactivity incorporated (^{14}C cpm / min / mg)
150
125
100
75
50
25
1
2
3
4
5
Trypsin (μg)

Table 26. Effect of colloidal chitin on the incorporation of radioactivity from UDP-N-acetyl[U-^{14}C]GlcNH$_2$ into particulate material by homogenates of the slime mutant and wild type mycelia of Neurospora crassa.

Assay condition	Radioactivity incorporated (cpm/min/mg protein)	
	slime mutant	wild type mycelia
control	125.4 ± 8.7	113.0 ± 16.7
+ colloidal chitin (0.5 mg)	164.2 ± 23.3	142.3 ± 9.3
+ 20 mM GlcNAc	324.4 ± 27.9	738.8 ± 133.2
+ colloidal chitin (0.5 mg) and 20 mM GlcNAc	350.8 ± 31.2	1953.0 ± 459.0

Table 27. Chitinase sensitive particulate material of homogenates of the slime mutant and wild type mycelia of Neurospora crassa after incorporation of radioactivity from UDP-N-acetyl[U-^{14}C]GlcNAc.

Assay condition	% chitinase solubilized material	
	slime mutant	wild type mycelia
control	13.9	16.8
+ 20 mM GlcNAc	36.1	93.5
+ colloidal chitin (0.5 mg)	48.3	14.1
+ colloidal chitin (0.5 mg) and 20 mM GlcNAc	62.3	----

mutant resulted in increases in the incorporation of radioactivity into particulate material. The curve of activity of the homogenate after trypsin treatment versus trypsin concentration was bell shaped (Figure 39). The homogenates of mycelia could be activated in a similar manner but the optimum trypsin concentrations were slighly lower (less than 1 µg trypsin). The maximal activity of the slime mutant was only approximately 10% of the activity obtained with the mycelial homogenates either in the presence or absence of GlcNAc. Colloidal chitin stimulated the trypsin activated incorporation of the slime mutant homogenate only 20% (Table 28). The trypsin treatment increased the proportions of chitin of the total particulate fraction in the slime mutant and mycelial homogenates (Table 29). On the basis of the chitin formed the specific activity of the latent form of chitin synthetase of the slime mutant homogenate could be estimated at 3.5 units/mg and 23.4 units/mg in the presence and absence of 20 mM GlcNAc respectively. The latent activity of the mycelial homogenate was approximately 49.6 units/mg and 550.5 units/mg under the same conditions. Therefore, slime mutant homogenate had only 5% of the latent chitin synthetase activity.

In contrast to initial incorporation rates of the slime mutant homogenate which varied from one preparation to the next, the trypsin activated activity was remarkably constant. This suggested that the initial activity was different than

Table 28. Effect of trypsin digestion on the incorporation of radioactivity from UDP-N-acetyl[U-^{14}C]GlcNH$_2$ into particulate material by homogenates of the slime mutant and wild type mycelia of Neurospora crassa.

Organism	Radioactivity incorporated (cpm/min/mg protein)			
	control	after trypsin		
		control	+ 20 mM GlcNAc	+ colloidal chitin (1.0 mg).
wild type mycelia	92.0 ± 35.5	1450.5 ± 56.5	7085.1	----
slime mutant	20.8 ± 99.5	165.0 ± 20.2	467.5	198.5

Table 29. Characterization of the particulate material formed from UDP-N-acetyl[U-^{14}C]-$GlcNH_2$ by the trypsin treated homogenates of the slime mutant and wild type mycelia of Neurospora crassa.

Organism	Assay condition	Radioactivity* in 40,000 x g pellet (cpm)		Soluble** radioactivity after chitinase digestion and ultrafiltration (cpm)	Neutral fraction from MB-3 column after chitinase digestion and ultrafiltration (cpm)	% chitin of original 40,000 x g pellet
		original	after 1N KOH digestion			
slime	after trypsin	280,000	229,455	87,690	75,764	27.1
	after trypsin +20 mM GlcNAc	250,000	205,755	155,503	125,124	50.1
wild type mycelium	after trypsin	253,782	---	84,850	---	<43.3

* the radioactivity in the 40,000 x g pellet does not represent total radioactivity incorporated in the assay but represents the aliquot taken for this experiment.

** chitinase digested material was ultrafiltered through UM-2 membrane to obtain a soluble fraction.

*** all the neutral fractions from MB-3 column were identified by chromatography as GlcNAc.

the trypsin activated activity. Indeed, the initial incorporation activity could be decreased by aging without any effect on the trypsin activated activity (Table 30).

5.12 Effect of PMSF on the incorporation of radioactivity from UDP-N-acetyl[U-^{14}C]GlcNH$_2$ into particulate material by homogenates of the slime mutant of Neurospora crassa

The slime mutant does not make chitin in vivo but does have measurable chitin synthetase activity under in vitro conditions. Since this chitin synthetase existed in an active and latent form, perhaps the active form found under in vitro conditions was the result of activation of the latent form during the preparation of the homogenate. In order to test this possibility PMSF, a general protease inhibitor, was added to the homogenization buffer. PMSF had no effect on the activity of the slime mutant homogenate; if anything, there was a slight stimulation of activity (Table 31).

