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Invertase And Other Exoenzymes In Neurospora I Association With Cell Walls Ii Biochemical And Histochemical Localization During The Asexual Life Cycle

Patricia Lai-yung Chang

INVERTASE AND OTHER EXOENZYMES IN NEUROSPORA

I. ASSOCIATION WITH CELL WALLS II. BIOCHEMICAL AND HISTOCHEMICAL LOCALIZATION DURING THE ASEXUAL LIFE CYCLE

by

Patricia Lai-Yung Chang
Department of Biochemistry

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TO

MY PARENTS

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GODFREY

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CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

GLOSSARY

DAB : 3,3'-diaminobenzidine

EDTA : ethylenediaminetetraacetate

NAD : nicotinamide adenosine diphosphate

NBT : Nitro-Blue Tetrazolium salts

PBS : phosphate buffered saline
0.9% sodium chloride in 0.01M sodium phosphate buffer at pH 7.1

RNA : ribonucleic acid

NSS : nonspecific staining

Note: The conventions used in this dissertation are adopted from "Conference of Biological Editors, Committee on Form and Style. 1964. Style manual for biological journals. 2nd. ed. Amer. Inst. Biol. Sci., Washington, D.C."

ABSTRACT

In fungal cells, secretion of exoenzymes necessitates the passage of macromolecules through the cell wall into the medium. This relationship between the cell walls and exoenzymes of Neurospora is now examined in detail. Histochemical and biochemical localization of a typical exoenzyme, invertase, through the asexual life cycle of the fungus revealed how this relationship varied in different physiological states.

Typical exoenzymes in Neurospora, i.e. ribonuclease, acid protease, amylase, trehalase, aryl-β-glucosidase, and invertase were found to be associated with the cell walls. The amount varied from a few to about 30% of the total cellular activity, depending on the particular enzyme studied.

Attempts were made to dissociate trehalase and invertase from the cell walls with chemical reagents and hydrolytic enzymes. These two enzymes were not significantly released from the cell walls by a detergent (Triton X-100), a sulfhydryl reducing agent (β-mercaptoethanol), a chelating agent (ethylenediaminetetraacetate), concentrated salt solution (1M KCl), and buffers ranging in pH from three to ten. Snail gut juice, containing a variety of hydrolytic

enzymes, released more than 90% of both enzymes. β-1,3-Glucanase, prepared from Bacillus circulans WL-12, also released similar amounts. Chitinase released about 80% of invertase and 60% of trehalase. Cellulase did not release any significant amount of either exoenzyme. Trypsin released only a few per cent of invertase and severely inactivated trehalase.

The association of invertase with cell walls was studied in six cytologically distinct stages of the Neurospora asexual life cycle. These stages were (i) conidia, (ii) mid-log phase hyphae [10 hours old], (iii) post log-phase hyphae [18 hours old], (iv) aerial mycelium [four days old], (v) submerged mycelium [four days old], and (vi) mycelium [two days old] induced to form conidiophores. The percentages of total invertase activity found in the cell wall fractions were 8, 18, 23, 8, 21 and 17, respectively. The specific activities of the cell wall fractions were 3, 85, 120, 40, 24, and 74 units of invertase per mg protein. respectively.

Histochemical localization of invertase in the above six stages was achieved with indirect immunofluorescent staining. In addition to confirming the biochemical changes of invertase distribution in cell walls, it also

showed the following: a) on germination, invertase activity was high in the young hyphal tube wall while it became depleted in the residual conidium; b) intense activity occurred at budding points of mature hyphae; c) the prominent vacuoles in senescent hyphae were devoid of enzyme activity.

Three conclusions are drawn from this investigation. First, significant amounts of exoenzymes are found in fungal cell walls. Second, the association neither depends on chemical bonds such as hydrophobic, disulfide, ionic, metal co-ordination linkages, nor on a single kind of covalent bond. Third, the association of invertase, a typical exoenzyme, varies with the physiological state of the fungus. The significance of the changing distribution of invertase during the Neurospora life cycle is discussed. A hypothesis is offered to account for the occurrence of exoenzymes in fungal cell walls.

CHAPTER I. INTRODUCTION

Prospective. To obtain enough food is the basic requirement for survival in all living organisms. It poses additional problems for micro-organisms such as fungi and bacteria because of their simple organization. Their small sizes limit specialization for food foraging purposes; their energy supply usually depends on complex organic requirements (i.e. they are chemoheterotrophic); they cannot absorb insoluble nutrients (i.e. they are osmotrophic); and they are enclosed by an additional permeability barrier, the cell wall, which prevents more direct contact with substrates in the extracellular milieu (Stanier, Doudoroff & Adelberg, 1970). The fungi and many gram-positive bacteria have solved these problems admirably by secretion of catabolic exoenzymes.

Secretion is a process by which substances produced in the cytoplasm are segregated and transferred to the cell's exterior (Mollenhauer & Morré, 1966). It occurs not only in micro-organisms but also in plants and animals. The substances secreted may include sugars, proteins, hormones, or mucilagenous material. The enzymes

so produced are called exoenzymes. They are usually catabolic catalysts that break down complex materials, e.g. proteins, polysaccharides, lipids etc. into simple forms suitable for passage through the cell wall barrier and uptake by the cytoplasmic membrane.

Before a product can be secreted, it needs to be synthesized in the cytoplasm, transported from the site of synthesis to the cell periphery, and extruded into the external medium. This scheme applies to all secretory processes and Lampen in 1965 reviewed the systems in micro-organisms. A conspicuous absence of information is noticed in one area; namely, how the secreted macromolecules, e.g. enzymes, pass the cell walls of micro-organisms in order to reach the exterior.

Cell walls confer shape and rigidity on a cell (Aronson, 1965). If they are lost, as by enzymatic digestion, the remaining organism is called a "protoplast" which becomes spherical, osmotically and mechanically fragile (Villanueva, 1965). The secretory products after extrusion from the cytoplasm are generally assumed to pass the cell wall by diffusion (Lampen, 1965; Matile, Jost & Moor, 1965; Manocha & Colvin, 1967). As will be seen later in this chapter on historical review, there is a fundamental enigma that challenges such an assumption.

Problem. The present project is an attempt to expose the relationship between exoenzymes and cell walls of fungi in two respects. First, do exoenzymes generally occur in fungal cell walls? If they do, is it a physical association, or are there chemical bonds formed between them? Secondly, does this relationship change with the age of the organism? If it does, what is the significance?

Approach. The model system used in this investigation is an Ascomycete fungus, Neurospora crassa. It was chosen because a considerable volume of information is available about its genetics, biochemistry, cell wall structure and exoenzymes (Burnett, 1968; Eberhart, 1961). In addition, it is easy to handle and the various stages in its life cycle are readily induced for the study of physiological changes.

During preliminary investigation, a significant amount of invertase was found in the cell wall fraction of Neurospora crassa mycelium. Further experiments were designed to:-

a. stimulate production of enzymes known to be secreted by Neurospora,
b. assay for any exoenzyme activity associated with the cell walls.

Six exoenzymes were studied in this way. They were associated with the cell walls to quite different extents.

Of these exoenzymes, invertase and trehalase had the highest per cent of their total activities in the cell walls. The enzyme-containing cell walls were then subjected to the actions of a variety of chemicals and lytic enzymes in an attempt to release the bound enzymes from the cell walls. Hydrolysis of covalent bonds appeared to be necessary. β-1,3-glucanase was the most potent 'lytic enzyme' to release cell-wall-bound exoenzymes.

The second part of this project employed biochemical and histochemical methods to trace the distribution of a typical exoenzyme invertase in six distinct stages of the Neurospora life cycle. The activities of invertase in the purified cell wall fractions varied characteristically with the age of the culture. Generally, younger cell walls had higher specific activities. This was qualitatively confirmed with an immunofluorescent staining technique on histochemical sections of Neurospora. This method also revealed the presence of intense enzyme activity at budding initials and the absence of any activity in vacuoles of old hyphae.

Historical Review. The following is a brief historical review of five topics related to exoenzymes and cell walls.

1. Secretion:-

Palade, Siekevitz & Caro (1962) found that in mammalian pancreas, secretion followed a definite sequence of events. Proteins synthesized by the polysomes at the rough endoplasmic reticulum were discharged into the reticulum cisternal space. They were then segregated by the Golgi apparatus into membrane-bound zymogen granules which were transferred to the cell periphery. The secretory products were extruded by reverse pinocytosis of the plasma membrane.

A similar plan of secretion seemed to prevail in plants (Mollenhauer & Morré, 1966) and in a Phycomycete fungus, Pythium ultimum (Grove, Bracker & Morré, 1970). However, there were some conspicuous differences in micro-organisms. Polysomes attached to endoplasmic reticulum had never been demonstrated in many fungi, including Neurospora (Burnett, 1968). Golgi apparatus, or similar structures in fungi called dictyosomes, have not been found

in a number of septate fungi, although they appeared quite often in the aseptate Phycomycetes, such as P. ultimum. On the other hand, membrane-bound vesicles containing enzyme activities, similar to the zymogen granules, occurred widely in micro-organisms, e.g. lysosomes in yeast (Matile & Wiemken, 1967), protease particles in Neurospora (Matile, Jost & Moor, 1965) and a vesicle-bound fraction of penicillinase in Bacillus licheniformis (Sargent & Lampen, 1970). An apparent exception was Bacillus subtilis in which none of its three exoenzymes was found in any intracellular pool (May & Elliott, 1968a). However, this did not necessarily preclude a vesicular location of inactive precursor proteins.

Membranous or vesicular structures associated with the plasma membrane have generally been inferred to play a direct role in secretion. Palade et al. (1962) showed that reverse pinocytosis by fusion of the plasma membrane with the vacuole membrane was responsible for the liberation of secretory material into the exterior. Grove et al. (1970) proposed that such a mechanism would also incorporate the vesicular membrane into the plasma membrane and contribute to the expansion of the cell surface. Matile et al. (1965) suggested a radically different process for protease secretion in Neurospora. The protease particle at the cytoplasmic periphery was proposed to be engulfed by the invaginating plasma membrane. The particle,

now with its extra coat of plasma membrane, was pinched off into the periplasmic space. There was no fusion of the particle membrane with plasma membrane, in contrast to the proposals by Palade et al.,(1962) and Grove et al.,(1970). Besides these relatively simple membrane-bound vesicles, other prospective organelles with more complicated membranous structures may include mesosomes (myelinoid figures of lamellar or vesicular membranous structures, as defined by Kozar & Weijer, 1969a), and lomasomes (membrane bound tubules or vesicles, as defined by Kozar & Weijer, 1969b). Mesosomes were often found at cell periphery in gram-positive bacteria during septa formation (Abram, 1965). Lomasomes were found in the periplasmic space of fungi, such as Neurospora, and sometimes even connected to the plasma membrane or embedded in the cell wall. Secretion was one of the several functions proposed for them but direct evidence has been lacking (Bracker, 1967).

After the secretory products had been extruded from the cytoplasm, little more was known about their fate. They were generally presumed to be free in the periplasmic space between the plasma membrane and cell wall (the intramural space according to Trevithick & Metzenberg, 1966a). In order to appear in the external medium, they must pass through the cell wall, a process usually assumed to involve diffusion.

2. Exoenzymes:-

Pollock (1962) defined exoenzymes as enzymes liberated by continuous secretion and not by cell lysis with resultant release of intracellular enzymes. Their functions may include destruction of host tissue by pathogens, e.g. α toxin of Clostridium perfringens (Stanier et al.,1970), synthesis of cell walls, e.g. chitin synthetase in Mucor rouxii (McMurrough & Bartnicki-Garcia, 1970), or preliminary hydrolysis of substrates as described in the beginning of this chapter.

Secretion of exoenzymes has several intracellular and extracellular requirements. Protein synthesis was necessary, as shown in Neurospora "protoplasts" by Trevithick & Metzenberg (1964) or in Bacillus subtilis by Elliott & May (1969). B. licheniformis was a notable exception because 25% of its penicillinase secretion was independent of protein synthesis, i.e. not inhibited by chloramphenicol (Sargent & Lampen, 1970). An energy source, such as glucose, was required for invertase secretion by yeast (Lampen, Neumann, Gascon & Montenecourt, 1967). Ca^{++} may be necessary for secretion as well as maintaining the stability of some bacterial proteases (Davis, Dulbecco, Eisen, Ginsberg & Wood, 1967). Secretion of exoenzymes may also depend on

environmental conditions. The presence of an extracellular inducer, e.g. protease secretion in a proteinaceous medium (Matile, 1965); the absence of a repressor, e.g. invertase secretion in a glucose-free medium (Metzenberg, 1962); or depletion of substrates, e.g. ribonuclease secretion in old cultures without appreciable autolysis (Suskind & Bonner, 1960) were among such conditions observed in Neurospora.

Subunits or multiple forms often occur in exoenzymes and, in some cases, may be involved with their secretion. The following three systems illustrate the wide range of relationship that can exist among them.

In the gram-negative bacteria, Escherichia coli, inactive subunits of alkaline phosphatase were secreted into the periplasmic space. Subsequent dimerization of the subunits gave the active form (Schlesinger, 1968). This mechanism not only conferred protection on the cytoplasmic content from hydrolysis by its own enzyme, but was also suggested to faciliate secretion across the cell membrane because of the subunit's smaller size.

In yeast, there were also a small (mol wt = 135,000) and a large (mol wt = 270,000) invertase (Gascón, Neumann & Lampen, 1968). However, they were both active

enzymes. Their similarity in kinetic behaviour, pH optima, substrate specificity and immunological property suggested a precursor-product relationship between the small and large forms. The small invertase, which made up only 5% of the total cell-bound activity, was intracellular and had no carbohydrates (Lampen et al.,1967). The heavy invertase, making up the rest of the cell-bound total activity, was outside the cell membrane (fully accessible to substrates), and contained characteristic cell wall polymers, i.e. 3% glucosamine and 50% mannan. These two major differences in location and molecular composition also suggested that the mannan component of the large invertase might link the enzyme to the mannan polymer in the cell wall. This would account for its larger size, carbohydrate content and exterior location as compared to the small invertase.

In Neurospora, invertase also existed in two different sizes, the light invertase ($S_{20,w}$ = 5.2) and the heavy invertase ($S_{20,w}$ = 10.3), but they were related in quite different ways (Metzenberg, 1964). Similar to the yeast system, both forms were active and the heavy form predominated (65-85% of total cell-bound activity). However, they occurred together intracellularly as well as in the culture medium and were interconvertible, the light form being the monomeric subunit. The invertase secreted by "protoplasts" or found inside them was mainly

the heavy form (Trevithick & Metzenberg, 1964). Since the light and heavy forms were not segregated as in yeast, it was unlikely that their interconversion was involved in extrusion through the cytoplasmic membrane or the cell wall. However, a characteristic component of Neurospora cell wall, hexosamine (2.4%), was found in the heavy form (Metzenberg, 1963a). The significance of this carbohydrate content with respect to secretion was not known.

Therefore, in recapitulation, the functions of exoenzymes are diversified and the secretion of each is governed by characteristic requirements and conditions. Moreover, despite a common presence of subunits or multiple forms, they do not conform to a general pattern of behaviour.

3. Fungal Cell Walls:-

The chemical constituents of fungal cell walls are very heterogeneous. Glucose polymer in various types of β-linkages is by far the most ubiquitous and abundant component. The less abundant ones include hexosamines, mannose, galactose, protein, lipid and ash. Their exact combinations and proportions varied with the strains, the morphology and the age of the fungus, as well as the laboratory where they were studied (Burnett, 1968). About 80% of Neurospora cell wall was made up of glucan, mostly in β-1,3-linkages but β-1,4- and α-1,4-linkages were also present. The rest of the cell wall consisted of chitin (ca. 10%); protein (maximum 14%); ash (2%); and glucuronic acid, galactosamine, glucosamine, mannose, all in very small quantities (Burnett, 1968; Galsworthy, 1966; de Terra & Tatum, 1963; Mahadevan & Tatum, 1965). Eddy (1958) found that in yeast, 60% of the wall was made up of glucan (50% insoluble, 10% soluble in alkaline). The insoluble glucan was shown by Peat, Whelan & Edwards (1958) to be mainly in β-1,3- and β-1,6-linkages. About 20% of the cell wall was mannan in α-1,6-, α-1,3-, and α-1,2-linkages (Kocourek & Ballou, 1969). The minor components included ca. 7% protein, 1% chitin and 0.05-1% phosphorous (Eddy, 1958).

These chemical constituents were usually organized into a microfibrillar network embedded in an amorphous matrix. Separation of the cell wall into layers were often observed. In Neurospora, as in Penicillium chrysogenum, the microfibrils were identified to be chitin and the bulk of the embedding matrix was made up of glucan (Manocha & Colvin, 1967; Troy & Koffler, 1969). Isolated cell walls were used for the above investigations, but if thin sections of intact Neurospora hyphae were examined, two layers were observed in the cell wall (Namboodiri, 1966; Shatkin & Tatum, 1959). The outer layer was loose,electron dense and easily lost during preparation. The inner layer was compact, electron transparent and composed of longitudinal elements. The chemical nature of these two layers has not been identified. However, three layers were recently observed by Hunsley & Burnett (1970) who studied thin sections of Neurospora hyphae from 50-hours old mycelium. The middle layer was electron dense. The additional layer observed by these workers may be due to secondary thickening of the aging organism because Namboodiri (1966) and Shatkin & Tatum (1959) used much younger cultures of ca. 20 hours old. In contrast to Neurospora, yeast cell walls had glucan as the microfibrillar network embedded in an amorphous mannan-protein matrix (Farkaš, Svoboda & Bauer, 1969). Electron microscopy on yeast cell walls often showed three

layers too: an outer granular layer, probably a mannan-protein with some chitin; an inner fibrillar layer of glucan; and an innermost thin membrane resistant to snail juice digestion (Matile, Moor & Robinow, 1969). On the other hand, in the dimorphic Mucor rouxii, the separation of cell walls into layers occurred only in the yeast forms (Bartnicki-Garcia & Nickerson, 1962).

M. rouxii is an interesting example to illustrate the dependence of morphological forms on cell wall structure and composition. This fungus grew in an unicellular yeast form in acidic medium or in the presence of CO_2 and reverted to a filamentous hyphal form when these conditions were removed. Subsequent analysis of the cell walls showed that there were more protein and mannose in the yeast forms. Under the electron microscope, they appeared more spongy and thicker with double layers whereas the hyphal cell walls had only a single compact layer (Bartnicki-Garcia, 1963).

Similarly, in a colonial mutant of Neurospora, the abnormal growth form (tufts of tightly restricted mycelium with frequent branching) appeared to be the result of a change in the proportions between polysaccharide-protein and β-1,3-glucan in its cell walls (Mahadevan & Tatum, 1965). The osmotic mutants of Neurospora, which failed to grow in medium of high osmotic strength, also differed from the

wild type in having less glucose (the alkaline insoluble portion) in their cell walls (Livingston, 1969). In one of these mutants, os-1 E1120, the cell walls appeared to be thinner, more fragile and porous than those of the wild type (Trevithick & Metzenberg, 1966b). The dependence of cell morphology on cell wall composition only emphasized the important role of this structural barrier.

The exact biochemical pathways and the cytoplasmic organelles involved in the biosynthesis of fungal cell walls have not been clarified. Chitin could be synthesized from glucose, involving transglycosylation of sugar-nucleotide intermediates onto a polymeric acceptor (poly-N-acetyl-glucosamine) by chitin synthetase (Burnett, 1968; Keller & Cabib, 1971). Biochemical pathways for the synthesis of the other wall components and the manner of their deposition outside the cell have not been resolved. Evidence from regenerating yeast "protoplasts" suggested that at least part of the cell wall was synthesized extracellularly (Necas & Svoboda, 1967). Golgi apparatus was shown to be involved in cell wall deposition in plants (Mollenhauer & Morré, 1966) and algae (Brown, 1969) but similar observations have not been recorded in the case of fungi, although Grove and his co-workers (1970) hypothesized that it was similarly employed in the Phycomycete fungus P. ultimum. The various vesicular organelles suggested for the secretion of exoenzymes, e.g.

lomasomes, mesosomes, were also suggested to be involved in cell wall synthesis (Kozar & Weijer, 1969a,b). However, Heath & Greenwood (1970) considered lomasome formation to be the mere consequence of an imbalance between cell expansion and plasma membrane formation. When the rate of hyphal elongation was limiting, excess plasma membrane precursors became sequestered into lomasomes. In the absence of definitive evidence, the roles assigned to these organelles are really a matter of individual preference. In contrast, another apical structure in septate fungi, the Spitzenkörper, seemed to have a close relationship with apical extension. It was a dark spot in the hyphal tip seen under phase-contrast microscope, disappearing when elongation of the hypha was inhibited, and reappearing just before growth was resumed (McClure, Park & Robinson, 1968). The same workers suggested that this "Spitzenkörper" was equivalent to the tip region containing many apical vesicles seen under the electron microscope. Recently, Grove & Bracker (1970) showed by electron microscopy that apical vesicles occurred quite regularly in representatives of all the fungal classes and indeed contained material similar to the cell wall substance in appearance. However, they also showed unequivocally that the small region within the apex, the so-called "Spitzenkörper", actually contained no apical vesicles, but this, of course, does not preclude its involvement with apical extension. Vesicles associated with bud and septum formation in yeast were also found to contain material

similar to the cell wall (Sentandrew & Northcote, 1969). Hence, their direct participation in cell wall synthesis seemed quite apparent.

The only facet of cell wall synthesis known with greater certainty is the pattern of cell wall deposition. Bartnicki-Garcia & Lippman (1969) labeled the dimorphic M. rouxii with tritiated N-acetylglucosamine. By means of autoradiography, they showed that maximum incorporation of the labeled compound occurred within ca. 5 μ of the apex in the filamentous hyphal wall. This pattern of apical intussusception of new wall material seems quite general for filamentous fungi (Burnett, 1968; Marchant & Smith, 1968; Grove et al., 1970). In contrast, the spherical yeast form of M. rouxii incorporated the labeled compound over the entire surface of the daughter bud. This is reminiscent of the diffuse addition of new material over the whole cell wall in some gram-negative bacteria. Certain gram-positive bacteria showed a third pattern of wall construction: new cell wall material was added only at the equatorial zone (Davis et al., 1967).

The concentration of cytoplasmic activities at the apical region, e.g. deposition of cell wall, biochemical differentiation (Zalokar, 1959b), and aggregation of vesicles (McClure et al., 1968), accentuates the importance of hyphal tips. As will be seen in the final discussion of this thesis, they may take on yet another important function in relation to macromolecular diffusion.

4. Cell Walls and Exoenzymes:-

Cell walls in micro-organisms, besides protecting the cell against deformation and mechanical injury, also appear to participate in the secretion of exoenzymes, the molecular sieving of macromolecules, and serve as an anchorage for certain enzymes.

When cell walls were removed from Bacillus subtilis, no extracellular enzymes were secreted or synthesized in an active form although protein and ribonucleic acid synthesis proceeded at normal rates (May & Elliott, 1968b). In the absence of intact cell walls, E. coli only secreted inactive subunits of alkaline phosphatase into the medium (Schlesinger, 1968). It seemed that cell walls might be involved with the synthesis, or the final assembly and activation of exoenzymes.

The occurrence of enzymes in fungal cell walls started to receive attention only in the last few years. Recently, Mahadevan & Mahadkar (1970) detected laminarinase activity (i.e. β-1,3-glucanase) in Neurospora cell walls. Its function was suggested to be the hydrolysis of cell wall glucan at budding points to prepare for the emergence of branches. McMurrough & Bartnicki-Garcia (1970) also detected high activity of chitin synthetase in M. rouxii cell walls. Its participation in cell wall synthesis was postulated.

Invertase was found in Neurospora cell wall fractions by many workers (Hill & Sussman, 1964; Sargent & Woodward, 1969; Chung & Trevithick, 1970); but its occurrence in yeast cell walls was studied much more thoroughly (see Lampen, 1968). Consequently, two models have been proposed to account for the mode of association between the yeast invertase and its cell walls.

Lampen in 1968 proposed the first model: the large invertase, a glycoprotein with 50% mannan, was bound to the cell wall surface mannan by either phosphodiester bridges or non-covalent chemical bonds. The evidence was this: phosphomannans were a cell wall component located at the surface of Saccharomyces cerevisiae, and a PR factor (enzyme splitting a mannosidic bond adjacent to a phosphodiester -linked mannose) that depolymerized phosphomannans could release the bulk of external invertase from intact cells. Kidby & Davis (1970b) proposed a second model in which no chemical bonds were involved between the invertase and the cell wall constituents. The enzyme was only physically confined within the cell wall surface layer. The structural impermeability of this layer was maintained by phosphodiester or disulphide linkages. If these linkages were disrupted, the permeability barrier broke down with subsequent diffusion of invertase molecules out of the cell wall. This model incorporated features of the Lampen's model as well as accounted for

the efficiency of β-mercaptoethanol to elute the bulk of invertase and acid phosphatase, another exoenzyme found in yeast cell walls, from Saccharomyces fragilis (Weimberg & Orton, 1966; Kidby & Davis, 1970a).

Similar studies had not been applied to other fungal exoenzymes. Therefore, the general validity of these proposals cannot be judged. Their implications in the association of invertase with Neurospora cell walls will be discussed in the last chapter.

A dilemma exists in the relationship between cell walls and exoenzymes. It is apparent from the above discussion that certain exoenzymes were found in the cell walls. At the same time, these exoenzymes also have to pass the cell walls a priori if they eventually appear in the external medium. Thus, the cell walls seem to have two conflicting functions -- to permit passage of and to retain exoenzymes.

The cell walls have been visualized as sieves with pores to allow for the egress of macromolecules (Trevithick & Metzenberg, 1966a). Therefore, the size of both the macromolecule and the cell wall openings determines the amount of a particular molecular species

that can pass through the cell walls. These deductions are compatible with the following observations. Trevithick & Metzenberg (1966a) found that the proportion of light invertase to heavy invertase was five times higher in the culture medium than inside the cell. Apparently, more light invertase, probably because of its smaller size, had been able to pass through the cell walls than heavy invertase. The same workers (Trevithick & Metzenberg, 1966b) also observed that the egress of heavy invertase was enhanced in cell walls with larger pores, i.e. proportion of light invertase to heavy invertase in the medium was reduced in an osmotic mutant whose cell walls were more porous than those of the wild type. These were taken as evidence to support the idea of cell walls acting as molecular sieves.

The molecular sieve concept received greater impetus in 1967 when Manocha & Colvin claimed to discover a system of three-dimensional, ramifying pores of 40-70 Å wide in Neurospora cell walls. Similar structures were also observed in Pythium debaryanum (Manocha & Colvin, 1968). This system was proposed to "serve as conduits for movement of macromolecular substances . . . ."