DISCUSSION

The estimation of chitin levels by determining the amounts of glucosamine released by acid hydrolysis does present some problems in regard to possible contamination by other hexosamines found in Neurospora crassa polymers. Galactosamine, quinovosamine (2-amino-2,6-dideoxyglucose) and non-chitinous glucosamine have been found in Neurospora crassa mycelium (Livingston, 1969). The galactosamine polymers in Neurospora crassa are completely soluble in alkali (Mahadevan and Tatum, 1965). This was confirmed in the [^{3}H]glucosamine incorporation experiments of slime cells where 1N KOH digestion solu-

Table 30. Effect of aging on the incorporation of radioactivity from UDP-N-acetyl[U-^{14}C]GlcNH$_2$ into particulate material by homogenates of the slime mutant of Neurospora crassa.

Experiment	Assay condition	Radioactivity incorporated (cpm/min/mg protein)	
		fresh homogenate	aged* homogenate
1	control	86.2	33.6
	after trypsin	106.7	136.5
2	control	99.5	40.4
	after trypsin	177.1	155.1

* aged at 4°C for 4.5 hours.

Table 31. Effect of PMSF on the incorporation of radioactivity from UDP-N-acetyl[U-^{14}C]GlcNH$_2$ into particulate material by homogenates of the slime mutant of Neurospora crassa.

Experiment	Assay condition	Radioactivity incorporated (cpm/min/mg protein)	
		buffer	PMSF buffer*
1	control	84.9	65.9
	+ 20 mM GlcNAc	296.5	408.0
2	control	91.2	121.6
	+ 20 mM GlcNAc	353.5	409.2

* the slime mutant was lyzed in normal buffer containing 3 mM PMSF. In order to keep the PMSF in solution, the buffer also contained 0.5% ethanol.

bilized material which released radioactive galactosamine upon acid hydrolysis. Therefore, pretreatment of the particulate fraction with 1N KOH eliminated any possible contamination by galactosamine. This technique gives an estimation of the maximum possible chitin present in each case; slime cells had, at most, only 7% of the chitin content of mycelia. Wiltse (1969) had previously found that an isolated "cell wall" fraction from the slime mutant (F.G.S.C. no. 326) completely lacked glucosamine or galactosamine after acid hydrolysis. Slime cells have been found to excrete "wall-like" material into the growth medium (Woodward and Woodward, 1968; Trevithick and Galsworthy, 1977). However, the examination of the growth medium failed to show the presence of any significant amounts of glucosamine in high molecular weight polymers.

Although these analyses indicated that the slime cells did not produce much chitin, they did not show us whether or not the glucosamine present in the particulate material was derived from chitin, or if chitin was excreted as small molecular weight material (not sedimented by centrifugation at 101,000 x g). To investigate these two questions, a comparison of the metabolism of [^{3}H]glucosamine between wild type mycelia and slime cells was conducted.

The major radioactive fraction of wild type mycelia after [^{3}H]glucosamine incorporation was the alkali resistant particulate fraction commonly designated as the "chitin fraction"

(Mahadevan and Tatum, 1965). Accordingly this fraction was completely solubilized by chitinase digestion. In contrast, the same fraction from the slime cells contained only a small proportion of the total radioactivity incorporated and was only partially solubilized by chitinase. The solubilized portion was not N-acetylglucosamine nor N,N diacetylchitobiose, the two products normally released from chitin by impure chitinase preparations (Jeuniaux, 1966). The alkali soluble fraction of the slime cells was also not chitin material as acid hydrolysis did not release glucosamine. The slime cells did not excrete any more radioactivity into the growth media (MW >5000) than did wild type mycelia. Also, this fraction was not susceptible to chitinase digestion. The slime cells therefore do not contain chitin.

One cannot rule out the possibility that the small amount of chitinase sensitive material found in the slime mutant was the polymer chitosan (deacetylated form of chitin) which after hydrolysis by chitinase would give [^{3}H]glucosamine, a product which would be bound by the MB-3 column. Interestingly, approximately 50% of the radioactivity found in this fraction was soluble in 1N HOAc, as is chitosan (Bartnicki-Garcia and Nickerson, 1962).

A comparison of the metabolism of [^{3}H]glucosamine in slime cells and wild type mycelia immediately indicated that slime cells could not transfer soluble radioactivity into the particulate fraction as well as wild type mycelia. The presence of significant amounts of galactosamine containing

polymers in the slime particulate fraction suggested that the chitin pathway up to the enzyme chitin synthetase was intact since UDP-GalNAc probably is the immediate precursor of the galactosamine containing polymers (Edson and Brody, 1976). UDP-GalNAc can be formed from UDP-GlcNAc via 4' epimerization in Neurospora crassa as it is in bacteria (Glaser, 1959) and mammals (Davidson, 1966). Indeed, the major radioactive soluble metabolite of [^{3}H]glucosamine was identified as UDP-GlcNAc. Smaller amounts of UDP-GalNAc were also present.

UDP-GlcNAc has been shown to be very sensitive to concentrations of ammonium formate greater than 1 N (Molnar et al., 1971). Therefore, the small quantities of GlcNAc and GlcNAc-1-PO_4 found could be artifacts arising from the breakdown of the major metabolite UDP-GlcNAc. UDP-GlcNAc has been shown to be the major soluble metabolite in the chitin pathway in Neurospora crassa (Schmit et al., 1975) as well as in Aspergillus parasiticus (McGarrahan and Maley, 1965).