The porosity of fungal and bacterial cell walls had only been studied in a few cases. However, what was

found seemed to be incompatible with the concept of macromolecules diffusing through cell walls *per se*. Gerhardt & Judge (1964) found that isolated cell walls from Baker's yeast and *Bacillus megaterium* were both heteroporous, permitting the penetration of polymers over a range of molecular weights. Molecular weights greater than 4,500 and 57,000 were excluded from cell walls of yeast and bacteria respectively; by calculation, this corresponded to maximal cell wall openings of 36 and 107 Å. However, they were unable to find comparable pore structures in their electron microscopy study of the isolated cell walls. Neurospora cell walls also appeared to have a continuum of pore size distribution with the molecular weight exclusion threshold of 4,750 for wild type and 18,500 for the *osmotic* mutant (Trevithick & Metzenberg, 1966b). Nevertheless, all the known molecular weights of exoenzymes in Neurospora were well over the molecular threshold of 4,750 in the wild type. Even the smallest one studied so far, i.e. ribonuclease, had a molecular weight of *ca*. 10,000 (Takai, Uchida & Egami, 1966) and that of heavy invertase was 210,000 (Meachum, Colvin & Braymer, 1971). The enigma is this. How do the cell walls allow diffusion of macromolecules that seem too large to penetrate most of their pores, and yet exert a fractionating effect that implies such diffusion has indeed occurred? This will be brought up again in the final discussion in conjunction with a proposed model structure.

5. Biochemical changes associated with fungal development:-

The general pattern of fungal development consists of germination of spores, rapid growth, differentiation, senescence, and finally production of reproductive structures. The biochemical changes involved are the results of interactions between environmental conditions and endogenous responses of the organism (Zalokar, 1965). Such changes may affect the accumulation and localization of enzymes, the relative proportion of multiple enzymatic forms, or the type of metabolic pathways and storage materials.

The metabolism of trehalose appeared to be closely linked with budding in yeast (Küenzi & Fiechter, 1969) and germination in Neurospora (Hanks & Sussman, 1969). Both processes coincided with increased activity of trehalase and depletion of trehalose content in the cell. The metabolism of pyruvate was drastically changed from fermentative to oxidative pathways with the onset of conidiation in Neurospora hyphae (Turian & Matikian, 1966). Such changes must have involved the appearance or disappearance of a variety of enzymes.

Differences in levels of enzyme activities are the most frequent kind of changes found in differentiating or aging cultures. In Aspergillus oryzae, alkaline phosphatase

was more active than acid phosphatase in young mycelium. In old mycelium, the reverse condition prevailed (Nagasaki, 1968). In Neurospora, much higher activities of aldolase, β-galactosidase, tryptophan synthetase were correlated with the aging of vegetative mycelium (Zalokar, 1959a). NAD glycohydrolase also accumulated in conidiophores and conidia of Neurospora (Stine, 1969).

Sometimes the proportion of enzyme components varies with age too. There were at least two alkaline phosphatases [1,2], two acid phosphatases [I,II], and two proteases [A,B] in Aspergillus niger (Nagasaki, 1968; Ohama, Tomonaga & Yanagita, 1966). In each of these three systems, the relative activities of the two components varied in a characteristic way with the age of the mycelium.

A less common finding is the change of enzyme localization with age. In young hyphae of A. niger, Nagasaki (1968) detected alkaline phosphatase in large particles purported to be the nuclei. In old hyphae, enzyme activity not only occurred in the large particles but became irregularly distributed in the cytoplasm as well.

Finally, the type of storage material also reflects the physiological status of the organism. In

Neurospora, increase in number and size of vacuoles, fat droplets, and metabolytes of pigments were the moribund features associated with senescence (Zalokar, 1965).

Thus, biochemical and physiological changes during the development of an organism were generally inter-related and enzymes appeared to play a central role.

A general introduction to the five topics related to the present investigation has just been presented, namely: secretion, exoenzymes, fungal cell walls, cell walls and exoenzymes, and biochemical changes associated with fungal developments. The following chapters will record the experimental procedures and findings of this project. The significance of the results and their relevance to current progress in related fields will be discussed in the final chapter.

CHAPTER II. GENERAL MATERIALS AND METHODS

The general materials and methods used routinely in the different sections of this project are described here. Those specific to a particular section will be dealt with in the relevant chapters.

A. Chemicals

All chemicals for routine laboratory use were of reagent grade or the purest grade that was commercially available. Special chemicals will be described in detail when first mentioned. Glass distilled water was used in all experiments. The concentrations of aqueous solutions of chemicals and reagents were expressed as w/v percentages unless otherwise stated.

B. Analytical Methods

1) Determination of protein.

Protein concentrations of fractions collected from column chromatography were measured by absorbance at 280 nm. Otherwise, it was determined with the Folin phenol reagent according to Lowry, Rosebrough, Farr & Randall (1951).

After the reaction was completed, insoluble samples, e.g. cell walls, were centrifuged at 6,000 rpm for 10 min in a GLC Sorvall Centrifuge. Only the clear supernatant fractions were taken for colorimetric readings at a wavelength of 750 nm. Bovine serum albumin dried overnight in a vacuum desiccator over NaOH pellets was used as the standard.

2) Determination of ribonucleic acid (RNA).

RNA was measured by a modified Mejbaum reaction according to Merchant, Kahn & Murphy (1964). Highly polymerized yeast RNA (A grade, Calbiochem, California) was used as the standard. A control was run for each sample by digesting a similar preparation with 27 units of ribonuclease A (Type III-A, Sigma Chemical Co., Missouri) for more than 12 hours at 37 C. RNA content in the acid precipitated fractions of both experimental and control samples were then determined.

3) Determination of reducing sugars.

Reducing sugars were measured with the dinitro-salicylate reagent according to Bernfeld (1955). Glucose and maltose were used as standards. (see also Appendix 4-c)

4) Enzymatic Assays.

The various enzymatic assays will be described in the appropriate chapters. Assay recipes are given in Appendix 4.

C. Growth of Neurospora Culture

1) Strain

Wild type Neurospora crassa wa-#1961 from the collection of R.L. Metzenberg in the University of Wisconsin, Madison, Wisconsin, was generally used unless otherwise stated. The genetic background of this strain was mainly Em 5297a and was selected for its high fertility and rapid growth.

2) Maintenance

Agar plates in petri dishes and agar slants in test tubes containing the Fries minimal growth medium (Beadle & Tatum, 1945) supplemented with 1.4% sucrose were used to maintain stock cultures.

3) Inoculum

All conidia used as inoculum were harvested aseptically from agar plate cultures about one week old. Conidia were scraped from the agar surface with an inoculation loop and suspended in 10 ml of water in a test tube. A sterile pipette was pressed against the test tube to break up the lumps of conidia and mycelium. The suspension was filtered through glass wool to remove strands of hyphae. The filtrate was centrifuged at 650 x g for 5 min and the supernatant discarded. The conidial pellet was resuspended in water and the amount of conidial suspension used for each experiment was standardized turbimetrically at a wavelength of 600 nm.

D. Purification of Cell Walls

1) Procedure

This was a modification of the method of Chung & Trevithick (1970). Mycelium harvested by filtration through a Millipore membrane (type HA, 0.45 μm, Millipore Corp., Massachusetts) was pressed dry with filter paper to yield the mycelium wet weight. It was then suspended in cold phosphate buffered saline (PBS, 0.9% sodium chloride in 0.01M sodium phosphate buffer at pH 7.1). The proportion was usually about 20 ml of buffer per gram of mycelium (wet weight). The cells in the suspension were disrupted in a metal container (ca. 40 x 80 mm) with a sonifier (Branson Soni Power S 125) at maximum output for a period of 3 to 25 min. The degree of breakage was checked every 3 to 5 min with a phase contrast microscope until more than 95% of cells was estimated to have been broken. The sample was kept below 10 C all the time by using an ethylene glycol-Dry Ice bath and turning on the Sonifier intermittently. About 1/5 of the treated sample was set aside for determinations of total protein and enzyme activity. The remaining portion was centrifuged at 650 x g in a model PR-2 Centrifuge (International Equipment Co.) for 30 min. The supernatant was removed by aspiration. The residue was washed by suspending it in twice the original volume of PBS followed by centrifugation for 15 min at 100 x g. This washing procedure was repeated

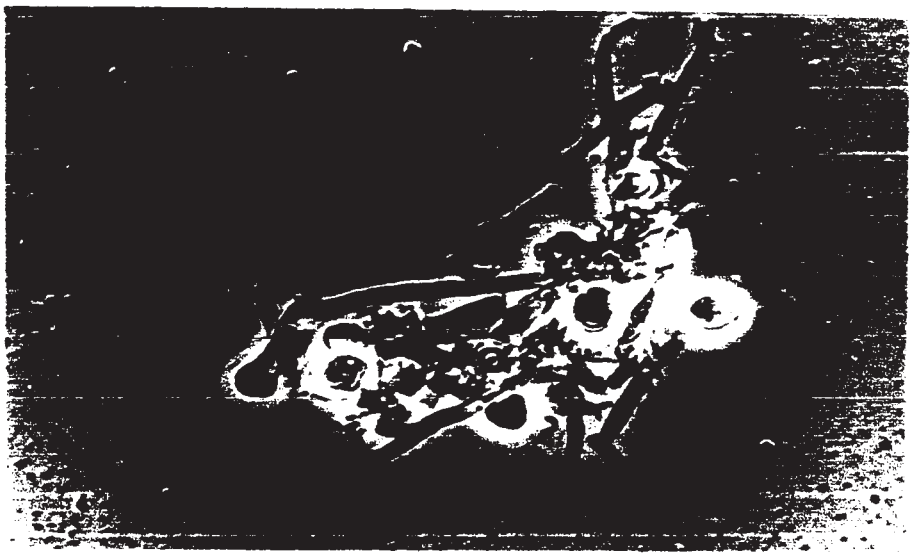
FIGURE 1

Preparation of Neurospora Cell Wall by Sonic Disruption

a) Mycelial homogenate immediately after sonic treatment.

b) Purified cell wall fraction after washing procedures.

x 1,000.



b

b

once with 1% Triton X-100 (to dissolve membranes), twice with 1M NaCl (to dissolve cytoplasmic proteins), and twice with PBS, a total of five times. The final white and loosely packed cell wall pellet was resuspended in an appropriate volume of PBS or water for subsequent determinations of protein and enzyme activity.

2) Purity of Cell Walls

The purity of the cell wall fractions thus prepared was checked by microscopic observation and chemical analysis. In a representative preparation from an 18-hour-mycelial culture, the hyphal filaments were almost completely broken after sonic treatment, as shown by phase contrast microscopy in Fig. 1a. A preparation of cell walls free from granular debris and cytoplasmic content (as detectable with phase contrast microscopy) was obtained after the washing procedures (Fig. 1b). Determination of RNA in the various fractions showed that less than 2% of total RNA was associated with the cell wall fraction. Cytoplasmic membranous material was not likely to contribute significantly towards contamination of the cell wall fraction washed with Triton X-100, a detergent known to dissolve cytoplasmic membranes. Furthermore, the small amount of invertase activity removable by Triton X-100 treatment (Chapter IV-B1, p.64) indicated that even if contaminating cytoplasmic membranes did occur, they could not account for the mural enzyme activity detected in the cell wall fraction.

E. Preparation of ^{14}C-labeled Cell Walls

A conidial suspension (0.1ml, absorbance at 600 nm = 20) was inoculated into 50 ml of growth medium containing Fries minimal growth medium, sodium succinate buffer (0.04M, pH 5.2), glucose (0.5%) and 0.05 mc D-glucose-^{14}C (U) with a specific activity of 2.8 mc/mM. This culture was incubated in a Metabolyte gyratory water bath (New Brunswick Scientific Co.) at 30 C and a speed of 200 rpm. The mycelium was harvested after 24 hours and ^{14}C-labeled cell wall fractions were prepared with the routine procedure described in section D.

CHAPTER III. ASSOCIATION OF EXOENZYMES WITH CELL WALLS

In preliminary experiments, an appreciable amount of invertase activity was found in the cell wall fraction of *Neurospora crassa*. Experiments were now designed to find out if this association with cell walls was general for all exo-enzymes.

According to Pollock (1962), the locations of enzymes in micro-organisms, such as bacteria, can be grouped into three categories: intracellular (inside the cytoplasmic membrane); surface-bound (outside the cytoplasmic membrane but cell-bound); and extracellular (easily separated from the cells by mild procedures). Considerable confusion exists in their designation because the surface-bound enzymes have been variously referred to as mural enzymes (Eberhart & Beck, 1970), exocellular enzymes (Weimberg & Orton, 1966), external enzymes (Lampen *et al.*, 1967), exoenzymes (Eberhart, 1961) and extracellular enzyme (Matile, 1964). The last three terms have also been used interchangeably to mean the free, and truly extracellular portion of the enzymes. For a clearer operational definition, the use of *extracellular* is restricted at present only to describe the enzymes that are free in the medium, or easily separated from the cells such

as by centrifugation or washing with water. Enzymes that are liberated on destruction of cell walls, e.g. during "spheroplasting", and whose activities are governed by factors in the medium, such as pH conditions and the presence of substrates or inhibitors that do not penetrate the cell membrane, will be specifically described as cell-bound or surface-bound exoenzymes, or mural exoenzymes if they are explicitly found in the cell wall fraction. Exoenzymes will refer generally to all the enzymes outside the permeability barrier (i.e. plasma membrane), regardless of whether they are still cell-bound or truly extracellular.

The exoenzymes studied in the following experiments were ribonuclease, acid protease (optimal pH at 4.2), amylase, aryl-β-glucosidase, trehalase, and invertase. Mycelia were grown under conditions favourable for production of the various exoenzymes and harvested for cell wall preparation when enzyme activities were detected in the culture medium.

A. Material and Methods

1) Growth Conditions and Assays for Exoenzymes

a. Ribonuclease [E.C. 2.7.7.26, ribonucleate guaninenucleotide-2'-transferase (cyclizing)]

Growth conditions were modified from those used by Takai, Uchida & Egami (1966). Neurospora crassa wild

type Em5297a (FGSC# 627) maintained on Fries minimal medium supplemented with 1.4% sucrose in agar plates was used. A conidial suspension (1 ml, absorbance at 600 nm = 5) was inoculated into 50 ml of growth medium in a 125-ml Erlenmeyer flask containing 500 µg of adenine, 2% sucrose and Vogel's minimal medium (Vogel & Bonner, 1956). Mycelia from duplicate experiments were harvested after growing for four days in a gyratory shaker bath at 30 C and a speed of 180 rpm.

Determination of ribonuclease activity was performed according to the method of Takai et al. (1966)with two exceptions: (i) EDTA was omitted from the reaction mixture, (ii) incubation lasted 30 instead of 15 min. A control was run for each sample by inactivating the enzyme solution before its incubation with 0.75% uranyl acetate in 25% perchloric acid. (For assay recipe, see Appendix 4-a).

A unit of activity was defined as the amount of enzyme that caused an increase in absorbance at 260 nm of 1.00 per minute at 37 C in the acid soluble fraction.

b. Acid Protease

[E. C. 3.4.4.-, an endopeptidase with optimal pH at 4.2]

Growth conditions were modified from those used by Matile (1965). An invertase deficient mutant inv-a (FGSC# 1857) of Neurospora crassa, obtained through the

courtesy of Dr. R.L. Metzenberg, University of Wisconsin, Wisconsin, was used. It was maintained on Fries minimal medium agar plates supplemented with 1.4% glucose. A conidial suspension (1 ml, absorbance at 600 nm = 5) was inoculated into 50 ml of growth medium in a 125-ml Erlenmeyer flask containing 2.7% glucose and Vogel's minimal medium. After incubation in a gyratory shaker bath for 19 hours at 30 C and 180 rpm, the culture was filtered through Whatman filter paper #1 in a Büchner funnel. The mycelial pad was washed with 50 ml of cold water by filtration and introduced into a 125-ml Erlenmeyer flask containing 0.4% peptone, 2.7% glucose and 50 ml of Vogel's minimal medium without NH_4NO_3. The above procedures were performed aseptically. The mycelial pad was dispersed by gentle shaking and the culture put back into the shaker bath at 30 C and 180 rpm. Duplicate flasks were harvested after 8 hours.

The activity of acid protease (optimal pH 4.2) was assayed by the method of Drysdale & Fling (1965) with two exceptions: (i) 0.05M sodium acetate buffer at pH 4.2 containing 0.5% horse globin was used instead of casein in a phosphate buffer, (ii) incubation lasted 30 min at 37 C instead of 15 min at 35 C. A control was run for each sample by inactivating the enzyme solution with 1M perchloric acid before its incubation. (For assay recipe, <u>see</u> Appendix 4-b.)

A unit of activity was defined as the amount of enzyme that liberated 1 micromole of tyrosine into an acid soluble fraction per minute at 37 C.

c. Amylase

Neurospora crassa wild type Em5297a was used (FGSC 627). A conidial suspension (1 ml, absorbance at 600 nm = 5) was inoculated into a 125-ml Erlenmeyer flask containing 50 ml of Vogel's minimal medium supplemented with 0.1% maltose and 1% starch. Duplicate cultures were incubated in a gyratory shaker bath at 30 C and 180 rpm for 24 hours before harvesting.

The method of Gratzner & Sheenan (1969) was used to assay for amylase activity. The reaction was stopped with an oxidizing chromogen after 30 min of incubation at 37 C. A control was run for each sample by inactivating the enzyme solution with the chromogen before its incubation. (For assay recipe, see Appendix 4-c)

A unit of activity was defined as the amount of enzyme that liberated 1 micromole of reducing sugar from starch per minute at 37 C.

d. Aryl-β-glucosidase

[E.C. 3.2.1.21, β-D-glucoside glucohydrolase]

Neurospora crassa wild type Em5297a was used (FGSC 627).

Growth and induction procedures were according to Eberhart & Beck (1970). Their discontinuous method was also used to assay for the enzyme activity. A blank was established by substituting the enzyme solution with water. (For assay -recipe, see Appendix 4-d.)

A unit of activity was defined as the amount of enzyme that released 1 micromole of p-nitrophenol from p-nitrophenol-β-D-glucopyranoside per minute at 37 C.

e. Trehalase

[E.C. 3.2.1.28, α,α'-glucoside 1-glucohydrolase]

Wild type Neurospora crassa was used (wa-#1961). A conidial suspension (1 ml, absorbance at 600 nm = 20) was inoculated into a 250-ml Erlenmeyer flask containing 100 ml of Fries minimal medium, galactose (2.7%) and sodium succinate buffer (0.04M, pH 5.2). The culture was harvested after growing for 20 hours in a gyratory shaker bath at 30 C and 200 rpm.

For assay of trehalase activity, 1 ml of reaction mixture containing enzyme solution, potassium phosphate buffer (50 μmole, pH 6) and trehalose (50 μmole) was incubated at 37 C for 60 min. Incubation was terminated by heating the mixture at 100 C for 2 min. The glucose liberated was determined by the glucose oxidase method (Huggett & Nixon,

1957). Absorbance of the final reaction mixture was measured at 420 nm. A blank was established by substituting the enzyme solution with water. (For assay recipe, see Appendix 4-f.)

A unit of activity was defined as the amount of enzyme that hydrolysed 1 micromole of trehalose into glucose per minute at 37 C.

f. Invertase

[E.C. 3.2.1.26, β-D-fructofuranoside fructohydrolase]

The Neurospora strain and growth conditions were identical with those for trehalase above.

The method of Metzenberg (1962) was used to assay for invertase activity. The glucose liberated was determined by the glucose oxidase method (Huggett & Nixon, 1957). Absorbance of the final reaction mixture was measured at 420 nm. A blank was established by substituting the sample with water. (For assay recipe, see Appendix 4-g.)

A unit of activity was defined as the amount of enzyme that released 1 micromole of glucose from sucrose per minute at 37 C.

In all enzyme assays on cell walls and mycelial homogenate, any incubation mixture with visible turbidity

was centrifuged at 6,000 rpm for 5 to 10 min with a GLC (Sorvall) Centrifuge. Only the clear supernatant fractions were taken for colorimetric measurements. In assays of aryl-β-glucosidase, trehalase and invertase, control samples with inactivated enzyme solutions had similar colorimetric readings as blank samples with water instead of the enzyme solutions. Therefore, only the latter was used as controls in later routine experiments.

2) General Experimental Procedure

Mycelia grown under the various conditions were harvested for cell wall preparation according to the procedures described in Chapter II-D. After the sonic treatment, enzyme activities were determined in the total mycelial homogenates and in the purified cell wall fractions.

For digestion of the cell walls with a hydrolytic enzyme preparation G-1 (see Chapter IV-A3, p.57, for enzymatic characteristics), 1-2 ml of each cell wall preparation was incubated with 0.5-1 ml of G-1 solution (1.3 mg protein/ml), and ca. 1 mg of Penicillin-Streptomycin mixture in a sterilized 8-ml screw-cap vial. It was agitated for 8-12 hr in a gyratory shaker bath at 30 C. Controls for detecting possible inactivation of enzyme activities during this

procedure were established by substituting the cell wall fractions with soluble fractions of the mycelial homogenates with a known amount of enzyme activity.

B. Results and Discussion

The activities of the various exoenzymes found in the cell wall fractions are shown in Table 1. In the assays for ribonuclease, acid protease and amylase activities, the substrates used were highly polymerized yeast RNA, horse globin and starch, respectively. These are macromolecules that may not gain access to enzymes within the cell walls, i.e. mural exoenzymes. Therefore, the cell wall fractions were first digested with a hydrolytic enzyme preparation, operationally designated as G-1, under conditions that released about 70% of cell wall material and more than 90% of cell-wall-bound invertase into a soluble fraction (see Chapter IV-A3, p.58). In this way, any mural enzyme would either be released from the cell walls or become more exposed to contact with its substrates. Both the untreated and the hydrolysed cell wall fractions were then assayed for the three exoenzyme activities. The experimental details for the preparation of G-1 from the culture fluid of Bacillus circulans WL-12 will be described in the next chapter.

TABLE I

Association of Exoenzymes with Neurospora Cell Walls

Mycelia from cultures grown under various conditions (III-A1) were suspended in 20-30 ml PBS and disrupted by sonic treatment. An aliquot was saved. The rest of the sample was used for cell wall preparation by extensive washing with Triton X-100 (1X), 1M NaCl (2X), PBS (3X) and recovered by centrifugation at 100 x g for 15 min after each wash. Enzyme activities were assayed in both the mycelial homogenates and purified cell wall fractions. Experiments in a) ribonuclease, b) acid protease, c) amylase, and d) aryl-β-glucosidase were performed in duplicate cultures A and B.

Prior to assays of a) ribonuclease, b) acid protease, and c) amylase, a portion of the cell wall fractions was digested by a wall-lytic enzyme preparation G-1.

	Mycelial wet wt (g)		Total Activity*		Cell Wall Activity* (untreated)			Cell Wall Activity* (G-1 treated)		
	A	B	A	B	A	B	Average % of Total	A	B	Average** % of Total
a. Ribonuclease	1.72	1.91	6.56	9.38	0.000	0.000	0.00	0.154	0.314	2.75
b. Acid protease	2.24	2.43	15.3	14.7	0.263	0.427	2.31	0.328	0.427	2.52
c. Amylase	0.80	0.79	8.22	9.46	0.143	0.212	1.99	0.822	0.863	8.89
d. Aryl-β-glucosidase	2.77	2.28	2.17	2.17	0.291	0.342	14.6			
e. Trehalase	1.67		3.02		0.80		26.5			
f. Invertase	1.67		623		184		29.5			

Each entry in the table was the average of two sample readings.

* Activity expressed in number of enzyme units as defined in Chapter III-A1.

** % calculated from a total activity which included the additional enzyme activity detected after G-1 treatment.

The percentages of total cellular activities associated with cell wall fractions varied significantly for the different enzymes (Table 1). In general, they could be classified into two groups. The first group had low percentages of the total cellular activities in the cell wall fractions and consisted of enzymes that hydrolysed macromolecules, i.e. ribonuclease (0%), acid protease (ca. 2%), and amylase (ca. 2%). The second group had high percentages of the total cellular activities in the cell wall fractions and consisted of enzymes that hydrolysed small molecules, i.e. aryl-β-glucosidase (ca. 15%), trehalase (ca.26%) and invertase (ca. 29%).

The relatively small amount of mural exoenzymes found in the first group may be the result of two possibilities. First, there was truly a low enzyme activity in the cell wall fraction. Second, much higher enzyme activity was actually in the cell wall fraction, perhaps comparable to that of the second group, but it was not detectable due to the inability of their macromolecular substrates to penetrate the cell walls. If this was true, division of the exoenzymes into the two groups would not be validated.

When the percentage of the total cellular activity associated with the untreated, intact cell wall fraction was compared with that of the G-1 digested cell walls, a dramatic

increase (from 1.99% to 8.89%) was evident in the case of amylase. A less dramatic but significant increase (from 0% to 2.7%) occurred for ribonuclease. No significant change was observed for acid protease. For all three enzymes, controls using the soluble fraction of mycelial homogenates showed that no inactivation of enzyme activities had occurred during the G-1 treatment under the specified conditions. In the case of amylase, activity was detected as the amount of reducing sugar liberated from starch (see Appendix 4-c). Neurospora cell walls, being composed mainly of glucan, would also yield a reducing sugar, glucose, when digested by a glucanolytic enzyme preparation such as G-1. Therefore, in this experiment, a control using heat-inactivated cell wall fraction digested similarly by the G-1 preparation was included. The amount of reducing sugar detected in this control has been subtracted from the total reducing sugar detected in the assay of amylase activity in the active cell wall samples to yield the experimental data as recorded in Table 1.

From the above results, it is concluded that some macromolecular substrates, i.e. RNA and starch, were unable to contact their hydrolytic enzymes in the cell walls per se. However, the broad division of the six exoenzymes into two groups, one of relatively low mural activity, and one of relatively high mural activity, seems to be still valid

because the low activity in the first group was not entirely due to the inaccessibility of the macromolecular substrates to the mural exoenzymes.

The probable significance of the cryptic portion of exoenzymes and the teleological interpretation about the two groups of exoenzymes will be discussed in Chapter VII, the general discussion.

CHAPTER IV. RELEASE OF CELL-WALL-BOUND INVERTASE & TREHALASE

In the experiments of the previous chapter, 25-30% of trehalase and invertase were found in the cell walls. The next step was to find out the nature of the forces responsible for keeping these macromolecules in their mural location. A variety of chemical reagents and hydrolytic enzymes were used in an attempt to release the two exoenzymes from their cell-wall association.

A. Material and Methods

All experimental manipulations were performed at 0-4 C except where otherwise indicated. Invertase and trehalase activities were assayed as described in Chapter III-A1.

1) Cell Wall Preparation

Mycelia were grown in Erlenmeyer flasks containing Pries minimal medium, galactose (2.7%), and sodium succinate buffer (0.04M, pH5.2) for 20 hours at 30 C in a gyratory shaker bath agitated at 180 rpm. Cell walls were purified as described in Chapter II-D. The concentration of cell walls used for the

following digestion experiments was 1-1.5 mg dry weight of cell walls per ml of water.

2) Treatment of Cell Walls with Chemical Reagents

(i) Buffers in a pH range of 3-10

Buffers with a range of pH values from 3 to 10 were obtained as follows:-
Each chemical reagent in the list below was made up in 1M stock solution. The appropriate pairs of stock solutions were mixed in proportions calculated from their pK_a values with adjustments until the required pH was obtained, as indicated by a glass electrode pH meter. The buffer solutions were diluted 1:6 with water before use (final concentration ca. 0.14M).

pH 3.0 -------	glycine - hydrochloric acid
pH 4.0 -------	sodium acetate - acetic acid
pH 5.0 -------	- do -
pH 6.2 -------	sodium monohydrogen phosphate - sodium dihydrogen phosphate
pH 7.0 -------	- do -
pH 8.0 -------	- do -
pH 9.0 -------	glycine - sodium hydroxide
pH 10.0 -------	- do -

Cell wall suspension (1 ml) was mixed with a buffer solution (0.5 ml) in a 12-ml graduated centrifuge tube and kept at 23 C for 30 min with frequent stirring on a vortex stirrer. The reaction was terminated by rapid chilling in ice and immediate centrifugation for 20 min at 900 x g. The supernatant fraction was saved. The cell wall pellet was resuspended to a final volume of 3 ml with PBS. Invertase and trehalase activities were assayed in both fractions.

(ii) Triton X-100 (Octyl phenoxy polyethoxyethanol)

For this experiment, the procedure of washing with Triton X-100 was omitted initially from the routine preparation of cell walls (see Chapter II-D). A portion of the final cell wall suspension was treated as follows:- A cell wall suspension (0.5 ml) was thoroughly mixed with 2% (v/v) Triton X-100 (0.5 ml) in a graduated centrifuge tube. After standing for 5 min in an ice bath, the suspension was centrifuged at 650 x g for 10 min and the supernatant was saved. The cell wall pellet was washed with 10 ml of water, centrifuged as before, and made up to 1 ml with water.

Invertase activity of the cell wall suspensions was determined with and without the above Triton treatment. Control samples established that no inactivation of enzyme activity occurred under such conditions.

(iii) EDTA (ethylenediaminetetraacetate)

The following reaction mixture was added to a sterile 8-ml screw-cap vial: EDTA (0.1M, 15 µl), cell wall suspension (2 ml), phosphate buffer (0.25 ml, 0.6M, pH 8.0) and water (0.74 ml). The vial was agitated in a gyratory shaker bath at 30 C at a speed of 280 rpm for 4 hours. The reaction mixture (2 ml) was centrifuged in a 12-ml graduated centrifuge tube at 650 x g for 10 min. The supernatant fraction was saved and the residue was made up to the original volume with water. The invertase activity of both fractions was determined. Estimations from control samples using water instead of EDTA showed that about 5% of invertase activity was lost under the experimental conditions.

(iv) Potassium chloride

Procedures were identical to those of EDTA treatment (iii). The incubation mixture was as follows: KCl (0.75 ml, 4M), cell wall suspension (2 ml) and phosphate buffer (0.25 ml, 0.6M, pH 8.0). Only a negligible amount of invertase was inactivated by this treatment.

(v) β-Mercaptoethanol

Procedures were identical to those of EDTA treatment (iii). The reaction mixture was as follows: β-mercaptoethanol (0.25 ml, 0.23M in 0.6M phosphate buffer

at pH 8.0), cell wall suspension (2 ml) and water (0.75 ml). About 15% of invertase activity was lost during this treatment.

3) Digestion of Cell Walls by Hydrolytic Enzymes

a. Special Chemicals

(i) Laminarin is a glucose polymer with predominant β-1,3-linkages from Laminaria cloustoni frond (Pierce Chemical Co., Illinois).

(ii) Pustulan is a glucose polymer with predominant β-1,6-linkages (8223M), a gift from Dr. E.T. Reese of Quartermaster Research and Engineering Centre, Natick, Massachusetts.

(iii) Chitin is N-acetylglucosamine polysaccharide with β-1,4-linkages. Colloidal chitin was prepared from poly-N-acetylglucosamine (Practical grade, Sigma Chemical Co., Missouri) according to the method of Berger & Reynolds (1958).

(iv) Sodium carboxymethyl cellulose is a derivative of glucose polymer with β-1,4-linkages (cellulose gum, type 7LF, Hercules Incorporated, Delaware).

b. Hydrolytic Enzymes

(i) Snail gut juice:- Suc d'Helix pomatia (Industries Biologiques Francaises, Gennevilliers) was processed according to the method of Trevithick & Metzenberg

(1964). It was dissolved in phosphate buffer (0.02M, pH 6.0) to a protein concentration 20% of that in the original snail juice.

(ii) Trypsin [E.C. 3.4.4.4]:- (lyophilized trypsin 193 U/mg, Worthington Biochemical Corp., New Jersey) was used as a 0.1% solution in Tris buffer (0.125M, pH 8.1) with 0.03M $CaCl_2$.

(iii) Chitinase [E.C. 3.2.1.14]:- (Calbiochem Co., California) was used as a 0.2% solution in phosphate buffer (0.03M, pH 6.3).

(iv) Cellulase [E.C. 3.2.1.4]:- (CSEI, Worthington Biochem. Corp., New Jersey) was used as a 0.1% solution in sodium acetate buffer (0.02M, pH 5).

(v) β-1,3(4)-glucanase [E.C. 3.2.1.6] :- This was prepared according to the method of Tanaka & Phaff (1965) from the culture fluid of Bacillus circulans WL-12, using purified Neurospora cell walls as the carbon source instead of yeast cell walls. The bacteria stock culture was kindly supplied by Professor H.J. Phaff, University of California, Davis, California. Cell walls were prepared from a supply of Neurospora mycelial debris, kindly donated by Dr. R.A. Cook, University of Western Ontario, Ontario. The method of cell wall purification described in Chapter II-D was used with slight modification to accommodate for the large quantities of material involved.

The last step in the enzyme preparation required elution of the enzyme mixture from a DEAE cellulose column, first with a convexly increasing gradient of phosphate buffer at pH 7.2, then with 1M acetate buffer at pH 5.0 (see Appendix 1). Fractions of the first protein peak containing lytic activities eluted in the first gradient were pooled and operationally designated as the G-1 fraction. Those eluted with the acetate buffer were designated as G-2. Their activity and specificity are as follows:

	G-1	G-2
Enzyme Activity Reported	β-1,6-glucanase	β-1,3-glucanase
Tanaka & Phaff (1965)		
Enzyme Activity Found		
1. β-1,3-Glucanase		
paper chromatography	+ + +	+ + +
enzyme assay	0.44 U/mg protein	4.80 U/mg protein
2. β-1,6-Glucanase		
paper chromatography	+	−
enzyme assay	0.042 U/mg protein	
3. Chitinase (17 hr, 37 C)	0.23 μM*/mg protein	−

*Chitinase activity was measured as the number of micromoles of N-acetylglucosamine liberated from colloidal chitin under the specified conditions (assayed as indicated in c(ii) of this section).

Hydrolysis of Neurospora cell wall at 37 C

	Control (0 hr)	G-1 (12 hr)	G-2 (24 hr)
1. Invertase activity per ml cell wall			
Supernatant	0.04 U	5.13 U	4.79 U
Residue	5.40 U	0.26 U	0.24 U
% in Supernatant	0.7	95.2	95.2
2. Radioactivity per ml cell wall residue (dpm)	291 682	70 877	51 492
% released	0.0	75.7	82.4

A unit of β-1,3-glucanase activity was defined as the amount of enzyme that liberated 1 micromole of reducing sugar per minute at pH 5.8 and 37 C from laminarin. A unit of β-1,6-glucanase activity was defined as the amount of enzyme that liberated 1 micromole of reducing sugar per minute at pH 6.5 and 37 C from pustulan, (assay recipes in Appendix 4-h,i). Glucose was used as the reducing sugar standard.

Although both G-1 and G-2 fractions could release the bulk of cell-wall-bound invertase, only the G-2 fraction, with its more specific mode of action, was used for critical studies.

The β-1,3-glucanase preparation used in this experiment had a specific activity of 4.8 units/mg protein and a concentration of 0.35 mg protein/ml sodium succinate buffer (0.05M, pH 5.8).

c. Substrate Specificity of Hydrolytic Enzymes

(i) on glucans (glucose polymers) :-

The method was according to Tanaka & Phaff (1965). The substrates used were laminarin (β-1,3-glucan), pustulan (β-1,6-glucan), sodium carboxymethyl cellulose (β-1,4-glucan) and soluble starch (α-1,4-glucan). Enzyme preparations of snail gut juice, various glucanase fractions or chitinase were allowed to digest the above substrates for 5 and 24 hours at 37 C in a gyratory shaker bath agitated at a speed of 200-300 rpm. The hydrolytic products were spotted on Whatman #1 paper and developed by descending chromatography with the upper phase of n-butanol:ethanol: water (4.5:0.5:5). Glucose, mannose and galactose were used as standards. Mono- and oligosaccharides were detected with an aniline hydrogen phthalate spraying reagent (Partridge, 1949).

(ii) on chitin:-

Chitinase, snail gut juice, and various glucanase fractions were allowed to react with colloidal chitin according to the chitinase assay procedure in the Worthington Biochemical Enzyme Manual (Worthington Biochemical Corp., New Jersey).

The hydrolytic product was estimated with N-acetylglucosamine as the standard.

(iii) on protein:-

Trypsin, snail gut juice and the various glucanase fractions were allowed to react with casein according to the procedure of Drysdale & Fling (1965). Tyrosine was used as the standard.

d. Digestion Procedure

Cell walls were incubated with snail gut juice, trypsin, chitinase, cellulase, or β-1,3-glucanase. Portions of the incubation mixtures were withdrawn at intervals and centrifuged. Invertase and trehalase activities in both the cell wall residue and supernatant fraction were measured.

A sterilized and stoppered Erlenmeyer flask (25-ml) containing 10 ml of cell wall suspension (ca. 1 mg dry wt/ml), 5 ml of hydrolytic enzyme solution, and 50 µl of penicillin-streptomycin mixture (BBL, Div. of Bioquest, Maryland; reconstituted with 0.1M phosphate buffer at pH 6.2) was incubated in a gyratory shaker bath at 30 C and ca. 200 rpm. Aliquots of 1-2 ml were aseptically withdrawn with sterilized pipettes and centrifuged in graduated 12-ml centrifuge tubes at 650 x g for 10 min. The supernatant was drawn up carefully and completely with a Pasteur pipette. The residue was made up to the original volume with water. Trehalase and invertase activities were measured in the supernatant and residue fractions.

In flasks used as controls, identical procedures were performed except that the buffered enzyme solution was replaced by the buffer alone.

Snail gut juice and cellulase preparations were found to have sucrose- and trehalose-cleaving activities. Therefore, in experiments using these two enzymes, an additional control flask containing all the experimental digestion ingredients was included except that the cell wall suspension had been heated previously at 100 C for 5-10 min. Any invertase or trehalase activities detected in this control series were attributed to the snail gut juice or cellulase and subtracted from the experimental values.

In the digestion of cell walls by β-1,3-glucanase, glucose was released from the cell wall polymers. This would interfere with the trehalase and invertase assays which involved a determination of glucose as a hydrolytic product from their substrates (trehalose and sucrose, respectively). Therefore, the glucose in the digested cell wall samples was measured prior to the enzyme assays and subtracted from the total glucose detected after the enzyme assays.

e. Digestion of ^{14}C-labeled Cell Walls

^{14}C-labeled cell walls were prepared as described in Chapter II-E. They were digested by snail gut juice,

trypsin, chitinase, cellulase, or β-1,3-glucanase. The amount of cell wall material liberated by these hydrolytic enzymes was estimated from the percentages of the total cell wall radioactivity released by them into the supernatant fractions.

A cell wall suspension (0.6 ml at a concentration of 1.1 mg dry weight and 291,682 dpm of radioactivity per ml) was added to 0.3 ml of enzyme solution and 6 µl of reconstituted penicillin-streptomycin solution in a sterile 8-ml screw-cap vial. After 24 hours of incubation in a gyratory shaker bath at 30 C and ca. 300 rpm,100 µl and 200 µl of the sample in each vial were withdrawn and spread evenly on separate pieces of glass fibre filter paper on a Millipore filter connected to a vacuum pump. The samples on the filter paper were washed by filtration with 10 ml and 20 ml of water respectively. The filter paper and the thin film of cell walls were put into a counting vial and dried for over 24 hours at 80 C. A toluene based scintillation counting fluid (10 ml) was added to each vial and the radioactivity of the samples was measured with a Beckman LS-150 Liquid Scintillation System set at Channel 1.

Control samples were obtained at 0 and 24 hours from similar incubations with phosphate buffered saline substituting for enzyme solutions.

The amount of radioactivity released from the cell walls was taken from the difference between the total dpm of the cell walls at 0 hour and the residual dpm of the cell walls after 24 hours of digestion.

f. Scanning Electron Microscopy of Digested Cell Walls

The cell wall residues left after hydrolytic enzyme digestions in Section 3d were washed twice with 10 ml of water by centrifugation at 650 x g for 10 min. The residual slurry of cell walls was thinly spread on a cover slip secured on a glass slide, and dried overnight in a vacuum desiccator. A fraction of the cover slip was cut and mounted on the specimen stub, coated with gold--palladium (40:60), and examined with a Cambridge Mark II Scanning Electron Microscope.

B. Results and Discussion

1) Treatment of Cell Walls with Chemical Reagents

(i) Buffers in the pH range of 3-10

No significant release of invertase and trehalase occurred over the range of buffers from pH 3 to pH 8. At pH 9, ca. 3 % of invertase activity and 8.0% of trehalase activity appeared in the supernatant. At pH 10,

ca. 4% invertase activity and 7.5% trehalase activity were also found in the supernatant. These values indicated very little release of cell-wall-bound activities when compared to the values obtained later by enzymatic digestions. Hence, the association of invertase and trehalase with cell walls did not seem to depend significantly on hydrogen bonds, at least those involving carboxyl or imidazole groups, since they would have been weakened by such drastic changes of $[H^+]$. (For preliminary data, see Appendix 2-a.)

(ii) Triton X-100 (1%)

Cell wall fractions washed once with Triton X-100 had ca. 8% of invertase activity in the supernatant compared to ca.5% in the control with no Triton treatment. This net release of activity probably represented the further removal from the cell wall fraction of cytoplasmic membranes which might contain invertase activity and were solubilized by the Triton treatment. Extending the treatment with Triton X-100 from 5 to 120 minutes did not alter the percentage of invertase released. Therefore, it was concluded that no significant release of the exoenzyme occurred, as compared to the enzymatic digestion to be described. This indicated that hydrophobic bonding, which would have been weakened in the presence of organic surfactants such as Triton X-100, was not a primary factor in the association of invertase with cell walls. (For preliminary data, see Appendix 2-b)

(iii) EDTA (0.5 mM)

(iv) KCl (1 M)

(v) β-Mercaptoethanol (19 mM)

These three reagents, in the final concentrations indicated in parentheses, caused about 6.0% of the invertase activity in cell walls to appear in the supernatant fraction. In Saccharomyces mellis, about 43% of acid phosphatase was eluted from intact cells in the presence of 1M KCl (Weimberg & Orton, 1965). In Saccharomyces fragilis, about 38 units of invertase was eluted by 20 mM mercaptoethanol from intact cells which originally had about 45 units of invertase activity (Kidby & Davis, 1970a). This amounted to a release of ca. 85% of total activity. The mere 6% of invertase released in the present series of experiments was in contrast to the high values obtained in yeasts. Thus, the above reagents failed to dissociate invertase from Neurospora cell walls to the extent that might be expected for acid phosphatase and invertase in S. mellis and S. fragilis, respectively. (For preliminary data, see Appendix 2-c.)

These findings led to the tentative conclusion that, under the specified conditions, metal ligands, salt linkages, or disulfide bridges could not account for the association of invertase with Neurospora cell walls. According to Manocha & Colvin (1967), no cysteine residues were found

FIGURE 2

Release of Invertase and Trehalase from Cell Walls by Snail Gut Juice

A 25-ml Erlenmeyer flask containing cell wall suspension (ca. 10 ml), hydrolytic enzyme solution (5 ml) and penicillin-streptomycin solution (6 µl) was incubated at 30 C in a shaker bath. Samples (1-2 ml) were collected at intervals and centrifuged (650 x g for 10 min) to separate the cell wall residue from the supernatant. Invertase and trehalase activities were determined in the two fractions. In the controls, the hydrolytic enzyme solution was substituted by a buffer solution.

● (In) = Invertase

○ (CIn) = Control for invertase

▲ (Tr) = Trehalase

△ (CTr) = Control for trehalase

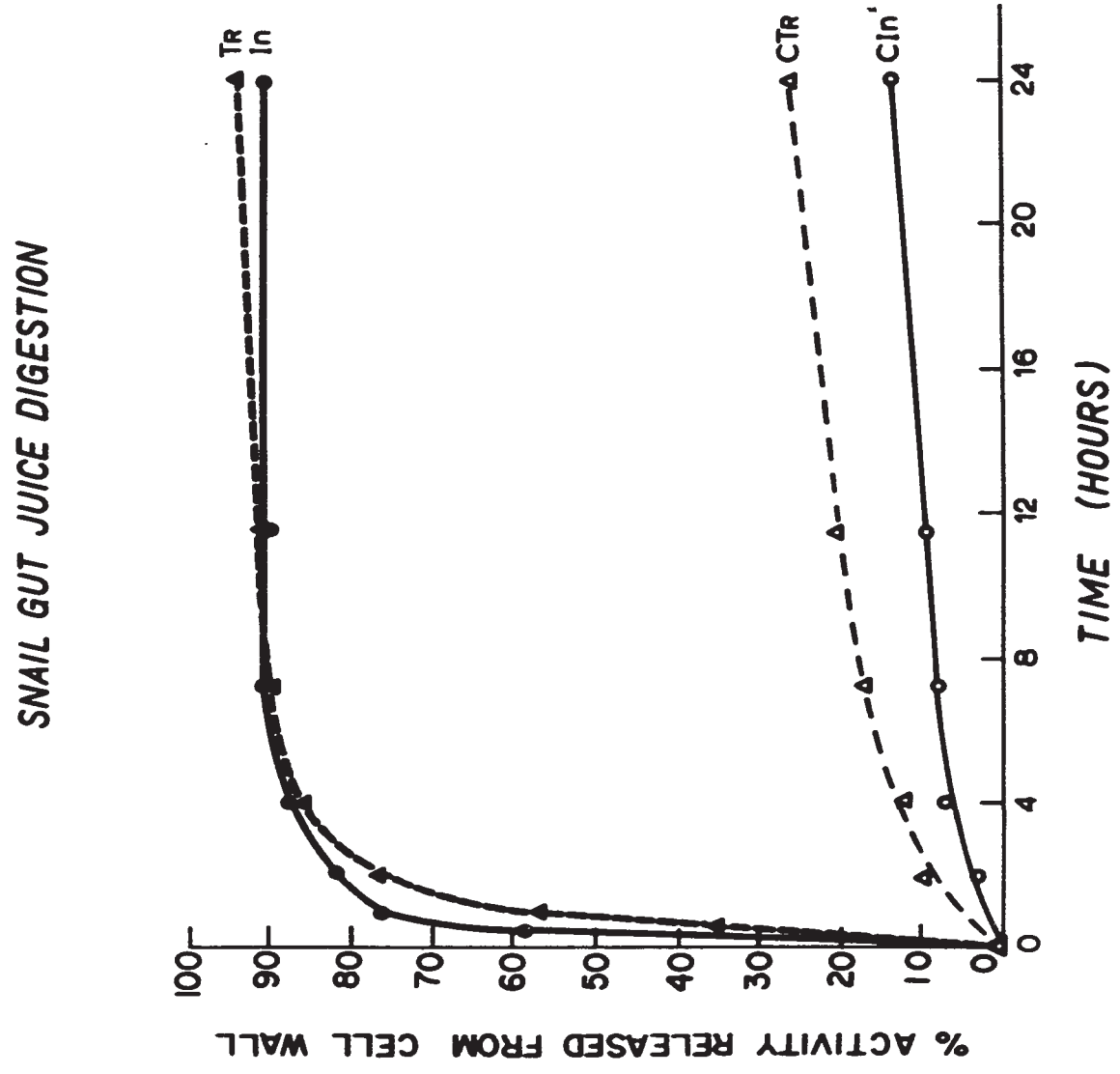

SNAIL GUT JUICE DIGESTION
% ACTIVITY RELEASED FROM CELL WALL
TIME (HOURS)
TR
In
CTR
CIn'
0
10
20
30
40
50
60
70
80
90
100
0
4
8
12
16
20
24

FIGURE 3

Release of Invertase and Trehalase from Cell Walls by Cellulase

Conditions of the experiment and notations are described in the legend of Figure 2.

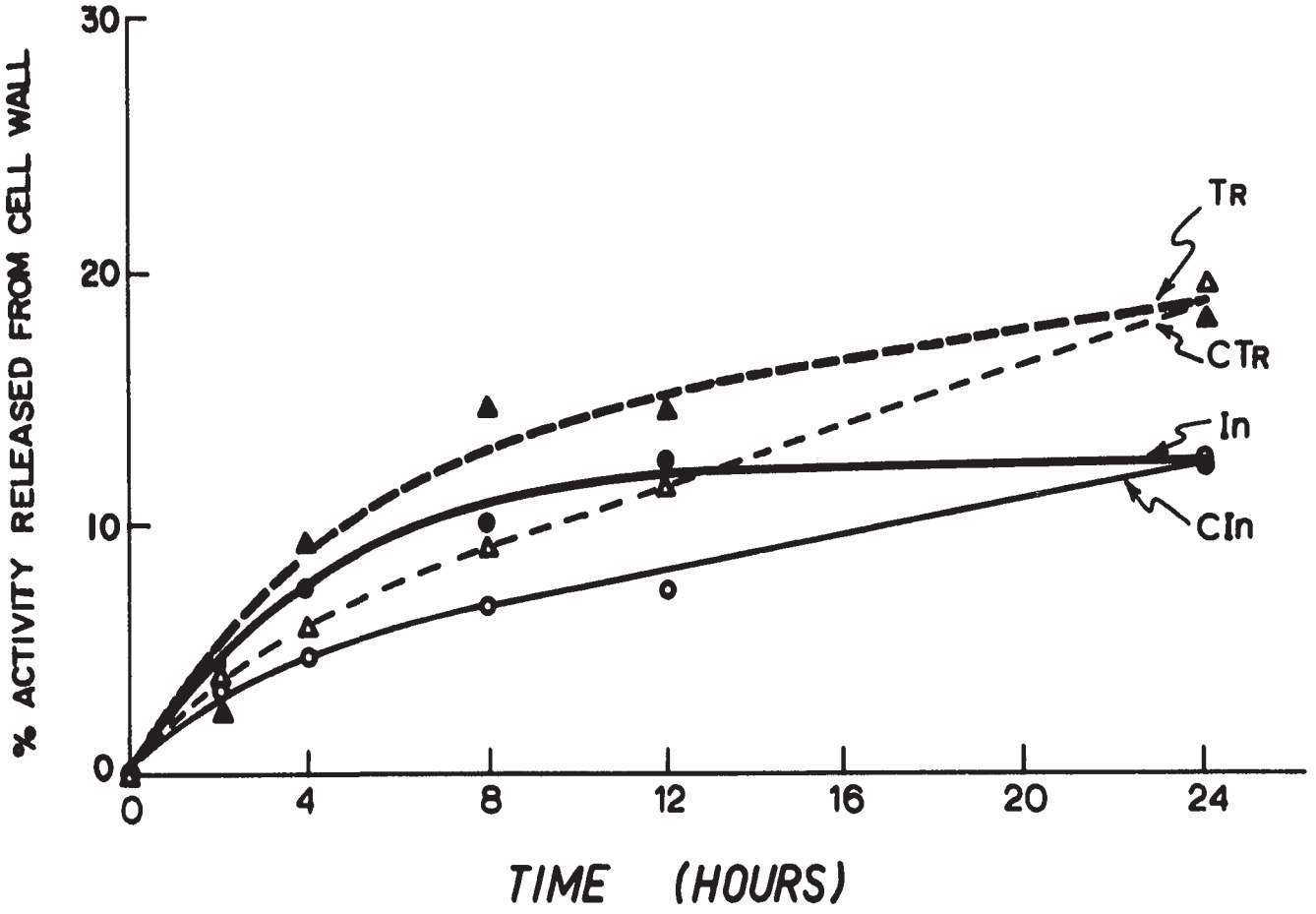

CELLULASE DIGESTION
% ACTIVITY RELEASED FROM CELL WALL
30
20
10
0
0
4
8
12
16
20
24
TIME (HOURS)
TR
CTR
In
CIn

FIGURE 4

Release of Invertase and Trehalase from Cell Walls by Trypsin

Conditions of the experiment and notations are described in the legend of Figure 2.

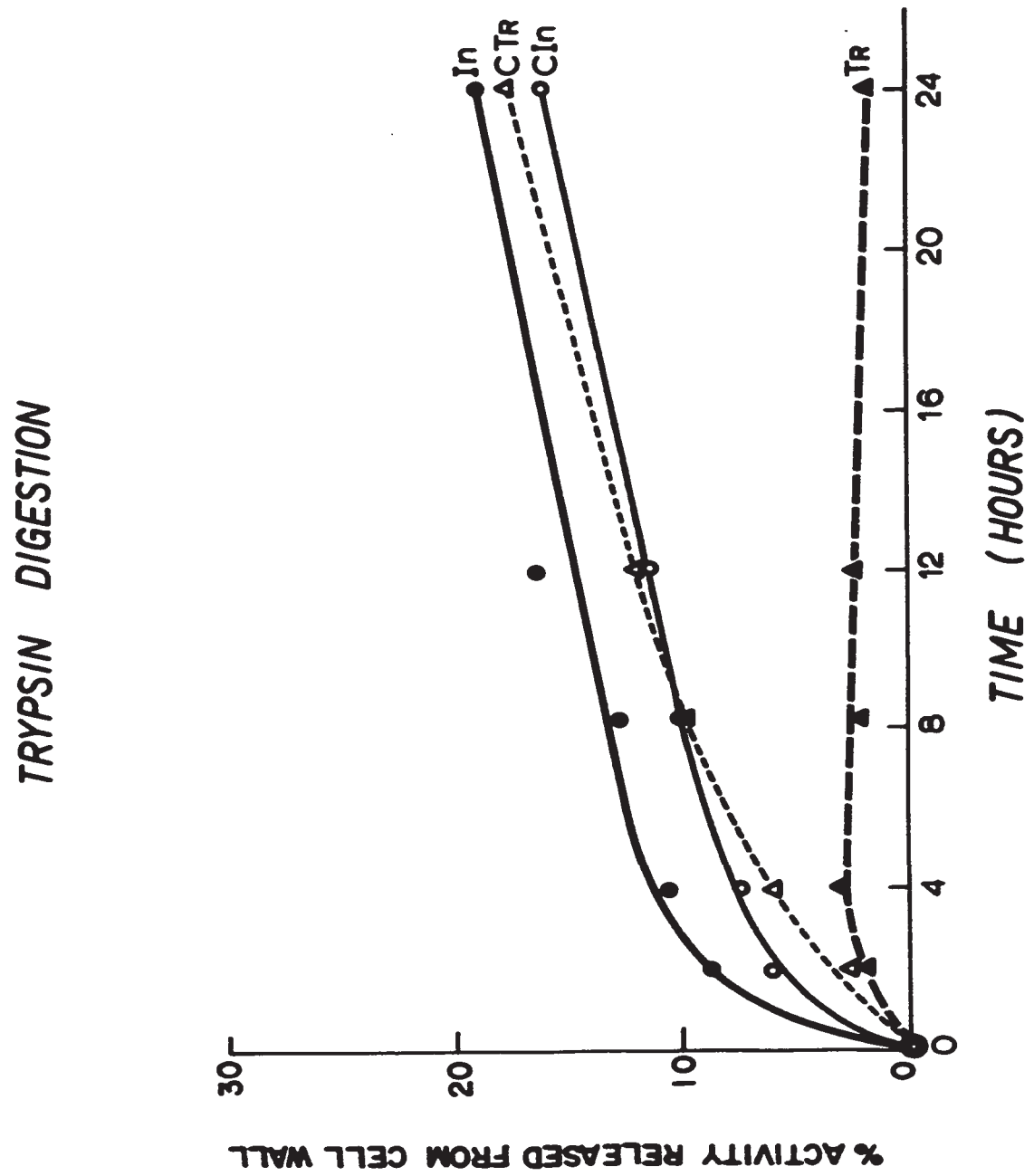

TRYPSIN DIGESTION
% ACTIVITY RELEASED FROM CELL WALL
30
20
10
0
0
4
8
12
16
20
24
TIME (HOURS)
In
CTR
CIn
TR

FIGURE 5

Release of Invertase and Trehalase from Cell Walls by Chitinase

Conditions of the experiment and notations are described in the legend of Figure 2.