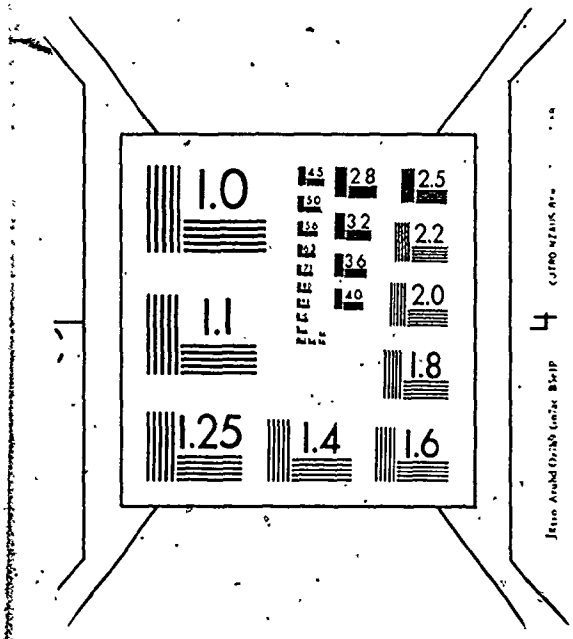
The radioactivity in the soluble fraction of the slime mutant also "turned over" into the particulate fractions but not into chitin. These results indicated a probable defect in the enzyme chitin synthetase. Deletions prior to the formation of glucosamine-6-phosphate could have also been partly responsible for the lack of chitin synthesis and they would not have been detected by the [^{3}H]glucosamine incorporation experiments since glucosamine enters the chitin pathway at the level of glucosamine-6-PO_4.

The growth of the slime organism in the presence of N-acetyl glucosamine as an alternate carbon source did not affect the chitin levels, another indication that a block existed towards the end of the chitin pathway, since N-acetyl glucosamine enters the pathway at the level of N-acetyl glucosamine-6-PO_4 (McGarrahan and Maley, 1965). Furthermore, the first enzyme of the chitin pathway, L-glutamine-fructose-6-PO_4 transamidase, had a specific activity greater than the wild type enzyme. The specific activities obtained for this enzyme were much lower than determined by Endo et al. (1970b) (3.5 units/mg) but similar to values obtained by Ghosh et al. (1960) (0.16 units/mg) for Neurospora crassa. Although early deletions in the chitin pathway can be eliminated, possible further blocks in glycolytic enzymes cannot be elimminated but are unlikely considering the excellent growth of the organism on sucrose.

Although the slime mutant of Neurospora crassa does not have chitin associated with it, homogenates from this organism do exhibit a small amount of chitin synthetase activity. Numerous reasons to explain this apparent paradox are possible: 1) since chitin synthetase existed in a latent form which could be activated by protease digestion, the chitin synthetase activity found in the homogenates could have resulted from non-specific activation during homogenization, 2) chitin synthesis requires primer chitin which was not present in the slime mutant, 3) GlcNAc plays an activator role in vivo and

3 3

OF/DE



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since the slime mutant has no source of GlcNAc (assuming it results from chitinase digestion of chitin), there is no chitin synthesis, 4) the slime mutant has a small amount of chitosan associated with it, 5) the *in vivo* chitin was lost due to chitinase digestion. Each possibility is discussed in some detail in the following paragraphs.

Chitin synthetase in the slime mutant exists in two forms, an active form and a latent form which can be activated by protease action. The active form could arise from the latent form *in vitro* by the non-specific action of proteases during homogenization. The addition of PMSF, a potent inhibitor of alkaline protease and carboxypeptidases of *Neurospora crassa* (Siepen *et al.*, 1975) had no effect. It should be kept in mind that the homogenization buffer contained EDTA which inhibits alkaline proteases and aminoproteases of *Neurospora crassa*. Even though the active form represented only a small proportion of the potential activity, linear rate curves were obtained upon incubation with UDP-GlcNAc indicating the lack of general protease activation during this time. Therefore, the active form of chitin synthetase measured in our analysis was most likely present in the cell.

The possible role of primer chitin in the chitin synthetase reaction is far from clear. In that respect, the slime cell which was completely devoid of chitin was still able to synthesize chitin *in vitro* indicating that primer chitin was not required. Even the addition of primer chitin to the

in vitro assay system did not have much stimulating effect.

The role of GlcNAc in *in vivo* chitin synthesis is unknown although under *in vitro* conditions it has been found to be an allosteric activator in numerous fungal systems (deRousset-Hall and Gooday, 1975; Ruiz-Herrera *et al.*, 1977). The apparent Km for UDP-GlcNAc in the *Neurospora crassa* chitin synthetase reaction was 1.43 mM (Endo *et al.*, 1970a) but it was unlikely that this concentration existed *in vivo* since UDP-GlcNAc was a potent inhibitor (K_i 5 x 10^{-6}M) of the first enzyme of the chitin pathway, L-glutamine D-fructose-6-PO_4 transamidase in *Neurospora crassa* (Endo *et al.*, 1970b). Since polyoxin D had no effect on the endogenous levels of UDP-GlcNAc (Endo *et al.*, 1970b), the level of UDP-GlcNAc *in vivo* must already be high enough to inhibit L-glutamine D-fructose-6-PO_4 transamidase. The UDP-GlcNAc concentration in *Blastocladiella emersonii* (Selitrennikoff *et al.*, 1976) has been estimated to be 0.17-0.63 mM; a concentration where GlcNAc would have a stimulatory effect. However, the addition of GlcNAc (0.1 M) to the slime mutant as a carbon source did not significantly increase the amount of glucosamine in the particulate fraction.

The slime cells did contain a small amount of material which could have been chitosan. If the formation of chitosan involves the initial formation of chitin followed by deacetylation as suggested by the results of Araki and Ito (1974), then perhaps this would explain the small amounts of chitin

synthetase found *in vitro*.

A more plausible explanation exists if one considers that the slime cells originated from conidia which contain a normal cell wall. The elimination of the wall from these cells could only occur by the degradation of the already existing wall. If the growth of the hyphae involves the continous digestion and resynthesis of wall material, then an increase in chitinase without a concomitant increase in chitin synthetase would result in the destruction of the wall. A normal level (mycelial level) of chitinase combined with the low amount of chitin synthetase in the slime mutant would result in the loss of the chitin fraction.

The slime mutant is the result of three morphological mutations referred to as osmotic, fuzzy and spontaneous germination. Wiltse (1969) found that the osmotic, fuzzy and the double mutant, fuzzy-osmotic grew normally and contained normal levels of chitin. The slime condition was therefore a result of the spontaneous germination locus. Although the slime cell did not form chitin, it also lacks other normal cell-wall components like β-1,3 glucan since it did not stain with Calcofluor. It is unlikely that the loss of this component was a result of chitin synthesis defects since regenerating protoplasts formed wall-like material in the absence of chitin synthesis (see chapter IV). The spontaneous germination mutation must also involve the other components of the wall.

The spontaneous germination mutant (Emerson, 1963) of Neurospora crassa undergoes lysis during conidial germination. Another mutant, the ts6 mutant of Aspergillus nidulans which is specifically blocked in chitin synthesis also undergoes lysis during conidial germination (Katz and Rosenberger, 1971a). Perhaps, the lack of chitin synthesis in the slime mutant is one reason for the protoplast-like growth pattern found in this Neurospora crassa mutant.

CHAPTER VI CHITIN SYNTHESIS IN NEUROSPORA CRASSA GERMINATING CONIDIA

It has been shown by Endo et al. (1970a) that polyoxin D had no effect on the rate of formation of germ tubes from Neurospora crassa conidia; yet, once the germ tubes had formed, polyoxin D inhibited their growth. In our reverting protoplast system, polyoxin D at concentrations equivalent to those used in the conidial system, inhibited the reversion process. This paradox could have been the result of either the lack of uptake of polyoxin D by the conidia or the lack of chitin biosynthesis during the germination process. Experimental evidence in support of either explanation exists. The K_i for polyoxin D in the chitin synthetase reaction was 100 fold less than the I_{50} for growing cells (Endo et al., 1970a) indicating that polyoxin D had difficulty entering the hyphae. On the other hand, Schmit et al. (1975) have indicated that chitin appeared to be incorporated into the wall of germinating conidia at low density or not at all during early germ tube formation. The experiments that follow were initiated to clarify this point.

RESULTS

6.1 Effect of chitin inhibitors on conidial germination

The initial observation of Endo et al. (1970a) was confirmed (Figure 40); a range of concentrations of polyoxin D from 2×10^{-3}M to 2×10^{-6}M had no effect on the rate of germination of Neurospora crassa conidia (although the germ tubes

Figure 40. Effect of polyoxin D on the germination of Neurospora crassa conidia.