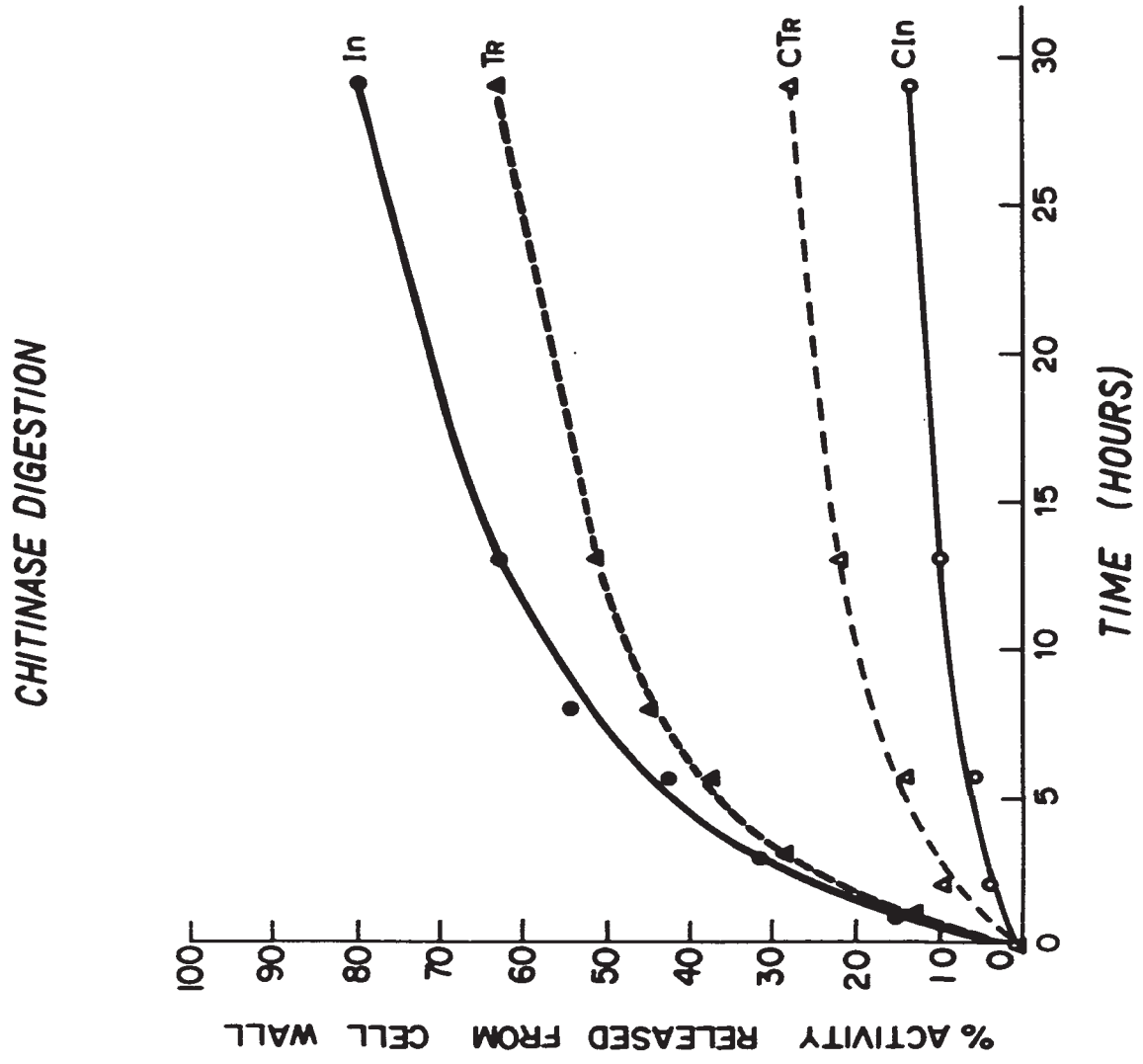

CHITINASE DIGESTION
% ACTIVITY RELEASED FROM CELL WALL
TIME (HOURS)
In
Tr
CTr
CIn
0
10
20
30
40
50
60
70
80
90
100
0
5
10
15
20
25
30

FIGURE 6

Release of Invertase and Trehalase from Cell Walls by β-1,3-glucanase

Conditions of the experiment and notations are described in the legend of Figure 2.

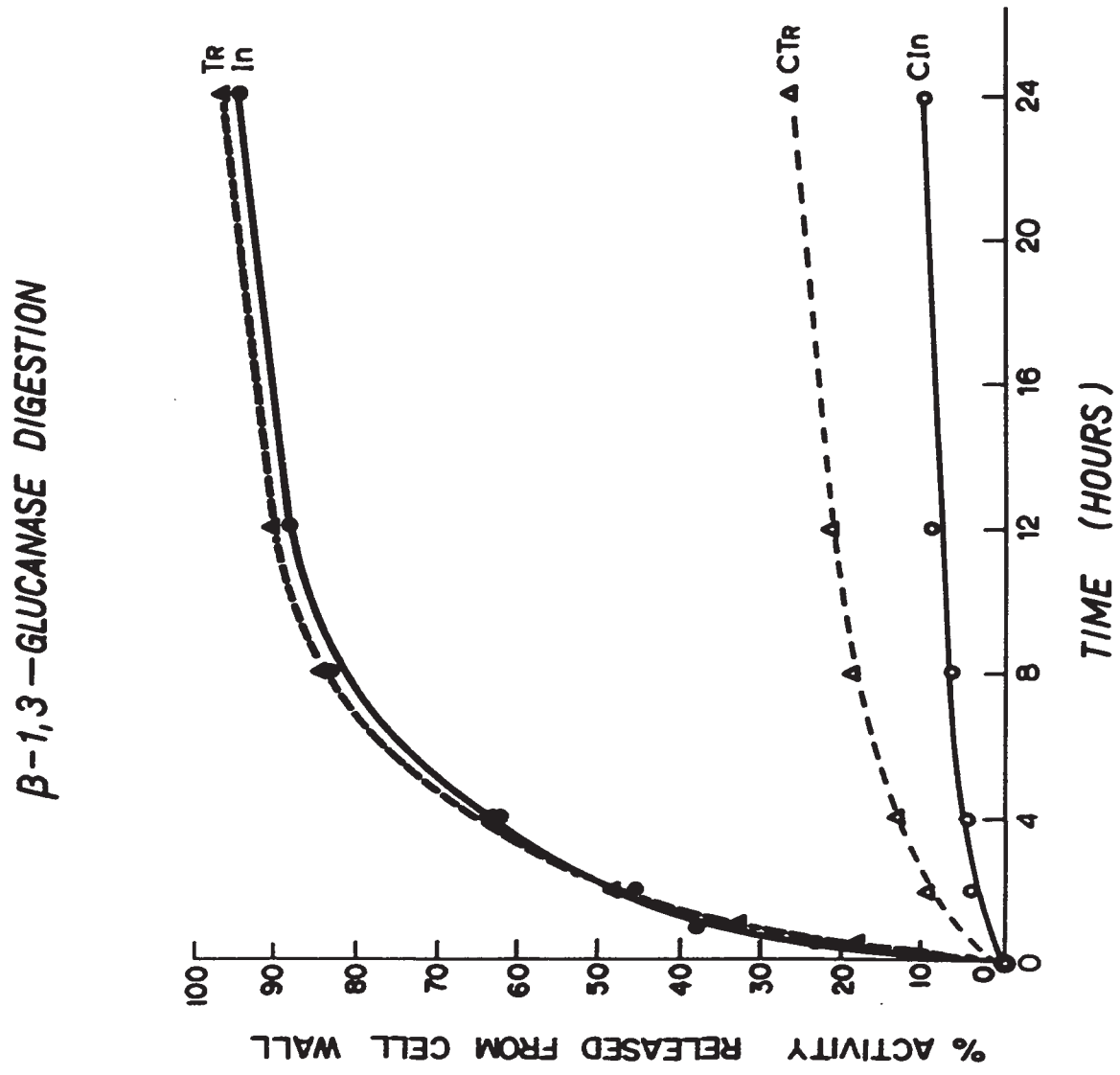

β-1,3—GLUCANASE DIGESTION
% ACTIVITY RELEASED FROM CELL WALL
TIME (HOURS)
TR
In
CTR
CIn
0
10
20
30
40
50
60
70
80
90
100
0
4
8
12
16
20
24

FIGURE 7

Release of Invertase from Cell Walls by Various Hydrolytic Enzymes

(SGJ) = Snail Gut Juice

(G) = β-1,3-Glucanase

(Ch) = Chitinase

(T) = Trypsin

(Ce) = Cellulase

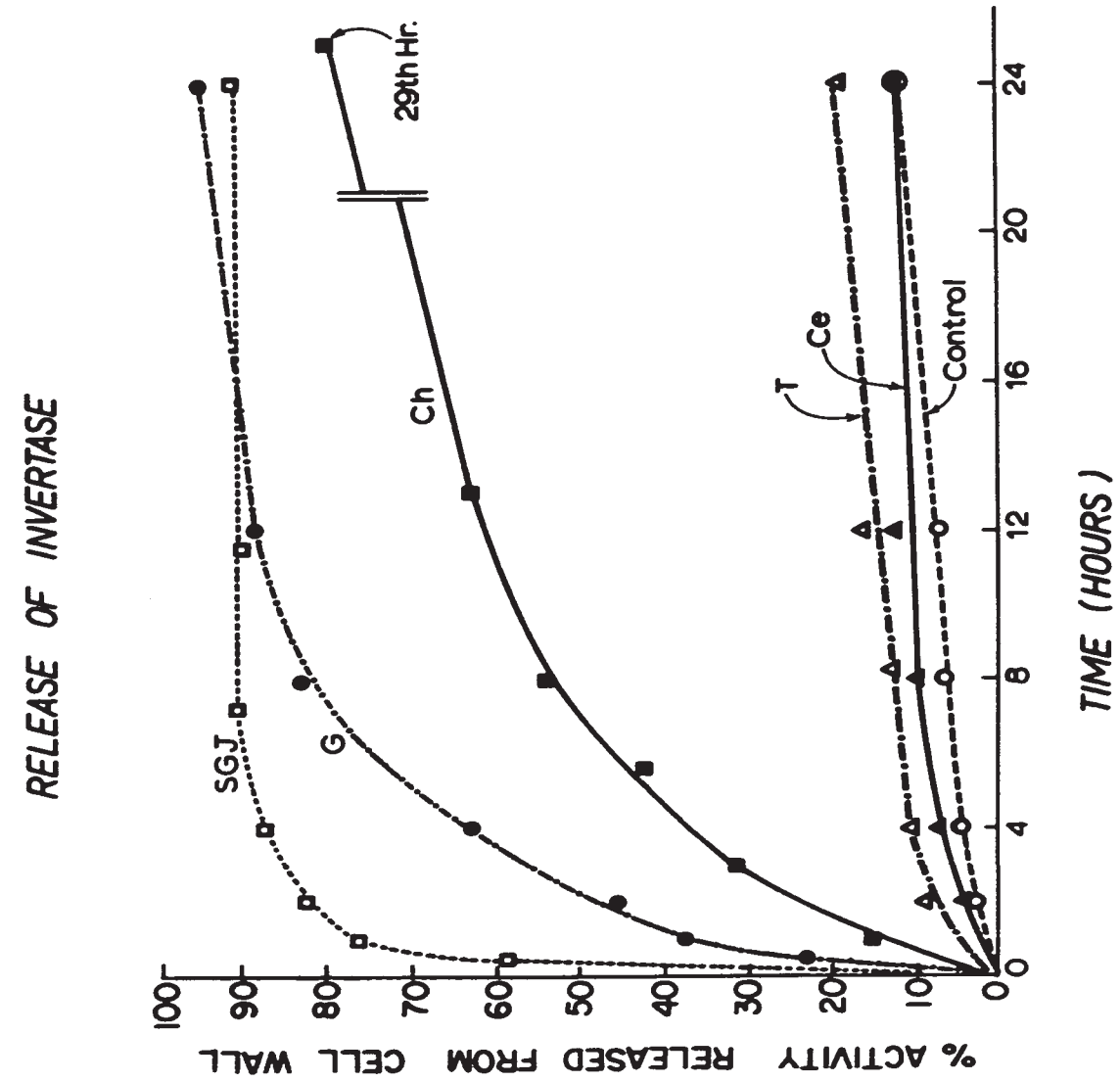

RELEASE OF INVERTASE
% ACTIVITY RELEASED FROM CELL WALL
TIME (HOURS)
SGJ
G
Ch
29th Hr.
T
Ce
Control
0
10
20
30
40
50
60
70
80
90
100
0
4
8
12
16
20
24

FIGURE 8

Release of Trehalase from Cell Walls by Various Hydrolytic Enzymes

(SGJ) = Snail Gut Juice

(G) = β-1,3-Glucanase

(Ch) = Chitinase

(Ce) = Cellulase

(T) = Trypsin

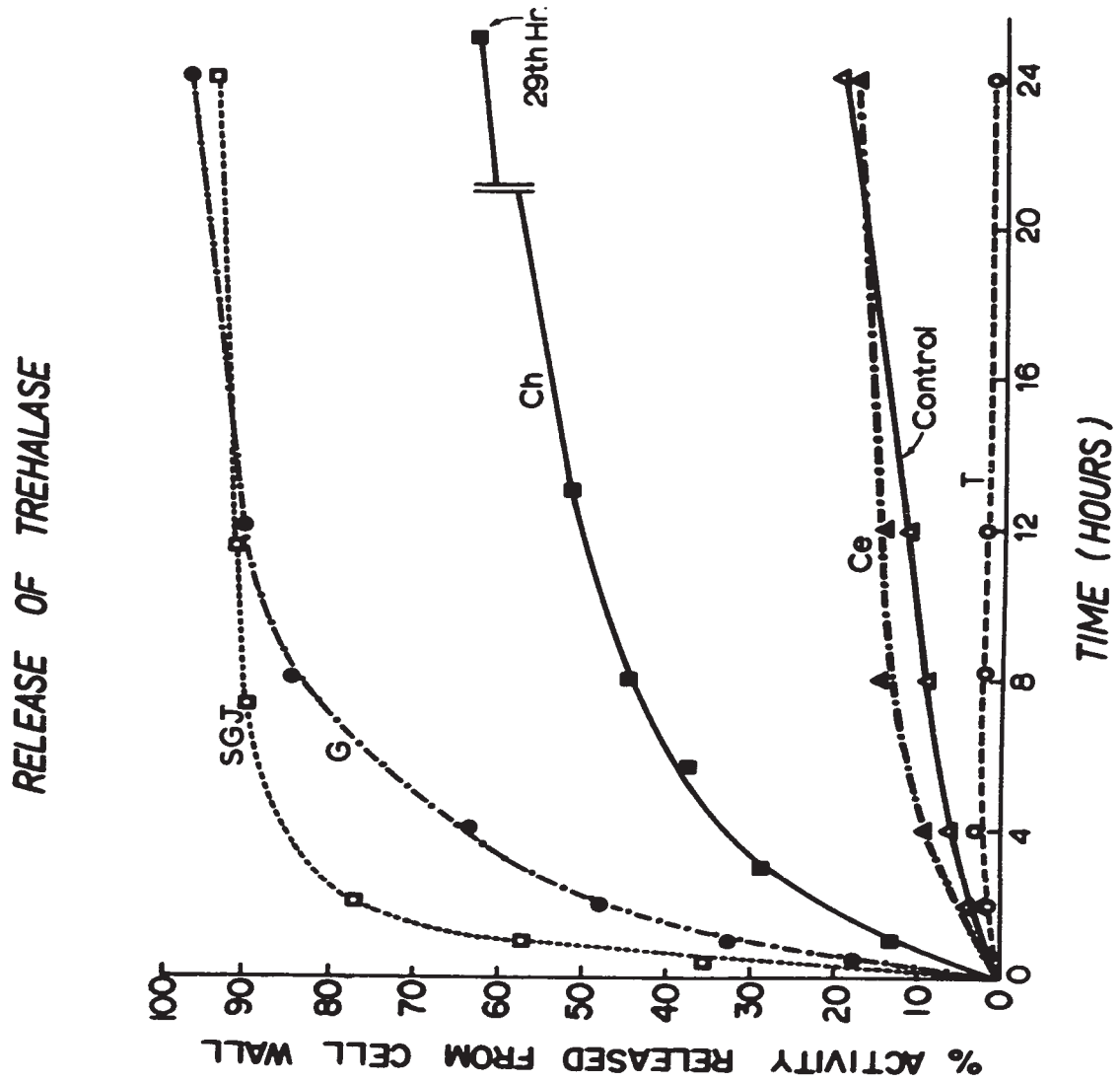

RELEASE OF TREHALASE
% ACTIVITY RELEASED FROM CELL WALL
TIME (HOURS)
SGJ
G
Ch
29th Hr.
Ce
Control
T
0
10
20
30
40
50
60
70
80
90
100
0
4
8
12
16
20
24

FIGURE 9

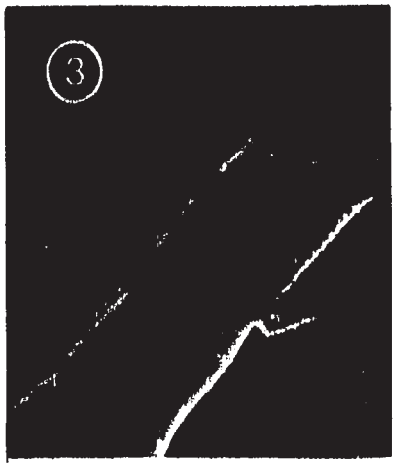
Scanning Electron Micrographs of

Neurospora Cell Walls After Digestion by Hydrolytic Enzymes

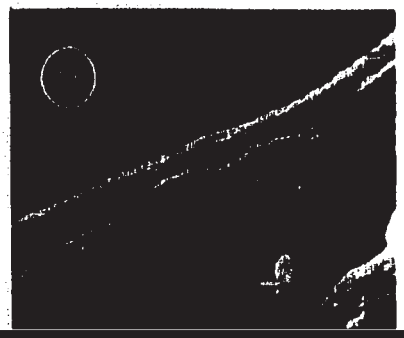
1. Control: no enzymatic treatment, surface amorphous and granular.
2. Snail Gut Juice (24 hr digestion): Structural elements such as granules and microfibrils are lost.
3. Trypsin (24 hr digestion): surface more granular.
4. Chitinase (24 hr digestion): slightly more prominent granules and underlying microfibrils.
5. Cellulase (24 hr digestion): no difference from control.
6. β-1,3-Glucanase (12 hr digestion): prominent reticulum with longitudinal thick strands and ramifying microfibrils.

7,500 X

1,500 X



7,500 X




1
2
3

7,500 X

7,500 X

7,500 X

of the ^{14}C-radioactivity in cell walls was solubiliz
cellulase. Therefore, the association of exoenzymes
Neurospora cell walls probably does not depend on β-
linkages as found in cellulose, although such polyme
seem to be present, as shown by the considerable amo
of radioactivity released by cellulase. This agrees
Galsworthy's data (1966) on the release of glucose f
Neurospora cell walls by cellulase. However, the ce
preparation used in this experiment appeared contami
with sucrose- and trehalose-cleaving activities. Th
contribution, if any, towards the release of radioac
or exoenzymes from the cell wall polymers is not kno

Fig. 4: Trypsin released only 19% of inve
into the supernatant, compared to almost 16% release
control. Its effect on trehalase could not be asses
in this experiment since the enzyme was severely ina
by trypsin. However, preliminary investigations sho
similar pattern of release as obtained for invertas
though none of the radioactivity in the ^{14}C-labeled
was released after 24 hours of incubation with tryps

Fig. 5: Chitinase was rather specific in h
lysing chitin with only a very slight hydrolytic act
towards laminarin after 24 hours of incubation. It
79% of invertase and 63% of trehalase to appear in t

supernatant fraction after 29 hours of digestion. for half of these enzymes to be released were 4.2 a hours respectively. However, only ca. 16.6% of rad activity was released from ^{14}C-labeled cell walls a 24 hours of incubation.

Fig. 6: β-1,3-Glucanase, specific agains hydrolysed Neurospora cell walls more slowly but al efficiently than snail gut juice. After 24 hours o 94% of invertase and 96% of trehalase finally appea in the supernatant fraction; half of them were rele after 2 hours in both cases. Similar incubation al ca. 82.4% of radioactivity from ^{14}C-labeled cell wa (cf. 73.5% by snail gut juice).

Fig. 7 is a composite graph to compare th ness of the various hydrolytic enzymes to release i from cell walls. They can be listed in the order o potency as: cellulase, trypsin, chitinase, snail gu and β-1,3-glucanase. The same order also applied t trehalase release (Fig. 8). Furthermore, it has be revealed that the amounts of exoenzymes released we proportional to the amounts of cell wall material h by the same treatment. An interpretation of these be presented in Chapter VII, the general discussion

b. Scanning Electron Microscopy

Scanning electron microscopy, a relatively technique for studying ultrastructure, has seldom bee to the study of fungal ultrastructure, primarily bec the rather low resolution (max. 20,000 X in the Camb Mark II model), compared to the conventional transmi electron microscopy which can generally offer 10- to fold higher magnification. The chief advantage of t technique is in its simple requirements. The sample only has to be dry and is ready for observation afte of conducting material has been applied, hence circu the many chances of introducing artificial modificat intrinsic to the techniques of transmission electron scopy, perhaps with the exception of the method of f etching (Haggis, 1966).

Conventional types of transmission electro microscopy, e.g. sectioning of tissue with subsequen fixation by glutaraldehyde, osmium tetroxide and per (Hunsley & Burnett, 1970; Shatkin & Tatum, 1959; Nam 1960), replica and shadowing in isolated cell walls & Colvin, 1967; Hunsley & Burnett, 1970), have all b applied to the study of Neurospora crassa cell walls culminating in the present state of knowledge concer

the structure and composition of Neurospora cell walls The purposes of this experiment were not to duplicate original and more meticulously persued findings but to (i) an independent approach to monitor any gross morph changes in cell walls resulting from enzymatic hydroly and (ii) a means of correlating the biochemical change with the ultrastructural composition of Neurospora cell as known from the literature.

Cell wall fraction digested by cellulase los ca. 10% of ^{14}C-radioactivity. However, the cell wall morphology was not visibly altered under the same cond (Fig. 9·5 cf. control in Fig. 9·1). In contrast, trypsi digested cell walls appeared more granular although no ^{14}C-radioactivity was released (Fig. 9·3). Manocha & Colvin (1967) and Hunsley & Burnett (1970) showed that proteolytic enzymes were able to digest away part of t cell wall matrix in the interstices of the fibrillar n This probably accounted for the rougher texture of cell wall surface observed here.

Potgieter & Alexander (1965) showed that chi alone released only ca. 5% of Neurospora cell wall in form of N-acetylglucosamine. However, if the cell wa had been previously digested by β-1,3-glucanase, twice much N-acetylglucosamine was released by chitinase.

Therefore, they suggested that part of the chitin w
accessible to enzymatic hydrolysis only after a mas
layer of glucan had been removed, such as by β-1,3-
The residues in Fig. 9·4 may represent the cell wal
the easily accessible chitin was removed. If so, m
the substance seen would be made up of a glucan mat
the inaccessible underlying chitin microfibrils.

Fig. 9·6. After β-1,3-glucanase digestio
thick reticulum layer with longitudinal strands and
orientated microfibrils of the cell wall was reveal
concurred with the recent observation by Hunsley &
(1970),who studied Neurospora cell walls by thin se
and shadow-casting techniques. The thick reticulum
were suggested to be a glycoprotein and the microfi
were shown to be chitinous with interstices partly
by a proteinaceous matrix.

Snail gut juice digestion removed all the
and fibrillar elements from the cell walls (Fig. 9·
Streiblová (1968) observed a thin cell wall layer i
closely adposed to the plasma membrane of the cell
resistant to snail gut juice digestion. Galsworthy
and other workers also observed fragments of Neuros
cell walls remaining after snail gut juice digestio
Hence, the flaky residues in Fig. 9·2 possibly corr

to these structures seen in yeast and Neurospora. Th residues were neither digested by phospholipase A (k donated by Dr. W. Magee, University of Western Ontar London), nor dissolved by acetone (personal observat Therefore, it was unlikely that they represented res contaminating cytoplasmic membranes (although such g contamination was improbable after the Triton wash d preparation of the cell wall fraction). Because sna gut juice contained all the enzymes necessary for di the three major components of the Neurospora cell wa (glucan matrix, proteinaceous matrix and chitinous m fibrils), these residual smooth flakes are tentative designated as a fourth structural component.

The effects of the various specific enzymes the ultrastructure of Neurospora cell walls have bee documented. Since the scanning electron micrographs the cell walls appeared compatible with the descript in literature, it is possible to interpret the ultr ural changes involved in the present biochemical inv gations with greater confidence. Thus, the release wall-bound exoenzymes was coincident with destructi part of the cell wall structure. However, the amou the released enzyme neither corresponded to the ext

destruction (as shown by the release of radioactivi nor to the destruction of a particular morphologica Chitinase was able to release ca. 80% of invertase trehalase although the amorphous matrix and part of fibrillar system were still intact. On the other h the dissolution of the glucan matrix, leaving some reticulum and microfibrillar network, also caused s release of the bound enzymes.

It is concluded that when any of the thre wall structural components was removed, i.e. inters protein matrix, fibrillar network (including microf and reticulum), or amorphous glucan matrix, cell-wa bound exoenzymes could be released, although to qui extents. However, the fourth structural component, innermost layer resistant to snail gut juice digest was devoid of any significant enzyme activities.

CHAPTER V. DISTRIBUTION OF INVERTASE IN NEUROSPOR DURING ITS ASEXUAL LIFE CYCLE

Neurospora crassa is a fungus in the Clas Ascomycetes. It can reproduce sexually by heteroth fusion of gametes or asexually by producing spores conidia. In the asexual life cycle, filamentous hy germinated from conidia under favourable conditions as in a flask of culture medium, and undergo an ini phase of rapid growth in which the cell mass increa logarithmically with time. Microscopically, the sm ovoid conidia are seen to give rise to germination that elongate to form unbranched slender hyphae. F logically, this is a stage of rapid transition fro to active metabolism.

After this logarithmic phase, the statio sets in when the growth rate drops abruptly, reflec less abundance of substrates and increased populati density. The hyphae can be seen to branch freely a join together to form an amorphous three-dimensiona ramifying mass, which is collectively called a myce According to Zalokar (1959a), the mycelium growing contact with air differed markedly in its biochemi

cytological characteristics from that which was compl submerged in a liquid medium. The differentiation be these two populations of mycelia was probably in resp to a difference of oxygen tension. In the submerged moribund features such as intense vacuolation of cyt accumulation of fat globules were prevalent.