control ● ——

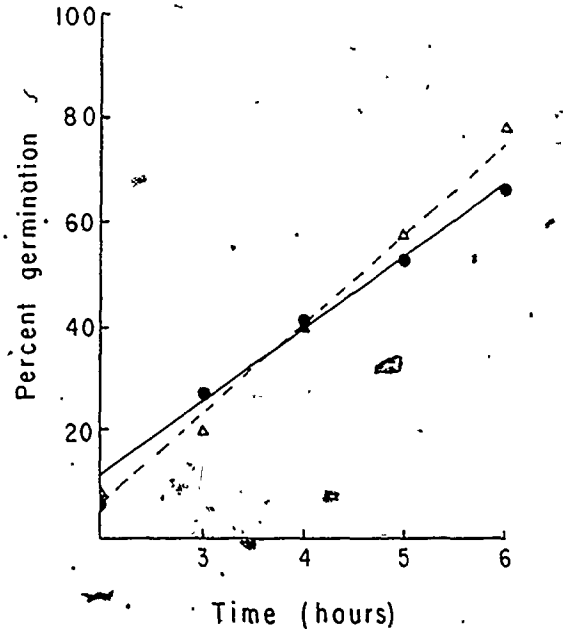
polyoxin D (2×10^{-4}M) Δ ---

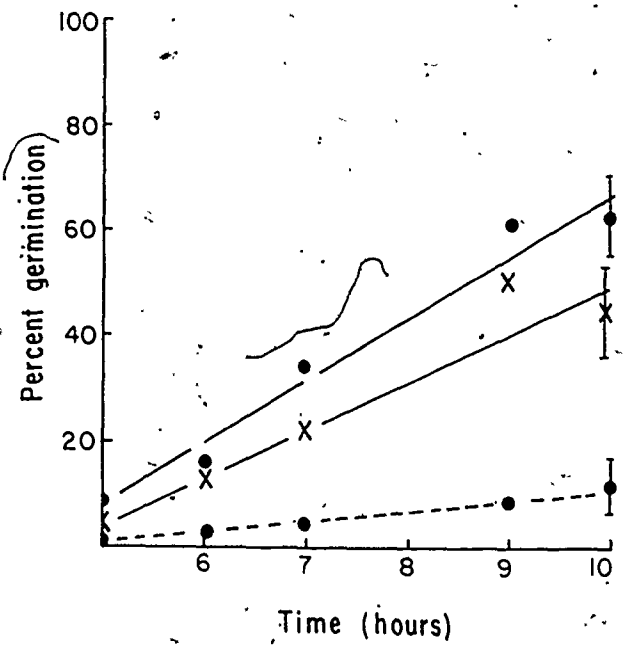
Figure 41. Effect of kitazin P on the germination of Neurospora crassa conidia.

control ● ——

kitazin P 3.78×10^{-5}M x ——

3.78×10^{-4}M ● ---


100
80
60
40
20
Percent germination
3
4
5
6
Time (hours)


100
80
60
40
20
Percent germination
6
7
8
9
10
Time (hours)

formed were distorted at the higher polyoxin D concentrations). Similar concentrations of polyoxin D inhibited hyphal chitin synthetase under *in vitro* conditions, 10^{-4}M giving almost complete inhibition (see Figure 37).

The effects of polyoxin D on germination in the presence of 5% DMSO (to increase membrane permeability) were impossible to interpret due to the large effects of DMSO itself.

Kitazin P (Figure 4), an organo-phosphorus fungicide, is also an inhibitor of chitin biosynthesis (Kakiki *et al.*, 1969; Misato and Kakiki, 1977). This compound significantly inhibited the germination of conidia at levels of 3.78×10^{-5}M and 3.78×10^{-4}M (Figure 41). In this experiment, the control conidia germinated at a slower rate than usual since older conidia were used. *In vitro* investigation of the effects of kitazin P on hyphal chitin synthetase activity showed that concentrations up to 3.78×10^{-4}M had little inhibitory effect (Table 32). Hence, the *in vivo* effect of kitazin P on conidial germination cannot be a result of interference with chitin synthesis.

6.2 Effect of polyoxin D on [^{3}H]glucosamine incorporation during conidial germination

The uptake of polyoxin D can be investigated indirectly by examination of its effect on [^{3}H]glucosamine incorporation into chitin during conidial germination provided that it has no effect upon the transport of [^{3}H]glucosamine into conidia, and that there is detectable chitin synthesis during conidial germination. Preliminary experiments showed that polyoxin D

Table 32. Effect of kitazin P on chitin synthetase activity of wild type mycelia* of Neurospora crassa.

Kitazin P concentration ($M \times 10^{-6}$)	Chitin synthetase activity (^{14}C cpm incorporated /15 minutes)	% of control activity
0	4575 ± 397	100.0 ± 8.7
3.78	4335 ± 250	95.0 ± 5.5
37.8	4840 ± 657	105.8 ± 13.6
378	3061 ± 287	66.9 ± 9.4

* standard assay system included 20 mM N-acetylglucosamine

concentrations of 2×10^{-5}M and 2×10^{-4}M had no effect on the total uptake of [^{3}H]glucosamine by the germinating conidia over a ten hour period (Figure 42).

The germinating conidia incorporated a large percentage of the [^{3}H]glucosamine into a fraction that sedimented at 40,000 x g. Both concentrations of polyoxin D (2×10^{-5}M and 2×10^{-4}M) significantly reduced the levels of radioactivity in this fraction, the degree of inhibition becoming less with time (Figure 43). The inhibition began to decrease between two and four hours after innoculation; the same time interval at which germ tubes were forming. Extrapolation of the control lines indicated that significant incorporation of radioactivity was evident as early as one hour. This was confirmed by examining the incorporation of radioactivity into the particulate fraction at one hour in the presence and absence of polyoxin D (2×10^{-5}M) (Table 33); polyoxin D inhibited up to 69%. Thus, polyoxin D was obviously taken up by germinating conidia to some degree.

The particulate fractions were analyzed for chitin. As shown in Table 35, the bulk of each sample was resistant to 1N KOH digestion but only partly susceptible to hydrolysis by chitinase. Approximately 85% of the chitinase digested products at six hours were identified as GlcNAc by paper chromatography (R_f - 0.57, system B). On this basis, chitin accounted for 30% of the particulate fraction at one hour, 43% at six hours and 54% at 24 hours (see chapter V).

Figure 42. Effect of polyoxin D on the uptake of [^{3}H]glucosamine by germinating Neurospora crassa conidia.

control ●

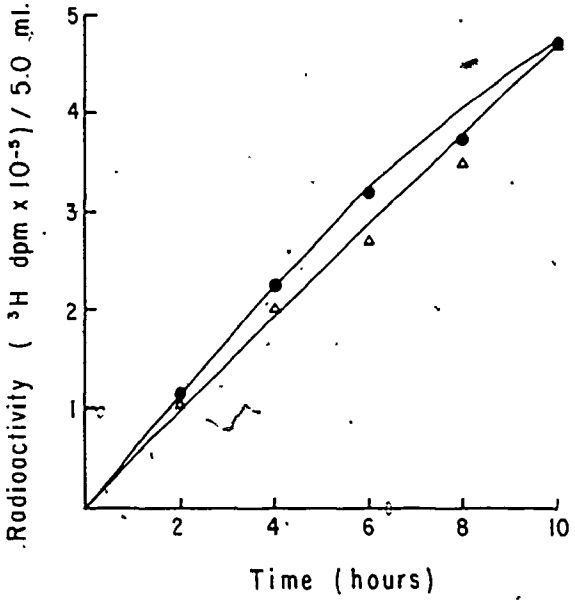
polyoxin D (2×10^{-4}M) Δ

Figure 43. Effect of polyoxin D on the incorporation of radioactivity from [^{3}H]glucosamine into the particulate fraction of Neurospora crassa germinating conidia.

control ● —

polyoxin D 2×10^{-5}M Δ – – –

2×10^{-4}M Δ —


Radioactivity (³H dpm x 10⁻³) / 5.0 ml.
5
4
3
2
1
2
4
6
8
10
Time (hours)

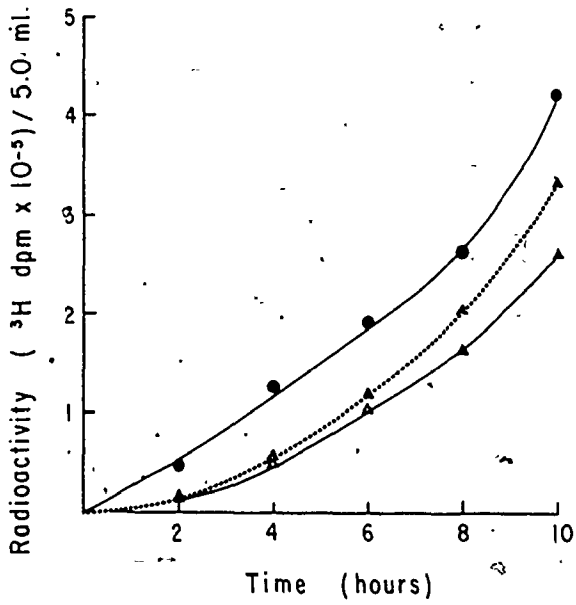

Radioactivity (³H dpm x 10⁻³) / 5.0 ml.
5
4
3
2
1
2
4
6
8
10
Time (hours)

Table 33. Effect of polyoxin D on the incorporation of [^{3}H]glucosamine into conidia at one hour.

Sample	Total [^{3}H] incorporated (dpm)	[^{3}H] incorporated into the 40,000 x g pellet	% inhibition of [^{3}H] incorporation into 40,000 x g pellet
control	54003	29500	----
polyoxin D (2×10^{-5}M)	54906	9086	69.2

Table 34. Effect of polyoxin D on the incorporation of [^{3}H]glucosamine into the particulate fraction of ethylene glycol treated conidia.

Time (hours)	[^{3}H] incorporated into the 40,000 x g pellet (dpm)		% inhibition of [^{3}H] incorporation into 40,000 x g pellet
	control	polyoxin D (2×10^{-5}M)	
1	10770	7833	27.3
2	17960	12319	30.4
3	24024	16769	30.2
24	503656	497784	1.2
48	1016924	974276	4.2

Table 35. Chitinase digestion of the particulate fractions from *Neurospora crassa* conidia after [^{3}H]glucosamine incorporation.

Sample	Time (hours)	[^{3}H] in 40,000 x g pellet (dpm)		[^{3}H] solubilized by chitinase (dpm)	% solubilized by chitinase	% inhibition by polyoxin D		
		original	after 1N KOH digestion			40,000 x g pellet	Chitinase sensitive fraction	Chitinase resistant fraction
control	1	30,289	22,346	9,208	30.6	----	----	----
	6	217,568	191,832	94,148	43.3	----	----	----
polyoxin D (2×10^{-4}M)	1	9,086	----	3,507	38.6	70.0	62.2	73.4
	6	105,015	107,411	69,138	65.8	51.7	26.6	70.9
ethylene glycol	1	11,003	8,766	2,754	25.3	----	----	----
	48	1,017,157	898,093	554,923	54.6	----	----	----

Polyoxin D (2×10^{-4}M) inhibited the chitinase sensitive fraction 62% at one hour but this was reduced to 26% by 6 hours. In contrast, the chitinase resistant fraction was inhibited 70% at either time point. The chitinase sensitive fraction formed by 6 hours was a mixture of germ tube and conidial material since only 50% of the conidia had formed germ tubes at this time. Since the conidial chitinase sensitive fraction was much more susceptible to inhibition by polyoxin D than the same fraction in the germ tube, the inhibition in germ tube chitin synthesis at 6 hours would be less than 26%. The poor inhibition of chitin synthesis may explain the lack of a visible effect of polyoxin D on germ tube formation during germination. The small effect on chitin synthesis could be the result of an insufficient in vivo polyoxin D concentration to inhibit chitin synthetase.