Later in the growth cycle, substrates even become limiting. Specialized erect hyphae called co phores differentiate from the mycelium. They cut of their ends in an acropetalous succession (developing the apex) strings of conidia. These conidia become they mature and can be dispersed over great distance the slightest air current to thrive again readily wh food and hot humid conditions prevail, hence their n the "bakery pest". After launching on suitable subs each conidium germinates and the cycle of growth and tiation will repeat itself.

In this part of the project, six morpholo or cytologically distinct stages were chosen from th life cycle of Neurospora to study the distribution c in the culture medium, in the total cell mass, and i cell walls. The purpose was to correlate the occur of the exoenzymes in cell walls with the changing ph states of the organism. The six stages were (i) co

(ii) mycelium in mid-logarithmic [mid-log] phase, (iii mycelium in post-log phase, (iv) aerial mycelium in st ionary phase, (v) submerged mycelium in stationary pha and (vi) mycelium induced to form conidiophores.

A. Materials and Methods

1) Culture and Growth Medium

The Neurospora strain and its maintainence were described in Chapter II-C. Standard growth media for culture used in all the following experiments containe Fries minimal medium (Beadle & Tatum, 1945) supplement with 2.7% galactose as carbon source and sodium succin buffer (0.04M, pH 5.2).

2) Culture conditions to produce Neurospora of dif physiological states

(i) Conidia

The method of Zalokar (1959a) was used t produce a large quantity of conidia. A conidial suspe (2 ml, absorbance at 600 nm = 20) was inoculated into Fernbach flask containing 500 ml of standard growth me solidified with 2% agar. After 7 days, the thick mat mycelium and conidia was scraped with a glass rod, dis

in water, crushed, and filtered as described in Chap
The final conidial pellet after centrifugation was a
in wet weight.

(ii) Mycelium in mid-log phase

A conidial suspension (2 ml, absorbar
at 600 nm = 20) was inoculated into 100 ml of standa
growth medium in a 250-ml Erlenmeyer flask. The cu
was grown at 30 C in a gyratory water bath shaker at
speed of 250 rpm and harvested after 10 hours by fil
through a membrane filter (type HA, 0.45 µm, Millipo
Massachusetts).

(iii) Mycelium in post-log phase

Procedure was similar to (ii) above b
culture was agitated at a speed of 200 rpm and harve
after 18 hours (50 ml in a 125-ml Erlenmeyer flask)

(iv) Aerial & (v) Submerged mycelium from stationary phase

The procedure was adapted from the me
by Zalokar (1959a). A standard culture medium (50 m
containing one drop of Tween 80 (polyoxyethylene sor
monooleate) in a 125-ml Erlenmeyer flask was inocula
with 0.5 ml of conidial suspension (absorbance at 60
= 20) and incubated in a gyratory shaker bath at 30
200 rpm for 3 days. The mycelium was harvested by f

through a Millipore membrane filter and washed with 5
of sterile water. The mycelial pad was dispersed in
flask containing 200 ml of the standard medium and 4
1% (v/v) Tween 80. This was incubated at 30 C as a s
culture. After 4 days, a translucent mycelium mat ap
on the surface of the liquid medium in contact with a
This mat was designated as the aerial mycelium. When
mined under the phase contrast microscope, it showed
dominance of hyphal filaments with fine granular cyto
content. Beneath this mat in the Roux bottle, there
dispersed wisps of mycelium entirely submerged in the
medium. These were referred to as the submerged myce
Under the phase contrast microscope, it showed a pred
of extensively vacuolated filaments and signs of cons
at some hyphal ends to form conidiophores. Actual co
was of course suppressed by the presence of Tween 80,
the mycelial mat from drying and differentiating into

(vi) Conidiophores

The method was adapted from that of St
Clark (1967). A standard growth medium (50 ml) in a
Erlenmeyer flask was inoculated with 0.5 ml of a coni
suspension (absorbance at 600 nm = 20). The culture
grown in a gyratory shaker bath at 30 C and 200 rpm f
48 hours. Portions of the mycelial culture (15-20 ml)
filtered on Whatman #1 filter paper in a 10-cm diamet
Büchner funnel connected to a vacuum pump. The filtra

was carefully controlled so that the mycelial mat wa
moist all the time. The final moist looking mat (ca
thick), together with the filter paper, was transfer
a petri dish and moistened with 0.2-0.6 ml of phosph
(0.1M, pH 6.0) containing 800 U/ml of penicillin-str
mixture (BBL, Div. of Bioquest, Maryland). The amou
buffer used was such that the mat was moderately moi
not dripping wet. This was critical for the success
induction of conidiophores. The mycelial mat was ma
1 mm thick instead of 2-3 mm as recommended so that
proportion of the mycelium was exposed to air and co
develop into erect conidiophores. The mycelial mats
harvested after 8 hours at room temperature. Most c
hyphae on the surface of the mat had developed into
phores with constrictions where the future conidia w
about to be split off. The mycelial mats (about six
in each experiment) were designated as "conidiophore
mycelium".

3) Experimental Procedure

The available culture medium from the above c
was saved. The harvested conidia or mycelia were di
by sonic treatment. An aliquot was set aside as
mycelial homogenate. The rest of the sample was us
cell wall preparation as described in Chapter II-D.

Table II.

Invertase Distribution in Neurospora crassa of Different Physiological Stages[a]

Neurospora cultures grown under different conditions (Chapter V-A) were harvested. Culture media were collected by filtration. Mycelia were washed with distilled water, blotted dry with filter paper to give the wet weight, and disrupted by sonic treatment. An aliquot of the mycelial homogenate was taken for total cellular activity determination. The rest of the sample was used for cell wall preparation by washing with PBS, 1% (v/v) Triton X-100, M-NaCl (twice) and PBS successively. The cell wall fractions were recovered by centrifugation at 100 x g for 15 min each time.

a: Each entry is the mean of two sample determinations in one experiment. Cultures in each physiological stage, except the conidia, were studied in duplicate experiments whose data are recorded separately.

b: Total activity was expressed in units of invertase activity, defined as μm glucose liberated from sucrose per minute at 37 C.

c: Specific activity was expressed as units of invertase activity/mg of protein.

	Conidia	Mid-Log (100 ml)		Post-Log (50 ml)		Stationary Culture - 4 days (200 ml)		Conidiophores & Mycelium	
(A) Culture Medium			Average		Average		Average		Average
i. Total activity[b]		27.3		30.3		171[d]			
		28.4		29.6		263[e]			
ii. Specific activity[c]				3.9		2.9[d]			
				3.8	3.8	4.0[e]	3.5		
iii.% of (B): $\frac{Ai}{Bii} \times 100$		13.6		16.9		17.9[d]			
		13.8	13.7	16.5	16.7	16.1[e]	17.0		
(B) Total Cellular Activity (100%)						Aerial Mycelium	Av.	Submerged Mycelium	Av.
i. Wet wt (g)	3.46	0.35		0.29		0.69[d]		0.26[d]		1.35	
		0.36		0.30		1.32[e]		0.39[e]		1.25	
ii. Total activity[b]	144	201		179		617[d]		429[d]		1450	
		206		180		963[e]		671[e]		1540	
iii.Specific activity[c]		6.5		8.6		10.7[d]		14.5[d]		16.2	
	0.63	6.6	6.5	8.9	8.7	11.0[e]	10.9	14.5[e]	14.5	16.2	16.2
(C) Cell Wall Fraction											
i. Total activity[b]	11.7	38.1		38.0		43.7[d]		96.1[d]		239	
		37.2		46.5		89.6[e]		137[e]		289	
ii. Specific activity[c]		82.0		128		40.8[d]		22.1[d]		74	
	2.93	88.8	85.4	113	120	40.7[e]	40.7	26.2[e]	24.2	75	74

protein concentration (see Chapter II-B) and invertase activity (see Chapter III-A1, p.43) of the culture m the total mycelial homogenate and the purified cell wa fraction were determined.

B. Results and Discussion

Table II: The specific activities and perce of invertase associated with cell wall fractions varie significantly in the different phases of the Neurospor asexual life cycle.

Conidia in the dormant stage had the lowest activity in the cell wall fraction. In young hyphae c mid-log phase (10 hr), invertase specific activity in total mycelial homogenate rose to 6.5 units/mg protein (ca. tenfold higher than conidial homogenate) and that the cell wall fraction was 85.4 units/mg protein (ca. fold higher than the conidia cell wall fraction). The percentage of total cellular activity associated with wall fractions also rose from 8% in conidia to 18.5% i young hyphae. The trend of increasing specific activi in total cells, in cell wall fraction and percentage c mural activity continued to the post-log phase mycelii (18 hr), reaching 8.7 units/mg protein, 120 units/mg p

and 23.4%, respectively; the last two data were the h
of their groups in all the stages investigated. The
phase under the specified growth conditions lasted 12
hours (Appendix 3).

The effects of aging can be inferred from
comparison of the mycelia in or near the log phase w
the older mycelia in a four-day-stationary culture.
specific activity of total cellular invertase contin
rise, with an almost twofold increase in the station
cultures. On the contrary, the specific activity of
cell wall fractions showed a two- to fivefold decrea

The lower specific activity in the cell wa
fractions of the stationary cultures may be the resu
decreased invertase activity and/or increased protei
The following comparison of post-log mycelium with s
mycelia is an attempt to delineate which of these tw
had been more dominant. Data of the post-log myceli
multiplied by four in order to normalize the culture
from 50 ml to 200 ml, similar to that of the station
cultures. Data of the stationary culture were poole
those of the aerial and submerged mycelia.

	Post-log (P)	Stationary (S)		
		Expt. I	S/P	Expt
Mycelial wet wt	1.18 g	0.95 g	0.8	1.7
Cellular activity	720 U	1046 U	1.4	164
Cell Wall activity	166 U	140 U	0.8	22

In both Experiments I and II, the ratios of cell wal between the stationary and post-log mycelia were ver to those of mycelial wet weight. Therefore, the inv activity of the cell wall fraction seemed to change comparable rate as the bulk of the mycelium. Hence, previously observed lower specific activity of the c wall fraction in the older stationary cultures could be caused by a lower mural invertase activity, but p by a higher non-invertase protein content than the c wall fractions of the log phase mycelia.

The effects of differentiation were demons by the two populations of mycelia of the same age, i the aerial mycelium and the submerged mycelium in th day-stationary culture. As in the case of aging, sp activity increased (to 1.5 times) in total cellular and decreased (to half) in the cell wall fraction of more senescent submerged mycelium. It would be inte to compare the data of these two mycelial population before in order to surmise what factor might have ca the lower cell wall specific activity.

	Expt. I			Expt
	Aerial (A)	Submerged (S)	S/A	(A) (S
Mycelial wet wt	0.69g	0.26g	0.38	1.32g 0.3
Cellular activity	617 U	429 U	0.69	963 U 67:
Cell Wall activity	44 U	96 U	2.20	90 U 13'

The ratios of cell wall activity between the submerg aerial mycelia exceeded both their mycelial wet weig total cellular activity ratios. Therefore, proporti higher invertase activity must be in the cell wall f of the submerged mycelium than that of the aerial my However, in spite of the increased mural activity, t specific activity of this cell wall fraction was onl half of that of the aerial mycelium. Hence, this co be the result of a lower mural invertase activity bu likely a marked increase of non-invertase protein in submerged cell wall fraction.

Some reservations about the data obtained the conidiophore induction experiment must be made. the normal life cycle, conidiophores should be chron gically older than the vegetative mycelium. In the experiments, the conidiophores were induced accordir the method of Stine & Clark (1967) from two-day-myce whereas the vegetative mycelium harvested in statior phase was already four days old. One more complicat

was that the conidiophores so induced were assayed
with the rest of the mycelial mat that made up the
portion of the sample material. Therefore, owing t
reverse order of age difference and the heterogeneo
condition of the sample, it was difficult to make a
accurate biochemical comparison between conidiophor
and the vegetative phase of the Neurospora life cyc
represented by the four-day-old culture. The only
made was that the conidiophores, together with a su
amount of 56-hour-mycelium, had the highest intrace
specific activity of invertase (16.2 U/mg protein).
contrast, the specific activity of the cell wall fr
of conidiophores and associated mycelium was interm
between that of the younger log phase mycelia and t
stationary phase mycelia. More accurate localizati
invertase in conidiophores by a cytochemical techni
be reported in the next chapter.

In conclusion, significant changes of inv
activity were observed during the Neurospora life c
Transition from dormancy to active growth was accom
by increased specific activity of the total cellula
invertase as well as the cell wall fractions. In c
older mycelia, either from aging or differentiation
activity of the total cellular invertase was also h
the younger mycelia but their cell wall fractions d
a concomitant increase. An interpretation of these
will be presented in the General Discussion, Chapte

CHAPTER VI. HISTOCHEMICAL LOCALIZATION OF INVERTAS DURING ITS ASEXUAL LIFE CYCLE

Results from experiments in the last chap that the Neurospora cell walls from different stage asexual life cycle differed significantly in their content and specific activity of invertase.

The cytochemical aspect of the same probl also investigated. Histochemical stains specific f were used to localize the enzyme with the objective (i) confirming the association of invertase with ce (ii) comparing the cytological distribution of inve throughout its asexual life cycle with the known bi data, and (iii) elucidating finer details of enzym bution that had evaded biochemical approaches. Tw methods were used: (a) a histochemical stain with diaminobenzidine (DAB) as the chromogen, and (b) a immunofluorescent staining technique. In the firs glucose produced by invertase activity was detecte second method, invertase in mycelial sections reac rabbit anti-invertase serum. The antibody-antigen so formed reacted further with a fluorescein-conju goat-anti-rabbit serum. Invertase occurrence was

visible as green fluorescence under a microscope eq
with a UV light source. This method was used to st
same six different stages in the Neurospora asexual
cycle as in the last chapter.

A. Materials and Methods

1) Histochemical Staining with 3,3'-diaminobenzi

This was according to the procedure of Chung &
Trevithick(1970). Invertase in 2 μm thick mycelial
was allowed to react with sucrose. The glucose that
produced reacted with DAB indirectly via an enzyme c
system and caused an insoluble brown deposit at site
reaction.

$$\text{Sucrose} \xrightleftharpoons{\text{Invertase}} \text{Fructose} + \text{Gl}$$

$$\text{Glucose} + O_2 \xrightleftharpoons{\text{Glucose Oxidase}} \text{Gluconic acid} +$$

$$H_2O_2 + \text{DAB} \xrightleftharpoons{\text{Peroxidase}} H_2O + \text{oxidize}$$

(brown c

The stained sections were observed under oil immers
with a Carl Zeiss Standard Universal Microscope.

2) Immunofluorescent Staining

a. Preparation of Antigen

Invertase was prepared from Neurospora according to the method of Metzenberg (1963a) with following modifications:-

(i) An inoculum (4 l) of 40 hours old mycelium fro culture was injected into a fermentor containing 8(sterilized growth medium.

(ii) Mycelium was harvested with a press filter, w with about 10 gallons of water and wrung dry in ch cloth to give a wet weight of 2.78 Kg. (The yield 100% more than that from 8-l carboy cultures).

(iii) The final purification step with carboxymeth cellulose column chromatography, which caused 50% of invertase activity, was now replaced by Sephade column chromatography. A 3 x 100 cm column of G-2(Sephadex was equilibrated with buffer B (sodium su buffer, 0.005 M, pH 5.0, containing 0.001M EDTA) f over 24 hr. The column was charged with ca. 100 m protein dissolved in 1.5 ml of buffer A (sodium su buffer, 0.1M, pH 5.0, containing 0.001M EDTA) at a specific activity of ca. 290 units of invertase ac per mg of protein. Elution with buffer B proceede

a flow rate of 0.2 ml/min and 3-ml fractions were co
The whole operation was performed below 4 C.

Absorbance at 280 nm and invertase activity
Chapter III-A1) of the eluted fractions were measure
(Fig. 11). About 100% invertase activity was recove
from fractions 50-82. Disc-gel electrophoresis fo
detecting proteins and invertase activity was perfor
according to Metzenberg (1964). A predominant singl
of protein (Fig. 10A), coincident with invertase act
was detected in fractions 59 and 67. Faster moving
of impurities appeared in fraction 76.

b. Preparation of Anti-invertase Rabbit Ser

Electrophoretically pure invertase (3 mg
1.8 ml of buffer B) dispersed in Freund's complete a
(1.8 ml) was injected subcutaneously into each of th
rabbits, followed by a booster shot of 1 mg in 2 ml
PBS after 8 weeks. Rabbit serum was collected 6 day
by cardiac puncture and processed according to the m
of Campbell, Garvey, Cremer & Sussdorf (1963). The
gave a single precipitin line against pure invertase
10B) by Ouchterlony's two-dimensional diffusion test
(Campbell et al., 1963). Aliquots were frozen and di
15-fold with PBS before use.

c. Staining Procedure

Conidia or mycelia harvested in the vari physiological stages were embedded in Tissue-Tek (Am Co., Division Miles Laboratories, Inc., Elkhart, Ind and kept at -20 to -30 C in an Ames Lab-Tek Cryostat Frozen sections (2-µm thick) were cut and laid on sl which had been soaked in PBS overnight. They were f in 90% alcohol and stained with the "sandwich techni according to the method of Nairn (1969). Rabbit ant invertase serum in 1:15 dilution was applied to the section. The mycelial invertase reacted with the ra immunoglobulins to form an antigen-antibody complex. After the unreacted rabbit serum was rinsed off, goa rabbit serum conjugated with fluorescein (Hyland Lal Los Angeles, California) was applied in 1:10 dilutic In order to reduce nonspecific staining from the flu conjugate, the goat serum had been previously adsorl with acetone-dried liver powder, either prepared acc to the method of Holborow & Johnson (1967) or purch from Difco. Excess goat anti-rabbit serum was wash and sites of invertase occurrence in the sample wer as green fluorescence under a fluorescent microscop (Carl Zeiss Standard Universal Microscope, excitati filter II, barrier filter 50/44).

FIGURE 10A

Disc-gel Electrophoresis of Purified Invertase

About 30 µg of protein from a representative fra of the first peak after G-200 Sephadex Chromatography applied to the cathodic end (left) of the gel. Prote was stained with Amido-Black and the single prominent coincided with invertase activity.

FIGURE 10B

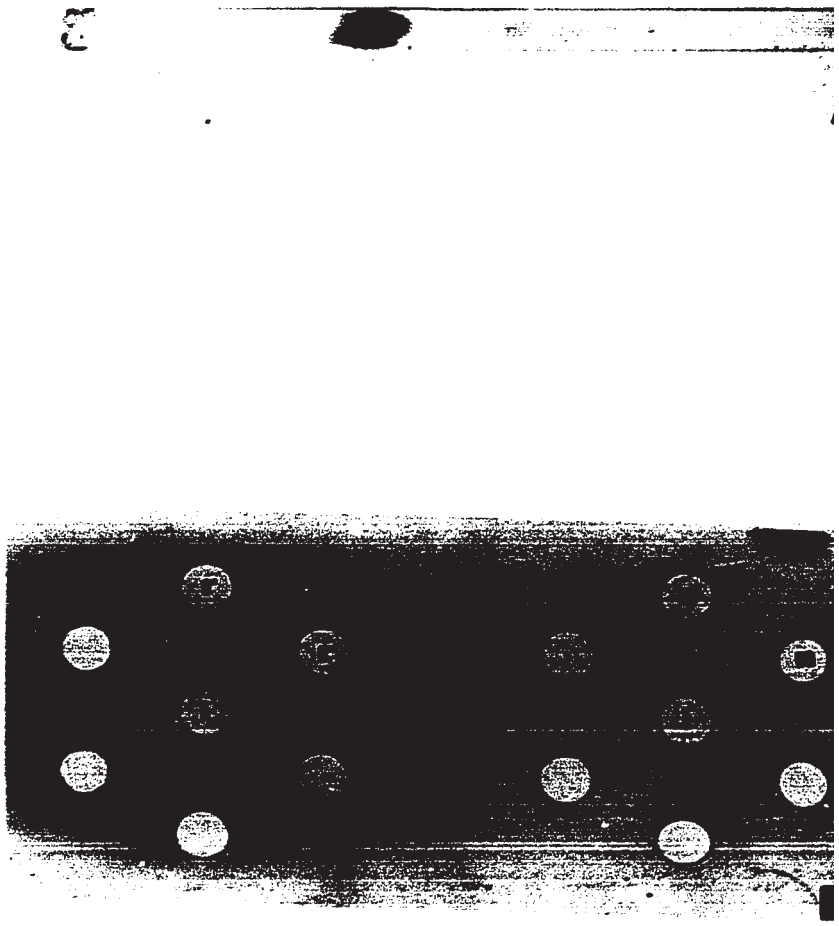
Ochterlony's Test
For Invertase and Anti-invertase Rabbit Seru

i: 5-10 µl of purified invertase (1 mg/ml).

r: anti-invertase rabbit serum.

n: normal serum from the same rabbit before immunizat

The four unlabeled wells on the left contained s of two other immunized rabbits which have not develop a strong immunologic reaction against this preparatic of invertase. The peripheral wells on the right were filled with the normal sera from the corresponding ra Procedures were according to Campbell et al., 1963.



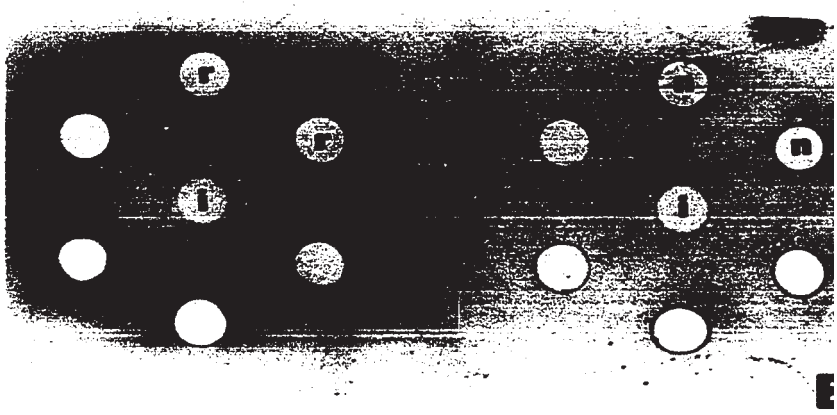


FIGURE 11

Purification of Invertase by Sephadex G-200 Chromato

Conditions of the experiment are described in Chapte

Protein concentration = (Absorbance at 280 nm/$\epsilon^{0.1\%}_{1\ cm}$

$\epsilon^{0.1\%}_{1\ cm}$ = extinction coefficient of in

= 1.86

Invertase activity = (Absorbance at 420 nm x C.F.

C.F. = Conversion Factor

= 1,333

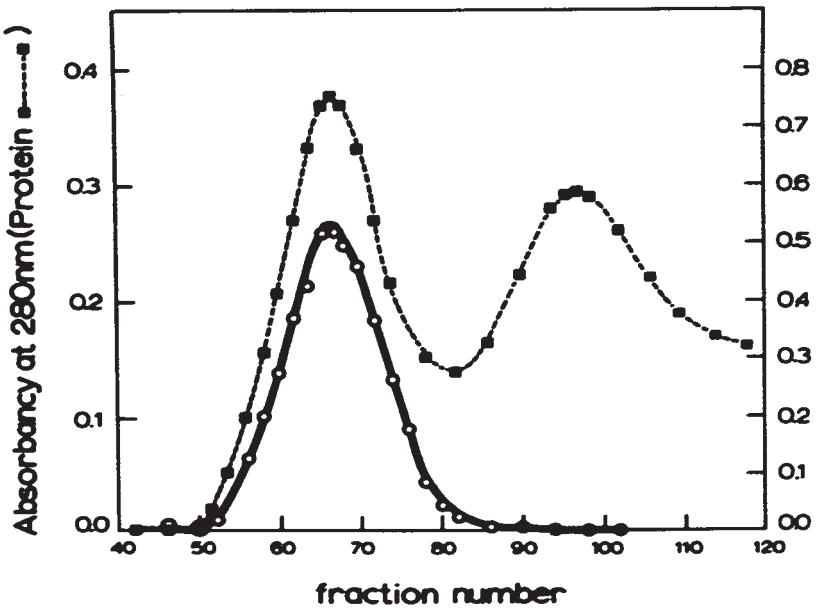

Absorbancy at 280nm(Protein ■----■)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
40
50
60
70
80
90
100
110
120
fraction number

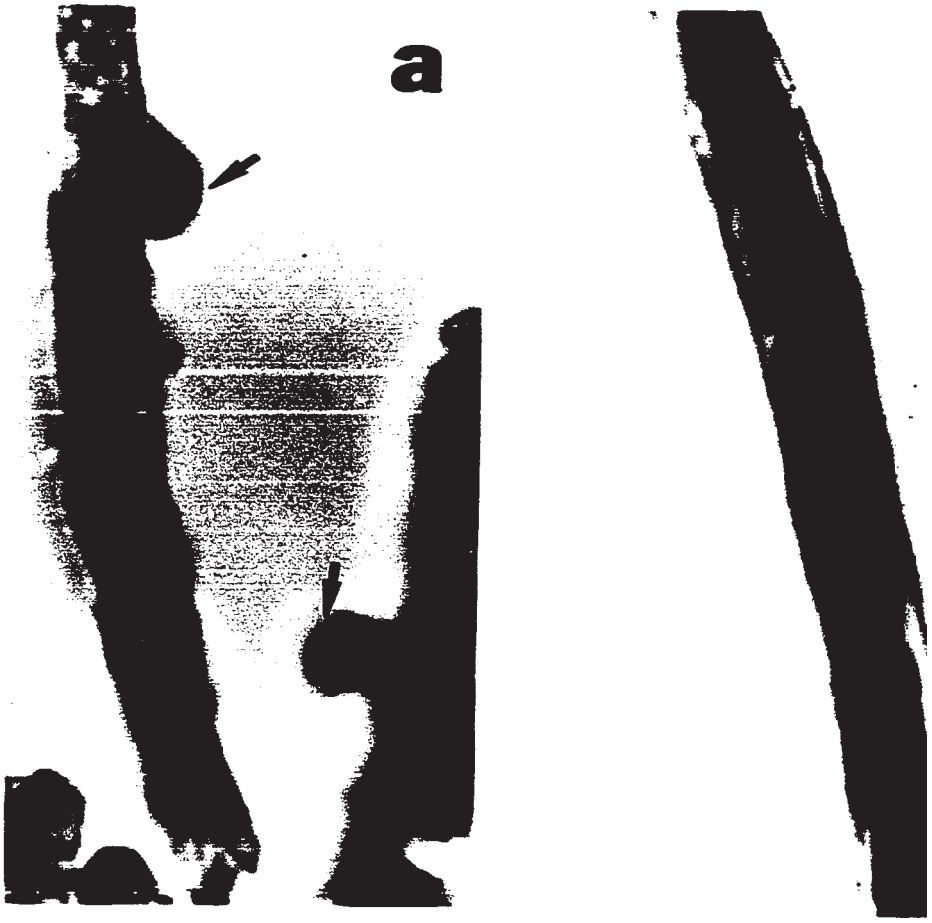
FIGURE 12A

Neurospora Hypha (2-µm section) in Post-log Phase
Stained for Invertase with DAB

a) Intense peripheral stain on the two budding points obliqu
facing each other.

b) Hypha showing invertase activity on the cell wall and cyt
and intense activity on the plasma membrane.