6.3 Effect of polyoxin D on [^{3}H]glucosamine incorporation in ethylene glycol treated conidia.

Bates and Wilson (1974) found that Neurospora crassa conidia grew as single cells in the presence of 3.22 M ethylene glycol and that removal of ethylene glycol by dilution after 48 hours of contact resulted in synchronous germ tube formation. The effect of polyoxin D on germination in this system was examined in the hope that the ethylene glycol treatment would increase the in vivo concentration of polyoxin D.

The results of Bates and Wilson (1974) were confirmed (Figure 44). The time of contact between conidia and ethylene glycol had an important effect on the number of conidia able

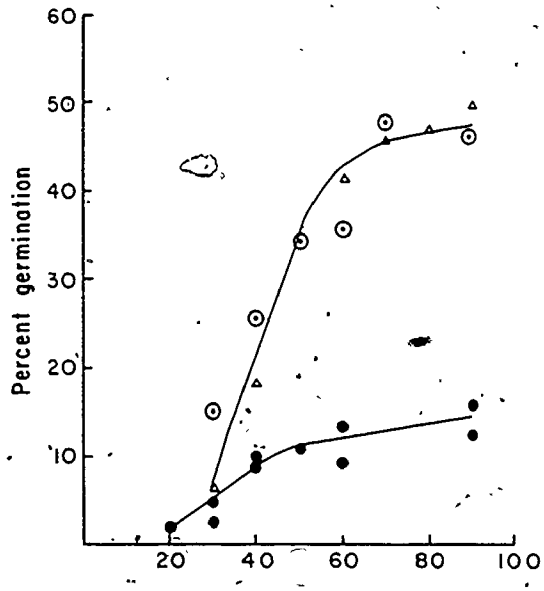
Figure 44. Effect of polyoxin D on the germination of Neurospora crassa conidia after growth in ethylene glycol medium.

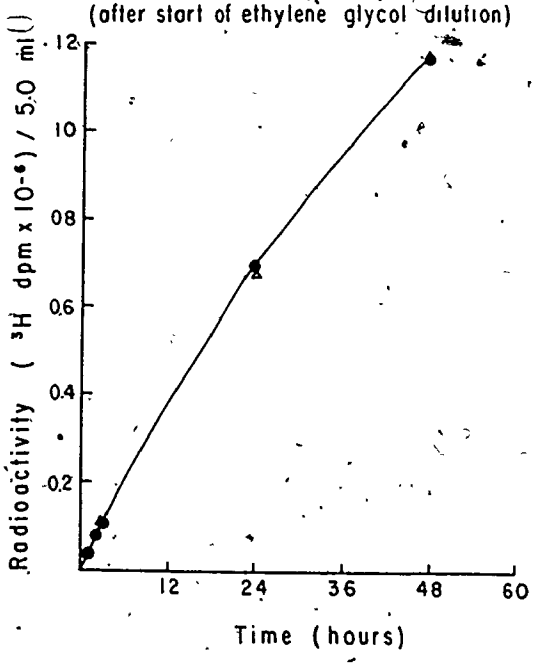
- treated with ethylene glycol for 24 hours ●
- treated with ethylene glycol for 48 hours ⊙
- treated with ethylene glycol for 48 hours in the presence of polyoxin D (2×10^{-5}M) Δ

Figure 45. Effect of polyoxin D on the uptake of [^{3}H] glucosamine by Neurospora crassa conidia in the presence of ethylene glycol.

control ●

polyoxin D (2×10^{-5}M) Δ


Percent germination
60
50
40
30
20
10
20
40
60
80
100
Time (mins.)
(after start of ethylene glycol dilution)


Radioactivity (³H dpm x 10⁻⁶) / 5.0 ml
12
10
08
0.6
0.4
0.2
12
24
36
48
60
Time (hours)

to germinate. At least 48 hours of contact was required to obtain synchronous germ tube formation but only 50% viability was obtained. The addition of polyoxin D (2×10^{-5}M) at time zero or at 24 hours had no effect on the ability of the treated conidia to form a germ tube (Figure 44).

The conidia grown in the presence of ethylene glycol took up substantial quantities of [^{3}H]glucosamine (Figure 45); this uptake was not affected by polyoxin D (2×10^{-5}M). Part of the radioactivity appeared in the 40,000 x g pellet. Although polyoxin D inhibited the incorporation of radioactivity into this fraction initially, the inhibition caused by the antibiotic was reduced to zero (Table 34) after 24 hours; well before the ability to form germ tubes was examined. The lack of effect of polyoxin D on germ tube formation in this system could be due to the lack of transport into the cell, since it had no effect on *in vivo* chitin synthesis.

DISCUSSION

The relationship between chitin synthesis and germ tube development in germinating conidia was investigated by examining the effects of chitin inhibitors on germination.

Kitazin P (3.78×10^{-4}M) strongly inhibited germ tube formation; yet this concentration had little effect upon mycelial *in vitro* chitin synthetase activity, indicating that disruption of conidial germination was not due to inhibition of chitin synthesis. Possibly, its interference with conidial germination was a result of a general membrane disruption, as previously demonstrated in *Pyricularia oryzae* (Kakiki *et al.*,

1969; Maedo et al., 1970).