Incubation lasted for 90 min. x 4,2


a

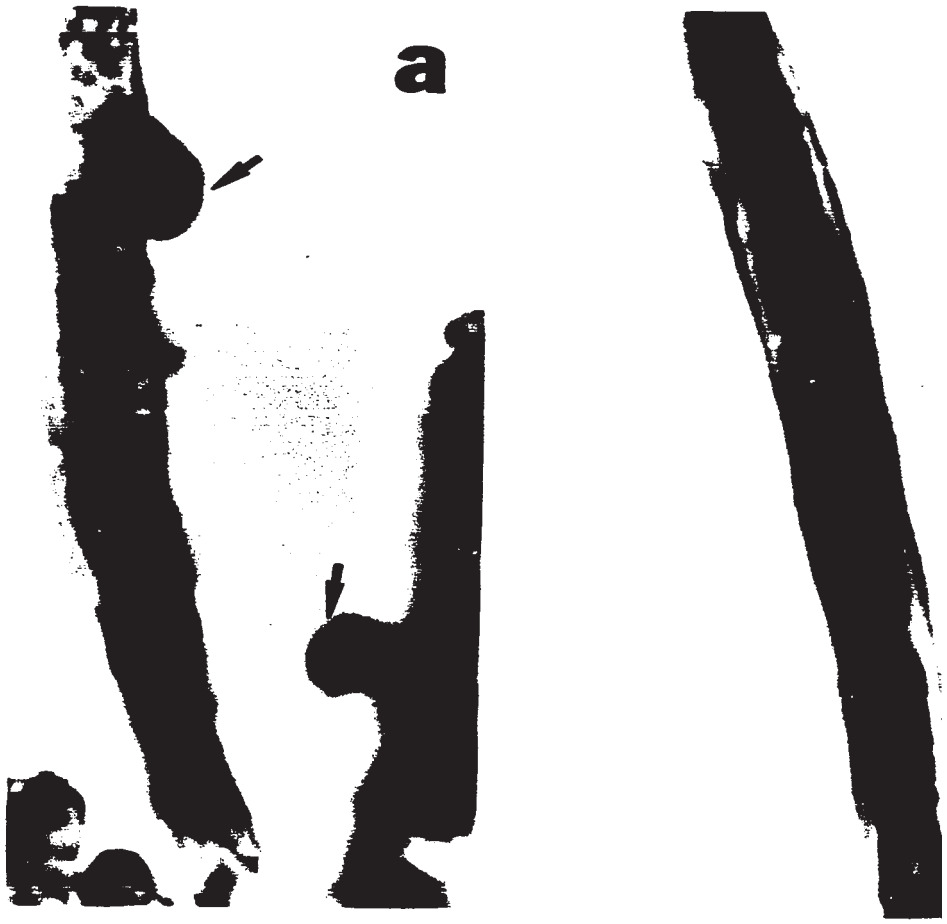

a

FIGURE 12B

Controls of

DAB Histochemical Stain for Invertase

(i): Experimental tissue section incubated with the co Reagent. Incubation lasted for 30 min.

(ii): Control tissue section incubated with an already developed chromogenic reagent by substituting suc with glucose, to test for nonspecific adsorption dye deposit on tissue section. Incubation lasted for 30 min.

(iii): Control tissue section incubated with water subst tuting for sucrose in the reagent, to test for chromogenic reaction due to endogenous glucose ir the tissue. Incubation lasted for 90 min.

X 2,000.

ii

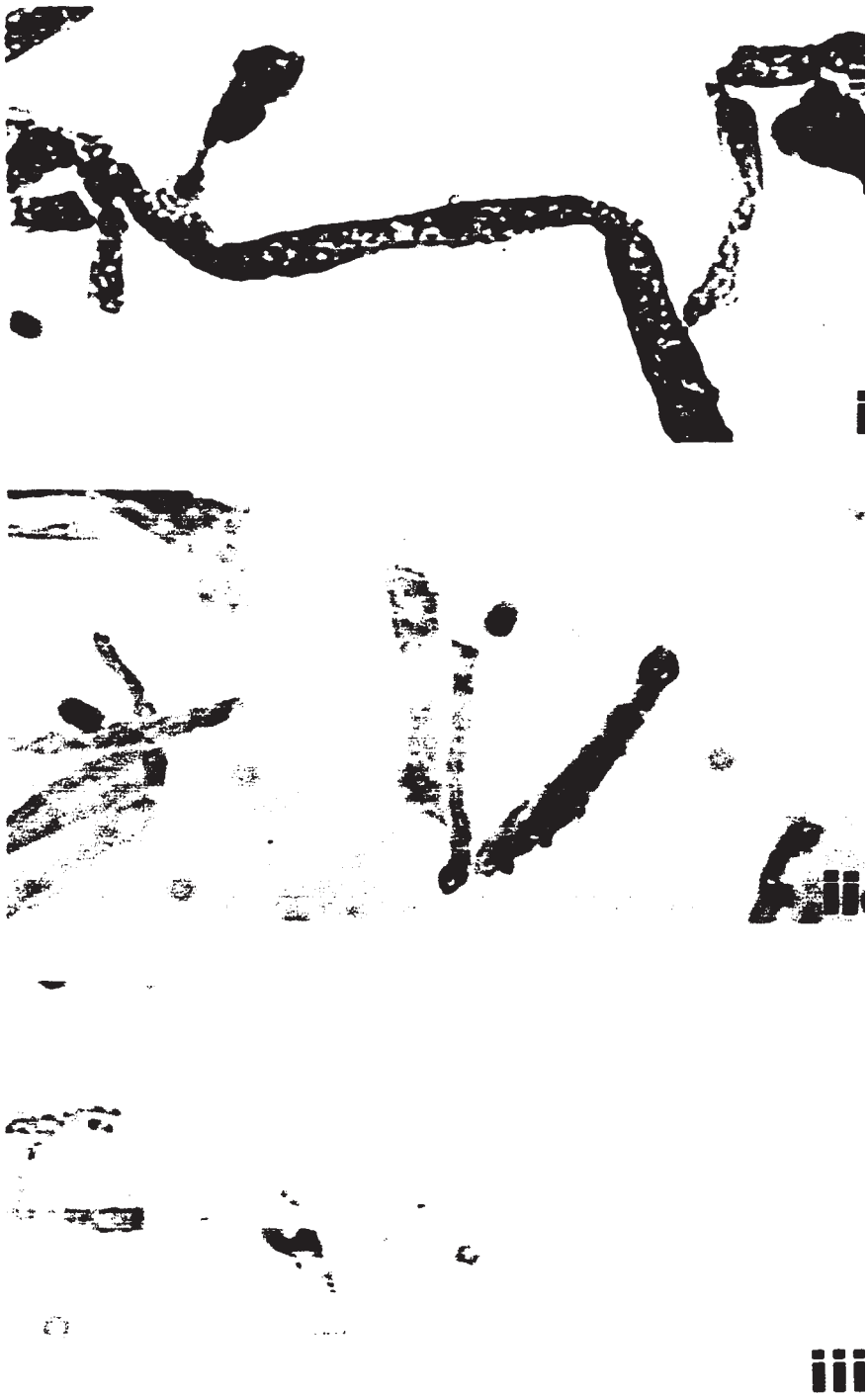


FIGURE 13

Germinating Neurospora Hyphae (2-μm sections)
Stained for Invertase with an Indirect Immunofluorescent Tec

(a) Conidia showing peripheral invertase activity.
(b) Germinating conidia with general cytoplasmic invertase a
and marked activity in the cell wall of the germ tube.
(c) Young hypha in early log phase, showing decreased cytopl
invertase but very strong activity associated with the c

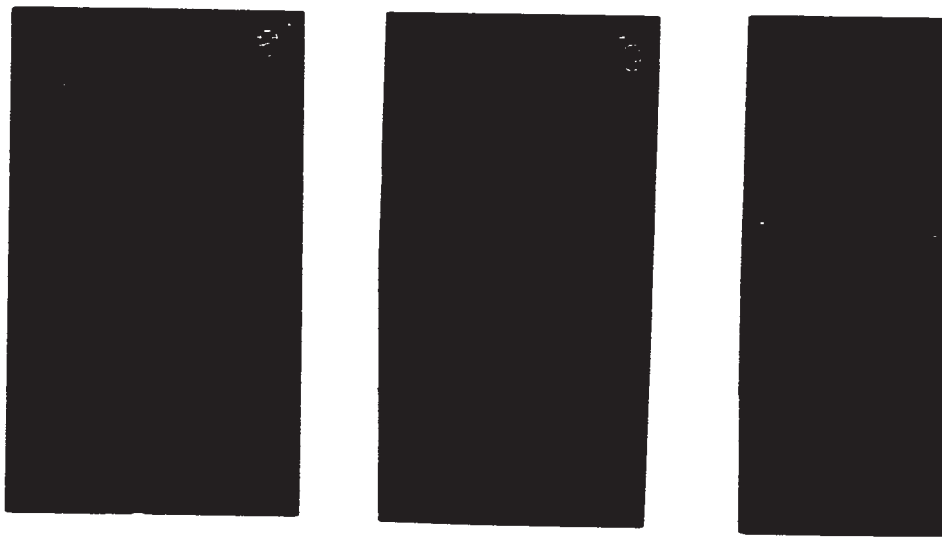
x 2,

FIGURE 14

Neurospora Hypha (2-μm section) in Post-log Phase
Stained for Invertase with an Indirect Immunofluorescent Tec

Intense activity was found at the cell wall and the budding
in lower right portion.

x 2,



Neurospora Hyphae (

Stained for Inverta

(a) Aerial hypha wi

intensely than

left with its s

(b) Submerged hypha

Neur

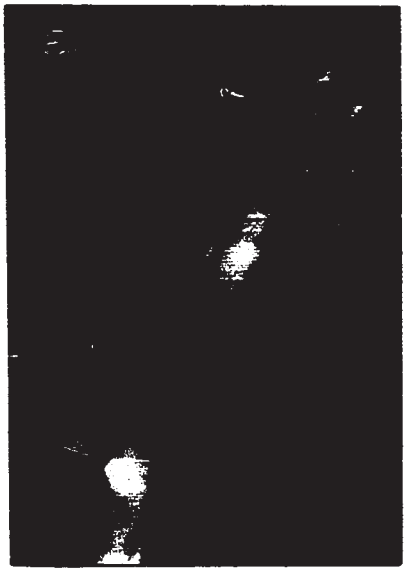
Stained for Inverta

Strong invertase ac

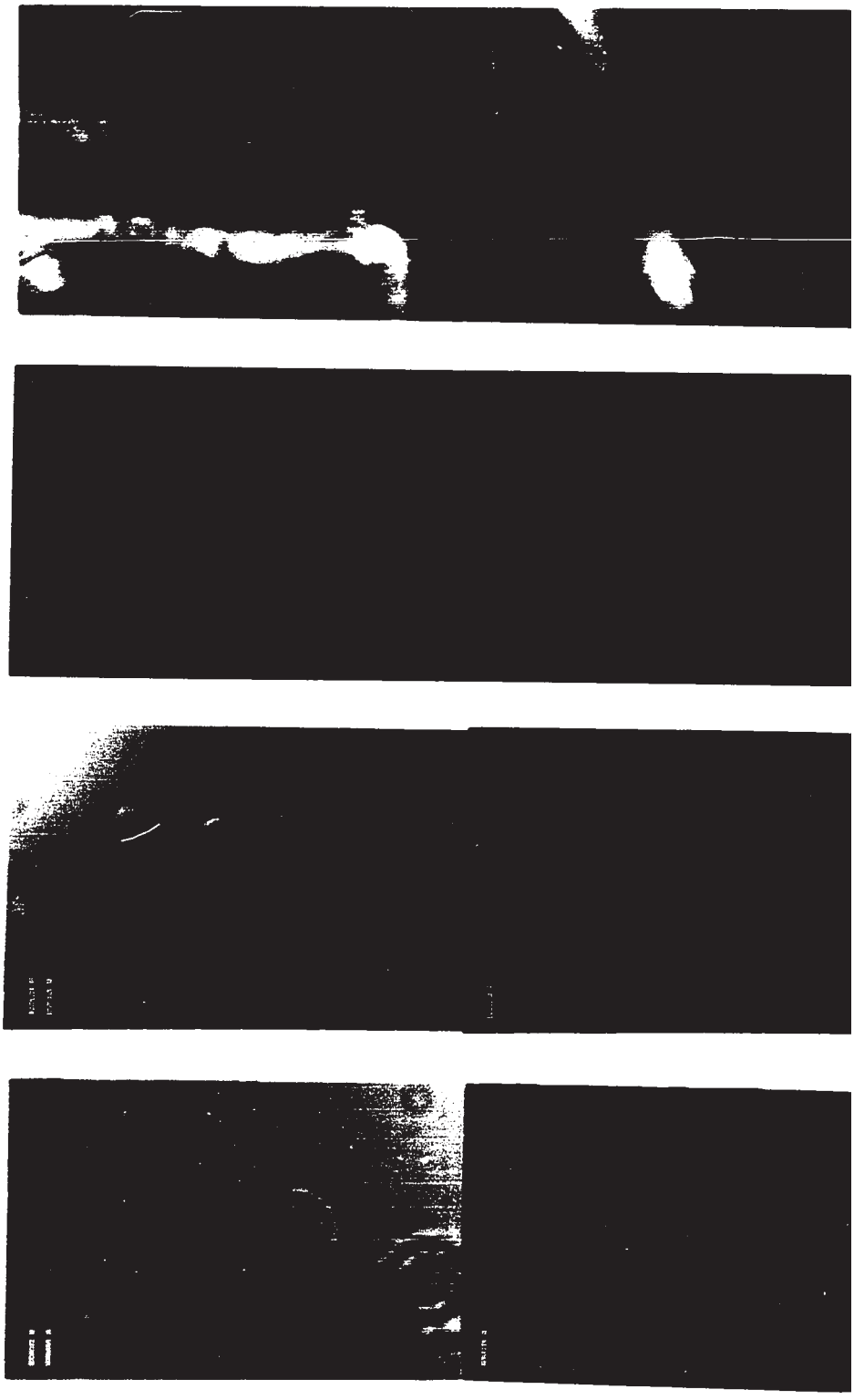
the cell walls.

c: newly detached c

x: unstained portio



a



The contrc
sections were: (i)
immunization agains
anti-invertase seru
was adsorbed with p
rabbit serum was us
conjugate was appli

B. Results and Disc

1) Histochemical

Fig. 12A. Se
phase were stained
first 10-20 min of
light brown patch o
By 30 min, a genera
a darkly stained pl
microscope. Incuba
to permit the cell
than the cytoplasmi
suitable for photog
the slides was chan
course of incubatio
the occurrence of i

walls was confirmed. In addition, budding points (showed strong activity in the cytoplasm and especia the plasma membrane and/or cell wall. The last two cannot be differentiated from each other at this re

The control sections supplied with a DAB r that had fully developed the chromogenic deposit (i replacing the sucrose in the incubation medium with showed no significant staining as compared to the e section over a period of 30 min of incubation (Fig. Therefore, nonspecific adsorption of the dye deposi the tissue section was not apparent. The control s supplied with water instead of sucrose also showed significant staining as compared to the experimenta section over a period of 30 min of incubation. Hov if the incubation was extended to 90 min as in Fig. slight nonspecific staining appeared in the cytopla plasma membrane, but to a much lower intensity than experimental sections and such nonspecific staining occurred on the cell walls. Hence, artifacts due t endogenous glucose in the tissue were negligible.

Nitro-blue tetrazolium salts (NBT) was a (tional chromogen for histochemical localization of oxidation:reduction enzyme systems (Jos, Frézal, R

& Wegmann, 1967; Seidler & Kunde, 1969; Altmann, 19
Sigel & Pette, 1969). In the present experiment to
invertase in Neurospora, this chromogenic reagent w
inferior to the DAB reagent, both in sensitivity an
specificity. Reaction color began to appear after
min of incubation with DAB, as compared with more t
hour in the case of NBT. With DAB, 90 min was suff
for development of an intense dark-brown stain whe
the NBT stain required at least 3 hr to develop a
intense but detectable color. The definition and
of localization demonstrated by NBT stain were poo
defined whereas those by DAB were comparable to th
immunofluorescent staining.

An invaluable asset of this staining meth
DAB is in the electron opaqueness of the reaction
so that the localization of enzymes can be readily
to the electron microscopic level. Furthermore, t
reaction product is osmiophilic and insoluble in o
solvents, two features that are most useful in ele
microscopy (Graham & Karnovsky, 1966).

Results obtained from DAB staining, such
wall and budding point activity of invertase (Fig.
were concordant with those from immunofluorescent
described below. The pattern of invertase localiz

during the germination stages obtained by this histo technique was very compatible with that shown in Fig by immunofluorescent staining. Only one of these me was employed to localize invertase activity during t rest of the Neurospora life cycle since the specific of each method and the compatibility of their result been established. Immunofluorescent staining was th method of choice in light microscopy because sites o reaction, as indicated by fluorescence, were more ea detected against a dark background than histochemica staining reactions.

2) Immunofluorescent Staining

The specificity of this indirect immunofluores staining depended on three primary factors: the puri the antigen; the specificity of the rabbit anti-seru and the absence of nonspecific staining (NSS) due to random adsorption of rabbit or goat serum on tissue of Neurospora.

As little as 1% impurity in antigen prepara can provoke independent immune responses and most an that meet the laboratory criteria of purity are cont by small amounts of immunologically unrelated antig (Davis et al., 1967). A sensitive method to detect s

specificity of anti-serum against an impure antigen
gel diffusion precipitin reactions. The double diff
technique is extremely sensitive and valuable in det
the number of antigen-antibody systems, which was in
by the number of precipitin lines between the antige
and the antibody well. Demonstration of a single pr
line is adequate for most immunohistochemical purpos
qualify the homogeneity of the immune system (Hopsu-
& Ekfors, 1969). Immunoelectrophoresis is a more pc
modification of this technique. The antigen in a sm
well is first allowed to migrate across an electric
before the double diffusion precipitin reaction occu
with the antiserum, which was placed in a long troug
parellel to the axis of the electric field after the
electrophoresis. This technique is usually used for
of complex composition, e.g. human unfractionated s
(Davis et al., 1967), tissue extract (Raunio, 1968)

In the present study, the antigen invertase
relatively pure protein as demonstrated by polyacry
gel electrophoresis (Fig. 10A), but slight contamin
by immunologically unrelated substances was possibl
Therefore, the double diffusion technique was appli
test for the specificity of this immune system. Ac
to the results from the Ochterlony's test, only a s
precipitin line was obtained between the antigen an

antibody (Fig. 10B). The absence of additional prec
line was taken to prove the monospecificity of this
In the event that additional immune-systems remained
undetectable by this technique, which is able to det
as little as 10 µg antibody per ml (Davis et al., 19
they were unlikely to cause significant error in the
staining specificity since the antiserum was used at
a high dilution (1:15).

The specificity of the immunological reacti
(between the invertase and anti-invertase rabbit ser
is demonstrated by two controls (Holborow & Johnson,
First, normal rabbit serum instead of anti-invertase
rabbit serum was used to stain the first layer of th
"sandwich". Lack of reaction indicated that negligi
random adsorption of rabbit serum to tissue sections
occurred (Fig. 17B). Second, rabbit antiserum previ
adsorbed with the antigen invertase was applied. La
significant fluorescent staining in this control (Fi
showed that the rabbit anti-invertase serum reacted
with the antigen (purified invertase) and did not cr
react with other Neurospora cellular constituents.

After the specificity of the rabbit serum h
been established, the last factor to be considered i
recurrent practical problem of nonspecific staining

due to random adsorption of fluorescein-conjugated globulins (Holborow & Johnson, 1967). Using dilut sera and adsorption with acetone-dried tissue powde two methods often capable of reducing such NSS. Th tactics were applied to the fluorescein-labeled goa antiserum, which was previously adsorbed with acetc liver powders and used at 1:10 dilution. As shown Fig. 17D, the goat anti-serum was not adsorbed to t tissue section when the middle layer of rabbit immu globulin was absent, thus showing the absence of NS the fluorescein-conjugated goat antiserum.

The above considerations and controls indi the high degree of specificity obtainable with the present indirect immunofluorescent method in that r specific staining arising from impure antigen, incc specificity of rabbit antibodies, or random adsorpt antisera did not contribute significantly to the st pattern in the experimental tissue sections.

Fig. 13. The localization of invertase wa through germination. The conidium showed enzyme ac on the periphery as an intense fluorescing ring and out the cytoplasm as a weaker diffuse fluorescence During germination, invertase fluorescence was four germ tube cytoplasm and markedly increased in the c

cytoplasm. Only the cell wall at the germ tube sh
fluorescence; the rest of the conidial cell wall h
the discrete peripheral stain (Fig. 13b). In the
log phase, the young hyphal cell wall became stron
fluorescent and the hyphal cytoplasm was weakly so
(Fig. 13c). In the meantime, the residual conidiu
to be quite depleted of activity.

Fig. 14. As the log phase came to an end
18 hr, stronger cytoplasmic activity was evident t
the conidia and younger hyphae (Fig. 13). This ph
was compatible with the higher specific activity f
the total mycelial homogenate (Table II). In addi
the budding point in the lower right portion of th
showed strong endogenous activity, similar to that
with the DAB stain in Fig. 12A. As before, the ac
seemed to reside in both the cytoplasm and the cel
of the incipient branch and no distinction can be
between the two. The significance of such bud act
will be discussed in the next chapter.

Fig. 15. The immunofluorescent staining
of representative hyphae from two mycelial populat
the same stationary culture of four days are shown.
the aerial hypha of Fig. 15a, the cell wall was mu
fluorescent than that of the post-log phase hypha
This was also reflected in the lower specific acti

well as the total activity per unit wet weight of t mycelium in the cell wall fraction. Again, relativ stronger fluorescent activity was observed in the h initial at the lower left portion of the hypha. Th this type of localized activity was not confined to close to the log phase. In the submerged hypha of the cell vacuoles, a prominent feature in this kind submerged mycelium, were devoid of activity. This especially remarkable as the total and specific act of the mycelial homogenate were relatively higher t of the aerial mycelium (Table II).

Fig. 16. Strong invertase activity was as with conidiophore cell walls as well as distributed evenly in the cytoplasm. This explicit localizatic invertase activity in the conidiophores showed that rather high total and specific activity observed in mycelial homogenate and the cell wall fractions of heterogeneous sample "conidiophores + mycelium" wer relevant to the conidiophores <u>per se</u>. The newly de conidia in this Figure 16 differed from the conidiu Fig. 13a in two main aspects. There was a discrete of fluorescence on the outside and the rest of the cytoplasmic activity was more diffuse. These diffe may be the result of their different ages. The di

cytoplasmic activity of the young and newly detached
could become more peripherally localized as they aged
form the well matured conidia shown in Fig. 13a. The
identity of the cellular component that accounted for
ring of fluorescent activity was not known. It could
the conidial coat that later became more dehydrated a
conidium aged. The subsequent shrinkage of the coat
cause it to fit more snugly outside the conidial cyto
On the other hand, it could be the residual cell wall
the conidiophore and the septa that used to demarcate
conidium from the next in the differentiating conidio

In conclusion, two methods have been successf
applied to localize invertase activity in Neurospora
confirm its occurrence in cell walls. The histochem
stain DAB was superior to NBT in sensitivity and in
specificity. Observations made with the immunofluor
method not only confirmed the biochemical localizati
invertase in Neurospora cell walls of different phys
logical states; they also indicated more subtle feat
such as activities in budding points, conidiophores,
the absence, in vacuoles, of any activity.

CHAPTER VII. GENERAL DISCUSSION

Several features about the relationship be
exoenzymes and Neurospora cell walls have been revea
the foregoing chapters. The different exoenzymes we
associated with cell walls to various degrees; coval
had to be disrupted when the bound enzymes were rele
and the extent of invertase association varied with
age of the mycelium. Most of the questions raised i
beginning of the investigation can be satisfactorily
now but a few still defy clear-cut solutions. The f
part of this discussion is devoted to the more posit
and direct answers with an interpretation of their t
logical significance. The second part deals with th
equivocal answers and an interpretation of them base
a proposed model of cell wall structure.

Section A

Exoenzymes and Neurospora Cell Walls

Of the six exoenzymes studied, ribonuclease amylase, and protease were found in small amounts in cell walls (2%, 2% & 9%, respectively) whereas signi portions of aryl-β-glucosidase, invertase and trehal (15%, 26% & 29%, respectively) were cell-wall-bound Table 1). These different percentages indicated tha enzymes were associated with the cell wall fractions various extents. Furthermore, the nature of their s strates appeared to correlate with the extent of enz association. Exoenzymes that acted on macromolecule e.g. ribonuclease (on polynucleotides), protease (on polypeptides), and amylase (on polysaccharides) were in small amounts in the cell wall fractions. Exoenz that acted on small molecules, e.g. aryl-β-glucosida (on substituted monosaccharides), invertase (on disa and trehalase (on disaccharides), were quite extensi associated. From this, it is postulated that when t substrates are small enough to diffuse into the cell it would be more economical for part of the corresp exoenzymes to remain cell-wall-bound. The enzyme pr is better conserved and its hydrolytic products are

closer to the cell membrane transport systems for up
On the other hand, this measure of economy would not
feasible if the substrates were too large to reach t
enzymes in a mural location. An appreciable 9% of a
was in the cell walls and yet appeared inaccessible
its macromolecular substrate starch. According to t
above postulate, this 9% might be responsible for hy
the smaller oligosaccharides, e.g. amylo-dextrins, w
are hydrolytic intermediates of starch and are prob
small enough to penetrate the cell walls.

Distribution of Invertase in the Asexual Life Cycle

The association of invertase with cell wal
to bear a significant relationship with the state o
growth of Neurospora. Generally, the more actively
hyphae had high specific invertase activity in thei
fractions (Table II). Histochemical methods reveal
budding points had very strong endogenous activity
conidiophores were moderately well supplied with th
whereas cell vacuoles were apparently devoid of it

The lowest invertase activity (total and s
was in the conidial wall preparation. A number of
enzyme systems in Neurospora (Zalokar, 1959a) also

the lowest activity in conidia, which was in accorda with the lower state of metabolic activity in dorman structures. Histochemically, invertase was found pr nantly at the conidial periphery. This was perhaps best strategic position to attack substrates during sequent germination. Metzenberg (1963b) studied the accessibility and enzyme inhibition of invertase in and came to similar conclusions. In his work, no di could be made whether the enzyme was in the cytoplas membrane, periplasmic space, or cell wall. However, least 8% of the total cellular activity has now been definitely found in the cell wall fraction (Table II

During germination and subsequent logarithm growth, the transition into a highly active metaboli state often entails an elevation of many enzyme acti In Neurospora, a spectrum of enzymes involved in bio pathways, e.g. NAD-, NADP-dependent glutamic dehydro (Stine, 1968), in respiratory activities, e.g. succi dehydrogenase (Zalokar, 1959a), cytochrome oxidase (1963), and in various hydrolytic reactions, e.g. tre invertase (Hill & Sussman, 1964), aryl-sulfatase (Sc & Metzenberg, 1970), increased markedly during these initial stages of the Neurospora life cycle. Theref the increasing specific activities of the total cell

invertase from the conidial stage to the mid-log pha
and then to the post-log phase (Table II) were compa
with the above general pattern of heightened activit
addition to a marked increase of cellular activity,
lar invertase activity and specific activity of the
fractions also rose during the logarithmic phase, pr
indicating active synthesis as well as secretion of
(although the possibility that higher specific activ
due to lower amount of Folin-positive material canno
out). The function of invertase is to supply the ea
metabolized glucose from sucrose, which otherwise ca
transported into the intracellular milieu (Marzluf &
1967). The increased synthesis and secretion of suc
enzyme are the logical answer to the need of an acti
growing organism.