Polyoxin D, an inhibitor of chitin synthetase had little effect on conidial germ tube formation even at high concentrations. However, it inhibited up to 70% the incorporation of [^{3}H]glucosamine into the 40,000 x g pellet. During the first ten hours of germination the degree of inhibition was reduced from 70% to 21% and 38% with polyoxin D, 2×10^{-5}M and 2×10^{-4}M respectively. A similar result was found in the ethylene glycol treated conidia where the inhibitory effect went from 30% to zero (polyoxin D, 2×10^{-5}M) over 24 hours. Although polyoxin D is a structural analog of UDP-GlcNAc and as such a competitive inhibitor of chitin synthetase, it was unlikely that the inhibition was reversed by increasing amounts of UDP-GlcNAc since Schmit et al. (1975) have shown that the level of this compound actually decreases during germination. The radioactivity in the 40,000 x g pellet consisted of chitinase digestible and chitinase resistant material; the chitinase solubilized fraction made up an increasing proportion of the incorporated radioactivity as germination progressed. This situation also occurred with ethylene glycol treated conidia. The inhibition of the chitinase resistant fraction by polyoxin D remained constant but the inhibition of the chitinase sensitive fraction was reduced and, therefore, the overall inhibition decreased.

At six hours polyoxin D (2×10^{-4}M) inhibited chitin synthesis a maximum of 26%. The lack of effect of polyoxin D on germ tube formation by Neurospora crassa conidia was not a

result of the absence of chitin synthesis at this time as suggested by Schmit and Brody (1976) but more likely a result of poor penetration by the antibiotic. Poor penetration of polyoxin D has been demonstrated in the yeast S. cerevisiae (Bowers et al., 1974). Also, Hori et al. (1974) suggested that the existance of resistant strains of Alternaria kikuchiana to polyoxin D was due to poor transport of the antibiotic.

One can argue that chitin synthesis plays no role during germination as it apparently does in germ tube elongation, thus explaining the lack of effect of polyoxin D on germination. Such an argument is unlikely, however, since polyoxin D has been found to inhibit germination in Mucor rouxii (Bartnicki-Garcia, and Lippman, 1972b) and Trichoderma viride (Benitez et al., 1976).

Although the specificity of polyoxin D has been demonstrated in numerous systems, one would expect it to act on other enzymes which involve UDP-GlcNAc since it is essentially a structural analog of this compound. Indeed, in germinating conidia of Neurospora crassa, polyoxin D inhibited the formation of a chitinase resistant fraction to a much greater extent than it inhibited chitin synthesis. Even though the inhibition of this fraction was 70%, no effect on germ tube formation was apparent. This fraction probably has no direct role in the formation of the germ tube.

The chitinase sensitive fraction from conidia was much

more susceptible to polyoxin D than the same fraction from the germ tube. If this conidial fraction was chitin, then the chitin synthetase of conidia was different from the enzyme located in the germ tube. A more plausible explanation would be that most of the conidial chitinase sensitive fraction was not chitin.

The chitinase sensitive fraction became an increasing proportion of the total radioactivity incorporated in germinating and ethylene glycol treated conidia. However, the ethylene glycol treated conidia did not form germ tubes and if this fraction was chitin as it was in the untreated conidia, then we have an increase in chitin synthesis without germ tube formation. Perhaps this occurrence prepares the ethylene glycol treated conidia for synchronous germ tube formation when the ethylene glycol is removed by dilution. This also indicates that other factors besides chitin synthesis are important in the formation of the germ tube.

CHAPTER VII GENERAL SUMMARY

The exact role of chitin synthesis in germ tube formation in fungi is not well understood but some evidence for its involvement has come from inhibitor studies and the study of various mutants.

In this study of Neurospora crassa protoplast systems, there was a direct relationship between the ability to form chitin and the ability to form a germ tube. The specific chitin synthetase inhibitor polyoxin D prevented protoplast reversion. Although it was difficult to say that the morphological effect was strictly due to the inhibition of chitin synthetase, this antibiotic had no effect on the growth of the slime mutant of Neurospora crassa, an organism which does not synthesize chitin. Also, polyoxin D had no significant effect on conidial germ tube formation or conidial chitin synthesis even though it did penetrate the conidia in sufficient concentrations to inhibit the incorporation of [^{3}H] glucosamine into non-chitinous polymers.

Both the slime mutant of Neurospora crassa and the majority of protoplasts obtained from mycelia were unable to form hypha. The non-reverting mycelial protoplasts had very low amounts of chitin synthetase either in an active or latent form. This condition was probably the result of membrane damage caused by the "snail gut" enzyme used to obtain the protoplasts. Their inability to revert to hyphal growth was more likely the result of damage caused by the snail enzyme rather than their lack of chitin synthetase. On the other

hand, the slime mutant also had low levels of chitin synthetase in an active or latent form which could be responsible for its form of growth.

These results provide evidence for the central role of chitin synthesis in the formation of the germ tube in Neurospora crassa and indicate that cell wall polymers have an important function in determining fungal morphology.

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