However, during the later stages of the
life cycle when senescent cytological features and s
growth rates were brought about either by aging or d
tiation, the specific activities of the total cellul
invertase also increased. A number of inducible enz
e.g. β-galactosidase (Zalokar, 1959a), aryl-β-glucos
(Eberhart, 1961), and de-repressible enzymes, e.g. i
trehalase (Metzenberg, 1962) in Neurospora has been
to increase in aging cultures, or in a medium with p
sugar substrates, or on the depletion of exogenous s
The increased cytoplasmic activities of these enzyme

been attributed to the accumulation of intracellula (Zalokar, 1959a), or the absence of repressor, sucl glucose in the production of trehalase (Hanks & Su 1969b). Therefore, the increased cytoplasmic speci activity of Neurospora invertase associated with s might be due to a depletion of accessible exogenou supply and a reduced metabolic rate, with subseque relief from intracellular catabolite repression, s by glucose. The mycelial mats that had been starve eight hours to induce conidiophore formation also very high specific activity in the total cellular (Table II). This is also explicable on the basis above proposition.

In spite of the increased cytoplasmic spe activity that accompanied senescence in Neurospora cell wall fractions showed a lower specific activi their younger counterparts. In both cases, i.e. s due to aging or differentiation, the decrease was to be the result of a lower mural activity relati that of the younger mycelia. Furthermore, a diff noted here between senescence caused by aging and caused by differentiation. In aging, the ratio (i invertase activity/mycelium wet weight) was quite in both the mycelia of post-log phase and those o older stationary phase (p.100, Chapter V). In di

the ratio (mural invertase activity/mycelial wet w
was much higher in the submerged mycelium than in
aerial mycelium (Chapter V, p.101). A preferentia
incorporation of invertase into the cell wall frac
the submerged mycelium was possibly indicated but
significance of such a difference is still not und

In another type of differentiation, veget
hyphae of Neurospora were induced to form conidiop
strong invertase activity occurred in both the cyt
and the cell walls of these newly differentiated s
(Fig. 16). Similar accumulation of NAD glycohydrc
Neurospora conidiophores was observed by Stine (19
and of trehalase in newly formed conidia by Hanks
Sussman (1969a). At this stage of the Neurospora
cycle, the organism was preparing for dormancy, or
prolonged deprivation of food. Investment of a ca
exoenzyme such as invertase would ensure a ready s
of suitable nutrient for germination when a substr
source was encountered. Several other exoenzymes
similar functions were also found on the conidial
besides invertase, e.g. aryl-β-glucosidase, maltas
cellobiase (Eberhart, 1961). If the teleological
pretation suggested for invertase is accepted, it
probably be applied to these enzymes as well.

The absence of invertase activity in vacuo presented an interesting contrast to the presence proteases, ribonuclease, amino-peptidase and estera the yeast vacuole (Matile & Wiemken, 1967). The di was readily rationalized by considering their funct The lysosomal enzymes in the yeast vacuole had the to degrade vital cytoplasmic components, such as pr ribonucleic acids, nascent peptides etc. It was on segregation of these enzymes that the rest of the c could be protected from uncontrolled and premature destruction. A similar precaution would be unnecess for invertase, whose function is mainly to cleave s into glucose and fructose, which are good sources c metabolic energy. However, recently, invertase was in yeast vacuoles (Beteta & Gascon, 1971). The enz characteristics of these vacuoles have not been ver to show that they were identical to those studied b Matile & Wiemken (1967). Beteta & Gascon (1971) al suggested that the vacuoles containing invertase se a similar function as the zymogen granules in mamma pancreas studied by Palade et al., 1964). If so, th "vacuoles" would correspond to "secretory vesicles" were quite different in nature from the moribund va in Neurospora hyphae, or the lysosomal vacuoles in (Matile & Wiemken, 1967).

A recurrent phenomenon observed in hyphae o
different physiological stages was the concentration
invertase activity at incipient branching points (Fi
12A, 14 & 15). This was not an artifact arising fro
intense staining of any dome-shaped structures, such
branch initials, that were not cut in a median secti
Primary growth tips had similar geometrical construc
but such intense localization of activity was never
whereas branch initials were frequently observed to
this type of localization. It may be a local respon
the greater need of energy supply at sites of cell w
synthesis (Zalokar, 1965). Initiation of new growin
fronts requires additional energy for the partial di
of old cell wall and deposition of new wall material.
aggregation of invertase is able to furnish the nece
energy supply by generating fermentable monosacchari
Aside from this probable physiological significance,
cause of the accumulation is not known but will be d
in the second part of this chapter in conjunction wi
hypothesis of cell wall structure.

Remarks

The greatest satisfaction during this proje
was derived from the revelation of the high degree o
efficiency and organization in living organisms. Ev

the finest detail of the cellular activity could be with a purpose. An enzyme was localized so that it with several basic principles: namely, the accessib of substrates, the economy of the cell, the need of enzyme in a particular area of the cell, and even a prognostic investment of enzymes in anticipation of future needs.

The next part of the discussion is concern results that are less amenable to an interpretation purpose or design. However, an attempt to account them, although speculative in nature, became even m interesting and challenging.

Section B

Besides their well acknowledged structural fungal cell walls are also intimately related to the functioning of exoenzymes. They may act as molecula to regulate a two-way transit of enzyme and substrat molecules, and as an anchorage for some of the exoen However, the experimental findings thus far did not any direct solution to the question: how did exoenzy traverse the cell walls that seemed too nonporous fo their passage?

A mechanism for this type of diffusion is suggested, based on a model of hyphal wall structure proposed by Burnett in 1968. It is an attempt to ra the phenomenon of exoenzyme association with cell wa Furthermore, it offers a theoretical reconciliation the various conflicting aspects of the molecular sie activity of fungal cell walls (See Chapter I, sect

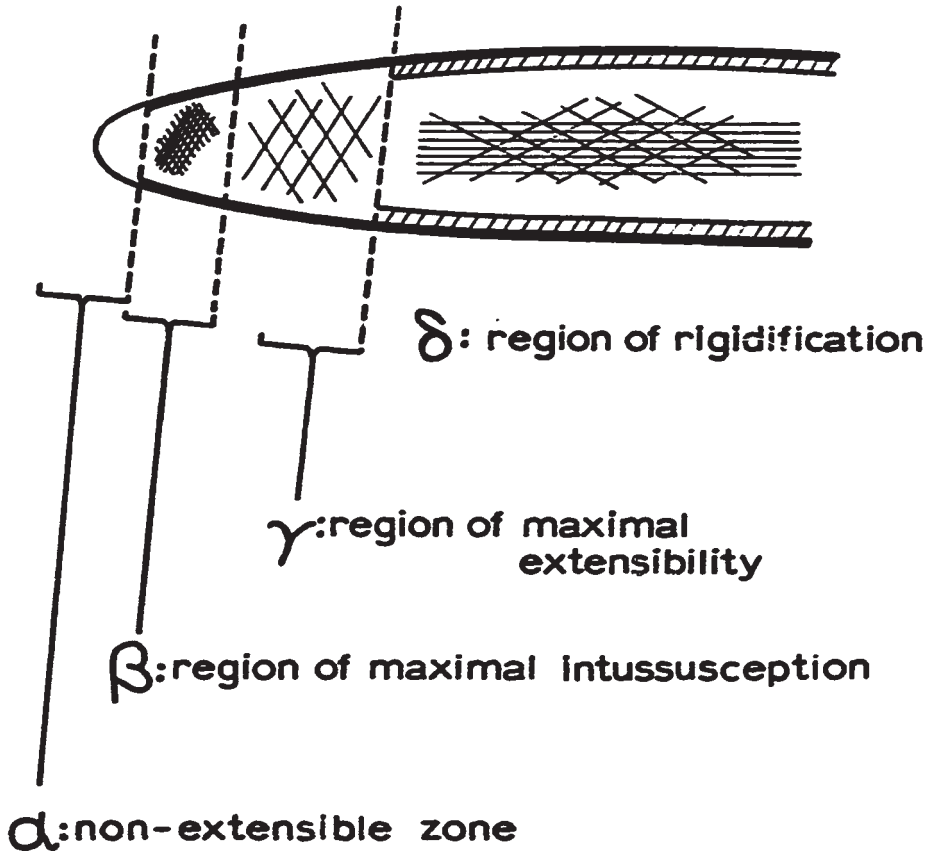
Hypothesis for Macromolecular Diffusion Through Cell

The structure and composition of fungal ce change with age. Secondary thickening and increased of chitin may occur in an aging mycelium (Aronson &

FIGURE 18

Model of Cell Wall Structure at Hyphal Tip

- adapted from Burnett, 1968.


δ: region of rigidification
γ:region of maximal extensibility
β:region of maximal intussusception
α:non-extensible zone

1959). However, the most interesting point is this. in a single hypha, perceptible regional differences e: Reinhardt (1892) first pointed out that hyphal extens: occurred exclusively at the tip region. This had bee repeatedly reaffirmed and extended in later experimen on hyphal tips. The accumulated data led to a propos: apical zonation in the hyphal cell wall by Burnett in The diagram in Fig. 18 is adapted from the original p (Burnett, 1968).

Fig. 18: α is the extreme non-elastic hyph which resisted distortion even by drastic osmotic cha in the medium. This zone was demonstrated in Neurosp and a number of other fungi by Robertson (1958, 1965) Zone β is the region of maximum intussusception and a of new cell wall material. Zone γ is the fastest gro region of the whole apex. It is highly elastic and m cally weak. When apices of some fungi were osmotical shocked with water, the shoulder region that showed t maximum amount of swelling and distortion corresponde this zone. In Neurospora this could also be the regi where lateral protrusion or disintegration began when hyphal tip was exposed to snail gut enzyme (Rizvi, 19 Robertson, 1965). Both β and γ zones are characteriz by transverse or randomly orientated (multi-net) micr Zone δ, extending back to the whole hypha, is rigidif

by more cell wall material, consisting of axially or microfibrils. According to this scheme, supported b many physiological experiments on fungal tip behavic apical region had a cell wall whose structure and cc are more flexible and less thickened than that in th of the hypha (i.e. α, β, γ against δ).

Exoenzymes are now suggested to pass throu cell wall, or rather the incomplete cell wall, in th apical region. According to this hypothesis, the pr of diffusion is visualized as follows. The cell wal this apical dome can be regarded initially as a very sieve so that passage of macromolecules is unimpeded because of extension growth in the apex, what has be elastic and porous apex (zones α, β, γ) gradually pass zone δ, a zone of cell wall thickening by microfibri addition. When more microfibrils are added, the ope of the sieve become proportionally smaller. In effe the pores of the molecular sieve are gradually being At the end of the rigidification process, the pore s are stabilized and have become too small for the pas of most macromolecules such as exoenzymes.

Interpretation of Data from Literature

Since the rigidification is probably a r process, the interstitial spaces between the pores ma be uniform. A continual distribution of pore sizes u a limit in the stabilized and rigidified cell wall is predicted. This is entirely compatible with the pres of definite upper threshold values for molecular diff and the heteroporous nature in isolated fungal and ba cell walls (Gerhardt & Judge, 1964; Trevithick & Metz 1966b).

As mentioned in the historical review in Ch Neurospora cell walls did exert a fractionation effec favouring the passage of the smaller invertase subuni into the culture medium (Trevithick & Metzenberg, 196 This is also accountable according to the present sch because the cell walls are still acting as a sieve, a a continuously closing one. Smaller molecules would stand a better chance of passing through. In the osm mutant, the proportion of light invertase in the medi decreased. Hence, more heavy invertase had been able pass the wall. This decrease of fractionation effect correlated with changes in the cell wall composition. of the changes noted was the increased ratio of galac

to glucosamine (Trevithick & Metzenberg, 1966b; Livin 1969). Chitin, an N-acetylglucosamine polymer, was c to be the microfibrillar element in Neurospora as wel other Ascomycete cell walls (Manocha & Colvin, 1967; & Koffler, 1969). According to the proposed scheme, situation in osmotic mutant could be visualized as fo because less of or abnormal microfibrillar material w available for rigidification in zone δ, the sieve clo down more slowly, thus allowing more heavy invertase pass through. After rigidification was completed, th stabilized pore sizes still remained larger than thos the wild type, thus accounting for the molecular weig threshold of 18,500 compared to 4,750 of the wild typ (Trevithick & Metzenberg, 1966b).

So far, the proposed mechanism of macromole diffusion seems to accommodate quite adequately the c of molecular sieving by cell walls as proposed by Tre & Metzenberg (1966a). Furthermore, it offers a mecha to overcome the impasse on macromolecular diffusion i by the small pore sizes of cell walls. Its relevance the present findings will now be examined.

TABLE III

Molecular Weights[a] and Per Cent Activities[b] of Exoenzymes in Neurospora Cell Wall

a: approximate mol wt from literature

b: data from Chapter III

c: % in parentheses = % activity detectable after degradation of cell wall fraction by lytic enzymes G-1

d: Over 90% of amylase activity was due to glucamylase (Gratzner, 1969) whose apparent molecular weight was ca. 25 000 as determined by agarose gel filtration.

e: The apparent molecular weight of intramural trehalase, assumed to be a globular protein, was determined by gel filtration but was not confirmed by ultracentrifugation.

Exoenzymes	% Activity in Cell Wall[c]	Molecular Weights	Reference
Group 1:			
Ribonuclease	0 (2)	10 000	Takai et al., 1966
Acid proteases	2 (2)	22 000	Matile, 1967
Amylase	2 (9)	25 000[d]	H.G. Gratzner, personal communication
Group 2:			
Aryl-β-glucosidase	15	168 000	Eberhart & Beck, 1970
Invertase (heavy)	29	210 000	Meachum et al., 1971
(light)		51 500	
Trehalase	26	300 000[e]	A.S. Sussman, personal communication

Interpretation of Present Findings

Three premises about fungal exoenzymes may be accepted. They are formed in the cytoplasm; some them are found in the cell walls; and they appear in medium. What is the connection among these events? is the presence of exoenzymes in the cell walls acco for?

1. Exoenzymes and Their Molecular Weights:-

The amounts of various exoenzymes associ with Neurospora cell walls differed significantly fr each other (see Table 1). If cell walls are molecular the molecular weights, and hence the sizes, of the e should determine the ease with which they pass throug sieve. However, if the cell walls responsible for di are a continuously closing sieve, some exoenzymes may trapped during their transit. During the process of rigidification, the cell walls whose pores are initia large enough to admit the macromolecules from the peri side have since become too small to let them out on t exterior side. This portion of trapped exoenzyme is postulated to correspond to that found in the cell wa On the other hand, the portion of exoenzymes that man escape before the cell wall is completely rigidified

account for the truly extracellular enzyme in the me

If the above assumption is correct, it fol that the larger molecules would be more easily trapp Therefore, the larger are the molecular weights of t exoenzymes, the more of them are expected to be in t cell walls. It is evident from Table III that the f group of exoenzymes present in small amounts in the wall fractions, i.e. ribonuclease, protease, amylase molecular weights about tenfold smaller than those c second group that were present in greater amounts, i aryl-β-glucosidase, invertase, trehalase. In the ca invertase, both heavy and light forms were found in cell wall fraction (Fig. 19). The exact percentages activity in the cell wall fraction were not critical they varied with the physiological state of the orga Inasmuch as the molecular weights of the enzymes wer approximate and unconfirmed by independent approache that they were not determined directly on the enzyme from the cell wall fractions (except invertase), any conclusions drawn therefrom must be tentative. Howe the direct correlation between molecular weights and of mural activity of these exoenzymes seemed to be m persistent than mere coincidence and was quite compa the premise derived from the hypothesis; namely, the portions of enzymes were trapped during cell wall ri

2. Binding of Exoenzymes to Cell Walls:-

An implication of the hypothesis -- that cell-wall-bound exoenzymes correspond to the enzymes trapped during cell wall rigidification -- is that th exoenzymes are related to the cell walls merely by ph juxtaposition and not by any chemical bonds between t As will be shown below, no direct verification of this premise could be obtained from the studies on the rel of exoenzymes from cell walls.

The failure to release cell-wall-bound inve by β-mercaptoethanol, 1M KCl, EDTA, Triton X-100 and buffers with a wide range of pH values indicated that association did not depend significantly on disulfide salt linkages, metal ligands, hydrophobic or certain of hydrogen bonds.

The efficiency of hydrolytic enzymes to rel the cell-wall-bound invertase and trehalase indicated covalent bonds were involved.

β-1,3-Glucanase released more than 90% of i and trehalase. Chitinase released ca. 80% of inverta 60% of trehalase. Trypsin released only a few per ce invertase. Cellulase had no significant effect. The

deductions could be made. (i) At least a few per ce
invertase was released after hydrolysis of either a
bond, or a β-1,3-glycosidic linkage in glucan. (ii)
substantial amount of invertase was released after e
a β-1,4-glycosidic linkage in chitin or a β-1,3-glyc
linkage in glucan was hydrolysed. (iii) Trehalase a
to be released in a similar manner.

Therefore, exoenzymes could be released by
hydrolysis of any of the three chemical constituents
(i.e. protein, chitin, glucan) of the cell walls. Th
possibilities exist to account for their release. Fi
the exoenzymes were only physically confined within t
matrix of the cell wall. The various hydrolytic enzy
that degraded the cell wall polymers caused a breakdc
its structural integrity. The loosened "molecular si
became permeable to the macromolecules once more. Se
the exoenzymes were chemically bonded to one or more
the cell wall polymers. The hydrolytic enzymes not c
disrupted the cell wall integrity but also "dissected
out fragments of the cell wall polymers that were che
bonded to the invertase molecule. The loosened enzym
molecules, together with the attached cell wall fragm
were able to diffuse through the partially degraded c
walls.

At this point, it is impossible to make a
between these two possibilities. However, in the mo
proposed by Kidby & Davis (1970b), invertase was als
within the yeast cell wall, which was impermeable to
because of disulfide and/or phosphodiester linkages.
this permeability barrier was broken, free diffusion
invertase became possible. Although no disulfide or
diester bridges have been found in Neurospora cell w
permeability barrier can be readily conferred by β-1,
β-1,4-linkages or even peptide bonds in glucan, chit
protein, respectively.

Another observation also favoured the firs
possibility. During routine storage of purified cel
a cycle of freeze-thawing released from a few to abo
per cent of the cell-wall-bound invertase into a sup
fraction. Hence, mere mechanical force was sufficie
liberate some invertase from its mural association.
no covalent bonds could have been involved, at least
fraction of enzyme that was so released.

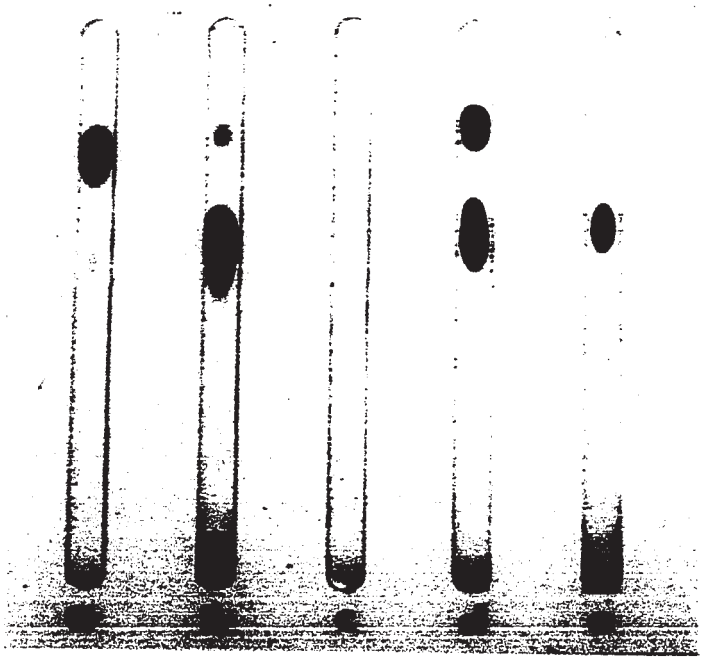
Furthermore, if cell wall fragments were s
attached to the liberated mural enzymes, as indicate
the second possibility, these attached enzymes would
larger than the pure enzymes and migrate slower in d
electrophoresis. However, no such difference was de
(Fig. 19).

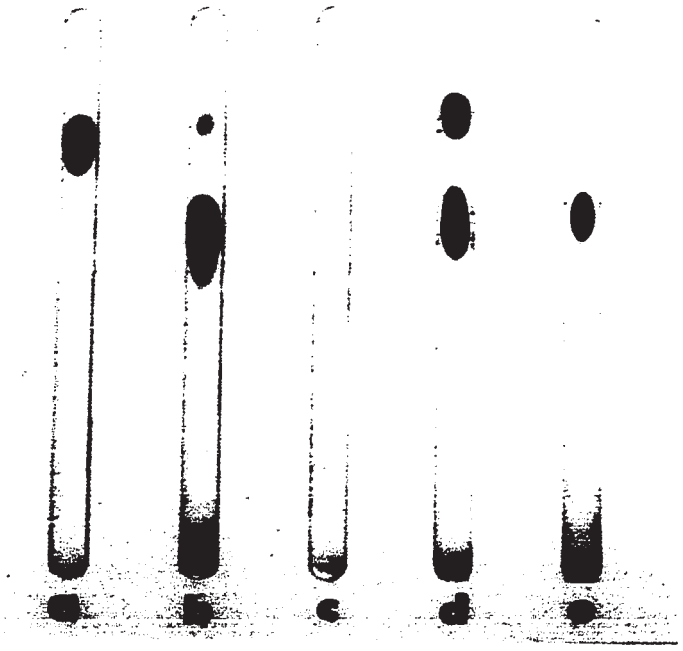
FIGURE 19

Disc-gel Electrophoresis of Invertase
Released from Purified Cell Wall Fractions

a: Purified invertase (heavy) containing about 0.2 U ac
b: Purified invertase after dissociation into subunits containing about 2.5 U activity, by incubation at 50 0.05M Tris-HCl (pH 7.5) for 30 min (Metzenberg, 196
c: Control of d in which the cell wall preparation was inactivated prior to the digestion incubation.
d: A purified Neurospora cell wall fraction was digeste with the wall-lytic enzyme preparation G-1 according to procedures described in Chapter IV-A3, p. 60 . Supernatant fraction containing ca. 0.4 U of inverta activity was applied.
e. Supernatant fraction of a cell wall preparation afte a cycle of freeze-thawing. About 0.04 U of invertas activity was applied.

All samples were applied on the cathodic end at the top and stained for invertase activity after electrophoresi as indicated in Chapter VI-A2, p. 106.




c
d

If there were indeed no direct binding betw the cell wall and the exoenzymes, the hypothesis rega the origin of the mural enzyme as being trapped in th wall molecular sieve could be viewed more favourably. was interesting to note that Kidby & Davis (1970b), w proposed the model for invertase in yeast cell wall, suggested that the size of the macromolecule may bear direct relevance to its ability to escape from the ce wall. "There is evidence to suggest that the S. frag invertase escapes (from the cell walls) more readily that of S. cerevisiae because the enzyme molecule is These features from the yeast model are quite compat with the present proposal that accounts for diffusion macromolecules through cell walls.

Although the proposed scheme did not receiv overwhelming confirmation from the above experimental it was able to explain the following situation most s factorily.

3. Exoenzyme and Branch Initials:-

In the histochemical studies, branch ini showed very strong localized invertase activity (see 12A,14 & 15). It was indicated in the first part of discussion to be teleologically important because gr

points needed more nutrient supply, hence the conce of enzyme activity. However, the primary hyphal ti also a very actively growing point but similar conc of enzyme activity was not observed. This apparent diction is actually a logical consequence of the pr scheme.

At the incipient branching point, existin wall must first be softened (i.e. undergoes plastic before secondary branches could be initiated (Rober It may involve a protein disulfide reductase in yea (Nickerson, 1963) or a laminarinase in Neurospora (& Mahadkar, 1970), which could hydrolyse the cell w components of the organisms. There must be a time during which the cell wall is being softened, under morphological changes until the characteristics of tip are acquired. During this interval, the tempo activity in this region is conceivably accelerated. probable increase in rates of synthesis and secreti may cause a local congestion of exoenzymes as long wall at the incipient branching point has not acqui characteristic porosity of a normal growing tip. S accumulation is proposed to account for the intense activity observed at the branching points of Neuros hyphae. This type of localized activity was never in branches which have matured into a longer filame

Presumably, these slightly more mature branches have taken up functions of normal growing tips, including of permitting macromolecular diffusion. Similar trar of cell wall structure is not expected to arise at tl primary hyphal apex. Therefore, no accumulation of e activity occurred. Hence, the presence of intense ir activity at incipient branches and its absence in the growth tip seemed to be adequately explained in terms the hypothesis.

An Assessment of the Hypothesis

Up to now, the hypothesis proposed to accor macromolecular diffusion through cell walls can neitl confirmed nor refuted. However, previous data and pr findings appeared quite compatible with the interpret based on it. The obvious merits of this mechanism ar its being the first proposal to explain satisfactori the diffusion of macromolecules through cell walls, a its accommodation of another model for mural locatior enzymes derived from completely different lines of e: mental evidences. Unfortunately, several other obse portend difficulties in accepting this hypothesis pe They are as follows:-

a. The proposal was based exclusively on
characteristic apical growth of filamentous hyphal
chiefly found in fungi. Many unicellular organisms,
as yeasts and bacteria, do not grow by apical exten
although they have well developed cell walls and ma
secrete exoenzymes. A possible modification to acc
the various growth forms is that diffusion of macro
may occur through any region where cell walls are b
i.e. regions of maximum elasticity and porosity.

b. The division of the apical region in f
fungi into zones α, β, γ and δ proposed by Burnett
was based mainly on experiments with sporangiophore
Phycomycetes. These were hyphae specialized to bea
and were more xerophytic (resistant to drying) than
vegetative ones. Therefore, more information on the
and physiology of apical walls in normal vegetative
is necessary to justify the use of this model.

c. This hypothesis implies that no macrom
whose molecular weights exceeded the porosity size
of cell walls could diffuse through them. However,
in 1967 showed that Neurospora acid proteases (mol
22,000) did penetrate cell walls. The enzyme activ

eluted from a column of cell walls plus glass beads later than the solvent front (V_e/V_o = 3.5). The mo exclusion limit for wild type Neurospora cell walls 4,750 as reported by Trevithick & Metzenberg (1966b Either Matile's report was truly at variance with t present proposed mechanism, or the apparent contrad arose from a difference of cell wall porosity in th heterotroph strain of Neurospora that Matile used f experiments.

d. According to the hypothesis, exoenzymes a bound to cell walls in a rather non-specific manner However, some experimental observations indicated t there were specific binding sites in cell walls for In the same report by Matile (1967), a fraction of peptidase (mol wt ca. 85,000) was regularly eluted than the solvent front although the bulk of the enz was excluded from a column of Neurospora cell walls glass beads. This enzyme in vivo was located outsi cell membrane (fully accessible to substrates). Ne theless, it was not secreted into the medium so tha penetration of cell walls was expected (Matile, 196 Therefore, its partial retention in the column coul from a reversible binding to cell walls. Specific sites are thus implied. The implication becomes ev evident in a gram-positive bacteria Staphylococcus

Part of its penicillinase, an exoenzyme, was bound to cell wall surface but the binding could be instantane displaced by certain inorganic anions or polyanions (& Gross, 1967). This portion of penicillinase appare was bound to the cell walls at specific ionic binding The above cases only stress the complexity of relatio between enzymes and cell walls so that their associa may well be the results of a variety of mechanisms.

e. If macromolecules such as exoenzymes cou penetrate cell walls at the tip, it seemed more effic for them to be discharged from the cytoplasm in the s region too. Apparently, this type of efficiency was practised in the secretion of proteases by Neurospora From the electron micrographs in the report of Matile his co-workers (1965), vesicles, possibly containing were seen leaving the cytoplasm not only at the hypha but also over the lateral surface of the hyphal filam However, Girbardt (1969) and other workers (McClure e 1968; Grove et al., 1970) observed numerous vesicles in the apical region and some even fused with the cel membrane. He suggested that these vesicles also tran exoenzymes to the tip where they were discharged intc periplasmic space. Therefore, the hyphal tip did seem have some role in secretion.

f. So far, no zonation of cell wall struct
at the apical region has been evident. This is conc
from examination of thin section electron micrograph
germinated hyphae in Neurospora (Manocha, 1968), in
rouxii (Bartnicki-Garicia, Nelson & Cota-Robles, 196
and in somatic hyphae of many fungi (McClure et al.,
Girbardt, 1969). However, in most cases, the type o
method and the resolution of the pictures did not pe
unequivocal identification of detailed cell wall str
Girbardt (1969) proposed a three-dimensional model s
for the apical region of a filamentous fungus Polyst
versicolor. No specific division of the cell wall i
zones was mentioned but in the model diagram (Abb. 1
the thickness of the cell wall was definitely decrea
to a minimum at the apex, a region called the "Spitz
containing numerous vesicles. Therefore, the requir
of thinner cell wall for extrusion of secretory mate
at the tip was arrived at in a more intuitive manner

During the final preparation of this manu
two groups of workers have independently discovered
structural difference in the apical cell walls of fu
Grove et al. (1970) showed that in the Oomycete P. i
the lateral hyphal wall was about 60 mμ thick but th
apical wall could be as thin as 10-20 mμ. Studying
sentatives from the fungal classes of Oomycete, Asc

and Basidiomycete, Hunsley & Burnett (1970) also conc
that the microfibrils in the apical wall had smaller
than those in the rest of the hyphal wall. Furthermc
the Ascomycete representative Neurospora, the hyphal
as revealed by shadow-casting technique, had only a f
granular layer. The thick strands of the reticulum l
and the outer amorphous layer of glucan characteristi
lateral walls were absent. These are the first insta
in which ultrastructural differences have been demons
in the apical wall of a variety of fungi. The thinne
dimensions and simpler organization in this region ar
compatible with the hypothesis that they may offer ea
passages for the secreted macromolecules into the mec

Concluding Remarks

The discussion above showed that the exper findings obtained in this investigation contributed small part to the understanding of the cell wall-exo relationship in fungi. These findings, together wit from other workers, seemed to fit into a jigsaw puzz appeared interesting and yet required many more piec work to reveal the whole picture.

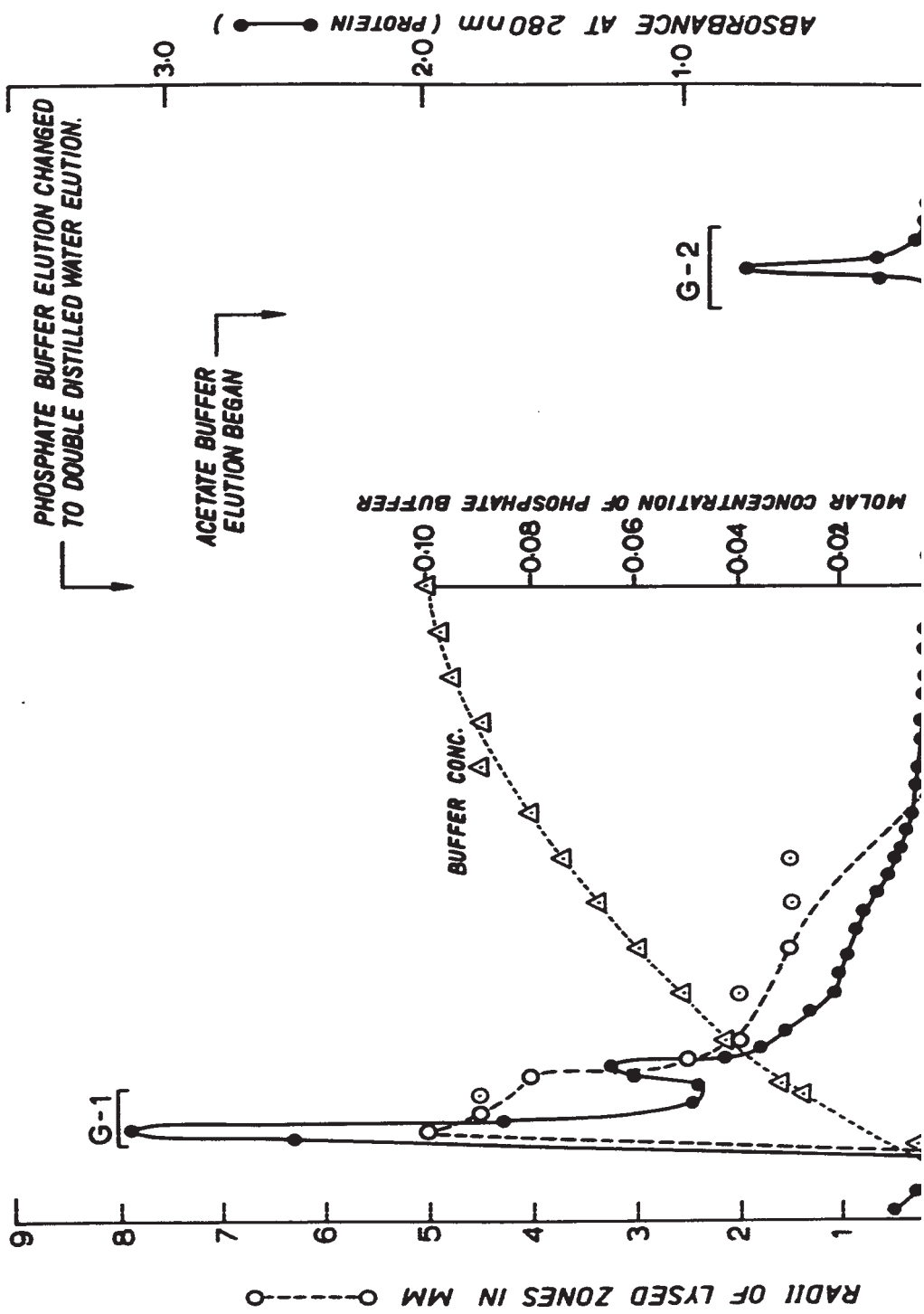
The final answer about the way micro-organ extrude macromolecules through cell walls must come concerted efforts of biochemists and electron-micros Techniques to obtain high resolution pictures of cel at the hyphal tip must be perfected to study any mor differentiation in this region. Histochemical stain exoenzymes have to be developed to meet the electron microscopy requirements. Peroxidase- or ferritin-la antibody stains and osmiophilic chromogens, e.g. dia benzidine, are prospective candidates. The accompli of these techniques, together with the armory of exi biochemical methods, will permit a final solution ab the origin of exoenzymes, their possible existence i apical vesicles, their discharge into the periplasmi and their retention by the cell wall or ultimate exo into the medium.

APPENDIX 1

DEAE Chromatography of Protamine-treated Dialysed Culture Fluid from B. circulans WL-12

The procedure was according to the method of Tanaka & Phaff (1965). DEAE cellulose (cellulose N,N'-diethylaminoethyl ether), a Biorad product (anion exchange cellulose, cellex-D #B-2567), was equilibrated with 1.0M Tris Buffer (pH 8.0), washed 10-20 times with water until the supernatant was clear, and packed into a 2.5 x 7 cm column for elution of 250 ml pre-treated culture fluid, first with a convex gradient of phosphate buffer (pH 7.2), then with acetate buffer (1M, pH 5.0). Fractions of 7 ml each were collected at a flow rate of ca. 4 ml/min.

Radii of lysed zones due to lytic activities of the collected fractions on Neurospora-cell-wall agar plates were measured after ca. 40 hr at 37 C. Fractions 9-14 were pooled and designated as G-1.


RADII OF LYSED ZONES IN MM
ABSORBANCE AT 280nm (PROTEIN)
MOLAR CONCENTRATION OF PHOSPHATE BUFFER
BUFFER CONC.
PHOSPHATE BUFFER ELUTION CHANGED TO DOUBLE DISTILLED WATER ELUTION.
ACETATE BUFFER ELUTION BEGAN
G-1
G-2

APPENDIX 2-a

PRELIMINARY DATA ON TREATMENT OF CELL WALL FRACTIONS WITH BUFFERS OF DIFFERENT PH

Enzyme	pH	Sample	Activity units/ ml cell wall A	Activity units/ ml cell wall B	% Activity released A	% Activity released B	Net % Activity released A	Net % Activity released B	% Activity recovered A	% Activity recovered B
INVERTASE	7.0 (Control)	Residue	4.53	8.15						
		Supernatant	0.53	0.38	10.5	4.5	0.0	0.0	100	100
	3.0	R	4.53	6.96						
		S	0.56	0.24	11.0	3.3	0.5	-1.2	100	84
	4.0	R	4.60	7.32						
		S		0.21		2.8		-1.7		88
	5.0	R	4.04	7.55						
		S	0.58	0.48	12.5	6.0	2.0	1.5	91	94
	6.2	R	4.08	7.55						
		S	0.56	0.33	12.1	4.2	1.6	-0.3	92	92
	8.0	R	4.16	7.92						
		S	0.55	0.36	11.7	4.3	1.2	-0.2	93	97
	9.0	R	4.08	7.50						
		S	0.64	0.57	13.6	7.1	3.1	2.6	93	95
	10.0	R	4.36	7.55						
		S	0.73	0.68	14.3	8.3	3.8	3.8	100	96
E	7.0 (Control)	R	0.088	0.260						
		S	0.011	0.010	11.1	3.7	0.0	0.0	100	100
	3.0	R	0.084	0.292						
		S	0.005	0.008	5.6	2.7	-5.5	-1.0	90	111
	4.0	R	0.086	0.278						
		S	0.010	0.012	10.4	4.1	-0.7	0.4	97	107

APPENDIX 2-b

PRELIMINARY DATA FOR THE RELEASE OF INVERTASE BY 1% TRITON X-100

Duration of Treatment (in min)	Activity units/ml cell wall		% Activity in Supernatant	% Activity recovered
	Residue	Supernatant		
0	6.00		(4.6)*	100
5	5.54	0.53	8.7	101
30	5.34	0.53	9.0	97.8
120	5.50	0.46	7.7	99.3

*Average percentage of three similar preparations: 4.56, 4.70, 4.50.

APPENDIX 2-c

PRELIMINARY DATA FOR THE RELEASE OF INVERTASE BY CHEMICAL REAGENTS

Treatment	Activity units/ml cell wall		% Activity in Supernatant	Net % Activity in Supernatant	% Activity recovered
	Residue	Supernatant			
Water (Control)	8.80	0.73	7.7	0.0	100
KCl (1 M)	8.02	1.28	13.8	6.1	97.5
β-Mercapto-ethanol (19 mM)	7.01	1.14	14.0	6.3	85.5
EDTA (0.5 mM)	7.85	1.23	13.5	5.8	95.2

APPENDIX 2-d

PRIMARY DATA ON THE RELEASE OF INVERTASE (INV) & TREHALASE (TRE) BY SNAIL GUT JUICE

Treatment	# hr	Sample	Activity units/ ml cell wall		% Activity released		Net % Activity released		% Activity recovered	
			INV	TRE	INV	TRE	INV	TRE	INV	TRE
CONTROL (no snail juice)	0	Residue	9.00	0.0635	6.4	6.5	0.0	0.0	100	100
		Supernatant	0.62	0.0044						
	2	R	7.80	0.0530						
		S	0.96	0.0100	10.9	15.9	4.4	9.4	91.1	92.8
	4	R	7.80	0.0530						
		S	1.19	0.0124	13.2	18.9	6.7	12.4	93.4	96.3
	7½	R	7.62	0.0510						
		S	1.26	0.0155	14.2	23.3	7.7	16.8	92.3	97.9
	11½	R	7.57	0.0500						
		S	1.42	0.0185	15.8	27.0	9.3	20.5	93.4	101
	24	R	6.85	0.0475						
		S	1.68	0.0225	19.7	32.1	13.2	25.6	88.7	103
*EXPERIMENTAL	½	R	3.36	0.0354						
		S	6.26	0.0266	65.0	42.9	58.5	36.4	100	91.3
	1	R	1.72	0.0202						
		S	7.94	0.0355	82.2	63.4	75.7	56.9	100	82.5
	2	R	1.12	0.0120						
		S	8.25	0.0380	88.0	76.0	81.5	69.5	97.4	73.6
	4	R	0.61	0.0050						
		S	8.60	0.0390	93.3	88.6	86.8	82.1	95.8	64.8
	7½	R	0.41	0.0025						
		S	8.70	0.0370	95.5	93.7	89.0	87.2	94.7	58.2
	11½	R	0.36	0.0023						

APPENDIX 2-e

PRIMARY DATA ON THE RELEASE OF INVERTASE(INV) & TREHALASE (TRE) BY CELLULASE

Treatment	# hr	Sample	Activity units/ ml cell wall INV	TRE	% Activity released INV	TRE	% Activity recovered INV	TRE
CONTROL (no cellulase)	0	Residue	6.15	0.0570				
		Supernatant	0.04	0.0000	0.6	0.0	100	100
	2	R	5.81	0.0532				
		S	0.20	0.0021	3.3	3.8	97.1	97.0
	4	R	5.68	0.0532				
		S	0.28	0.0033	4.7	5.8	96.3	99.1
	8	R	5.35	0.0502				
		S	0.39	0.0050	6.8	9.0	92.7	96.8
	12	R	5.14	0.0467				
		S	0.41	0.0060	7.4	11.4	89.7	92.4
	24	R	4.60	0.0387				
		S	0.64	0.0093	12.2	19.4	84.7	84.2
EXPERIMENTAL	2	R	5.67	0.0535				
		S	0.25	0.0015	4.2	2.7	95.6	96.5
	4	R	5.42	0.0507				
		S	0.43	0.0051	7.3	9.1	94.5	97.9
	8	R	5.30	0.0500				
		S	0.59	0.0085	10.0	14.5	95.1	102
	12	R	4.92	0.0482				
		S	0.70	0.0082	12.4	14.5	90.8	98.9
		R	5.01	0.0463				

APPENDIX 2-f

PRIMARY DATA ON THE RELEASE OF INVERTASE (INV) & TREHALASE (TRE) BY TRYPSIN

Treatment	# hr	Sample	Activity units/ ml cell wall		% Activity released		% Activity recovered	
			INV	TRE	INV	TRE	INV	TRE
CONTROL	0	Residue	6.58	0.0670	0.0	0.0	100	100
		Supernatant	0.00	0.0000				
	2	R	6.05	0.0617				
		S	0.39	0.0015	6.0	2.4	97.9	94.3
	4	R	5.91	0.0587				
		S	0.48	0.0038	7.5	6.1	97.1	93.3
	8½	R	5.65	0.0567				
		S	0.63	0.0061	10.0	9.7	95.4	93.7
	12	R	5.17	0.0510				
		S	0.66	0.0068	11.3	11.8	88.6	86.3
	24	R	4.77	0.0452				
		S	0.90	0.0095	15.9	17.4	86.2	81.6
RIMENTAL	2	R	5.55	0.0517				
		S	0.53	0.0010	8.7	1.9	92.4	78.6
	4	R	5.52	0.0470				
		S	0.65	0.0014	10.5	2.9	93.8	72.2
	8½	R	5.75	0.0462				
		S	0.83	0.0010	12.6	2.1	100	70.4
		R	4.75	0.0387				

APPENDIX 2-g

PRIMARY DATA ON THE RELEASE OF INVERTASE (INV) & TREHALASE (TRE) BY CHITINASE

Treatment	# hr	Sample	Activity units/ml cell wall		% Activity released		% Activity recovered	
			INV	TRE	INV	TRE	INV	TRE
CONTROL (no chitinase)	0	Residue	7.41	0.0647				
		Supernatant	0.03	0.0000	0.4	0.0	100	100
	2	R	7.15	0.0580				
		S	0.29	0.0063	3.9	9.8	100	99.4
	5.6	R	7.10	0.0555				
		S	0.44	0.0091	5.8	14.1	101	99.8
	13	R	6.53	0.0472				
		S	0.71	0.0134	9.8	22.1	97.3	93.7
	29	R	5.83	0.0417				
		S	0.91	0.0176	13.5	29.7	90.6	91.6
EXPERIMENTAL	1	R	5.93	0.0532				
		S	1.06	0.0081	15.2	13.2	94.0	94.7
	3	R	4.72	0.0422				
		S	2.17	0.0169	31.5	28.6	92.6	91.3
	5.6	R	3.93	0.0370				
		S	2.91	0.0222	42.5	37.6	91.9	91.5
	8	R	3.11	0.0343				
		S	3.62	0.0272	53.8	44.2	90.5	95.0
	13	R	2.46	0.0300				
		S	4.13	0.0315	62.7	51.2	88.6	95.0

APPENDIX 2-h

PRIMARY DATA ON THE RELEASE OF INVERTASE (INV) & TREHALASE (TRE) BY β-1,3-GLUCANASE

Treatment	# hr	Sample	Activity units/ ml cell wall INV	Activity units/ ml cell wall TRE	% Activity released INV	% Activity released TRE	% Activity recovered INV	% Activity recovered TRE
CONTROL (no β-1,3-glucanase)	0	Residue	5.40	0.0530				
		Supernatant	0.04	0.0003	0.7	0.6	100	100
	2	R	5.03	0.0450				
		S	0.25	0.0050	4.7	10.0	97.0	93.8
	4	R	5.45	0.0442				
		S	0.31	0.0070	5.4	13.7	105	96.1
	8	R	4.71	0.0375				
		S	0.37	0.0087	7.3	18.8	93.4	86.7
	12	R	4.95	0.0375				
		S	0.53	0.0109	9.7	22.5	100	90.8
	24	R	4.82	0.0342				
		S	0.57	0.0128	10.6	27.2	99.1	88.2
EXPERIMENTAL	½	R	3.84	0.0400				
		S	1.20	0.0091	23.8	18.5	92.6	92.1
	1	R	3.26	0.0332				
		S	2.02	0.0165	38.2	33.2	97.0	93.2
	2	R	2.60	0.0235				
		S	2.77	0.0222	51.6	48.6	98.7	85.7
	4	R	1.90	0.0166				
		S	3.34	0.0292	63.7	63.7	96.3	85.9
		R	0.84	0.0071				

APPENDIX 2-i

Release of Radioactivity from ^{14}C-Labeled Cell W by Hydrolytic Enzymes

^{14}C-Labeled cell wall fractions were incubated the various hydrolytic enzymes for 24 hr. Sample A and Sample B (200 ul) were withdrawn. Control sampl similarly incubated with PBS substituting for hydrol enzymes. The difference of radioactivity in the cel residue between the control at 0hr and the enzyme-di cell wall fractions at 24 hr was taken to be the amc radioactivity released.

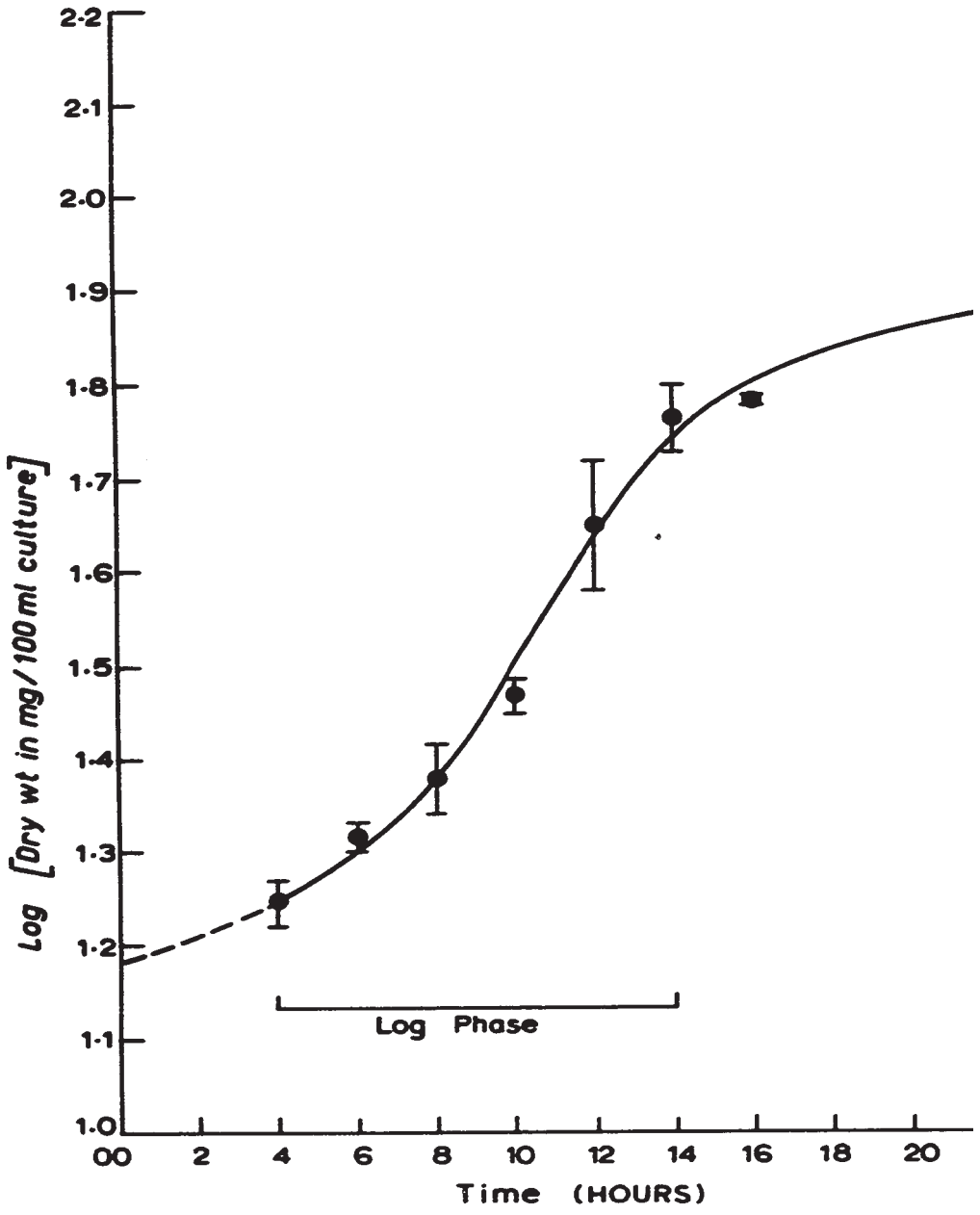
Hydrolytic Enzyme	Residual Radioactivity (dpm)		Average release
	A	B	
Control (0 hr)	29 408	57 857	0.0
Trypsin	31 162	57 540	(0)
Cellulase	27 098	51 623	9.4
Chitinase	24 705	47 951	16.6
Snail Gut Juice	7 907	15 156	73.4
β-1,3-Glucanase	5 437	9 723	82.3
Control (24 hr)	28 445	55 366	3.8

APPENDIX 3

Logarithmic Growth of Neurospora crassa

In each experiment, a 1-liter Erlenmeyer f
containing 500 ml of Fries' Minimal Medium supplemen
with 2.7% galactose at pH 5.2 was inoculated with a
suspension (5.4 ml, absorbance at 600 nm = 20) and i
in a gyratory shaker bath at 30 C and 200 rpm. Dupl
aliquots (5-10 ml) were withdrawn aseptically at tim
intervals, washed, filtered and dried in an oven at
for over 48 hr. The average dry weights were calcul
100 ml of culture.

Each point in the graph represents the mea
logarithmic values from two experiments.


2·2
2·1
2·0
1·9
1·8
1·7
1·6
1·5
1·4
1·3
1·2
1·1
1·0
Log [Dry wt in mg/100 ml culture]
Log Phase
00
2
4
6
8
10
12
14
16
18
20
Time (HOURS)

APPENDIX 4

a. Assay of Ribonuclease

Principle : Measure acid-soluble digestion product commercial RNA at 260 nm.

Reagents :
0.10 ml enzyme solution
0.25 ml sodium phosphate buffer (pH
0.40 ml Double Distilled Water (DDW)
0.25 ml RNA (3 mg/ml aq) prepared ju

Procedure : Incubate the mixed reagents at 37 C fo

Stop the reaction with 0.25 ml of uran acetate (0.75% in 25% $HClO_4$).

Centrifuge. Supernatant (0.2 ml) + 5 m

Read absorbance at 260 nm.

b. Assay of Acid Protease

Principle : Measure acid-soluble tyrosine in diges products from protein.

Reagent a : 1-nitroso-2-naphthol (0.1% in 95% etha

Reagent b : 2.6 M nitric acid containing 0.05% NaN (made just before use)

Procedure : Incubate 0.9 ml horseglobin (0.5% in 0 acetate buffer at pH 4.2) with 0.1 ml solution at 37 C for 30 min.

Stop the reaction with 1 ml perchloric

After 10 min at room temperature, cent

Heat (0.5 ml supernatant + 0.25 ml Reag 0.25 ml Reagent b) at 55C for 30 min.

Cool and add 2.5 ml 1,2-dichloroethane thoroughly and centrifuge to break the

Measure absorbance of upper phase at 4

e. Assay for glucose with Glucose Oxidase

Principle : glucose + O_2 + H_2O $\xrightarrow{\text{Glucose Oxidase}}$ H_2O_2 + glucor

H_2O_2 + reduced chromogen $\xrightarrow{\text{peroxidase}}$ oxidized chrc

Reagents :
- 8.30 ml o-dianisidine (0.03% at pH 3)
- 1.25 ml Tris buffer (1 M, pH 8.1)
- 0.10 ml peroxidase (1 mg/ml aq)
- 0.50 ml glucose oxidase (1 mg/ml, ca.

(Mix before use and protect from light)

Procedure : Incubate 1 ml of glucose sample with 2.5 the mixed reagents at 37 C for 30 min.

Stop the reaction with 2 drops of 6N HC

Read absorbance at 420 nm.

f. Assay of Trehalase

Principle : Measure the production of glucose from

Reagents :
- 35 ml potassium phosphate buffer (0.1
- 7 ml trehalose (1 M)
- 24.5 ml DDW

Procedure : Incubate 0.5 ml of enzyme solution with of the mixed reagents at 37 C for 60 mi

Stop the reaction by heating in boiling bath for 2 min.

Assay for glucose with the glucose oxid method (e).

g. Assay of Invertase

(i) Biochemical assay:-

Principle : Measure production of glucose from su

Reagents : 0.50 ml acetate buffer (1 M, pH 5.0
0.25 ml sucrose (2 M)
8.75 ml DDW

Procedure : Incubate 50 µl of enzyme solution wit
0.95 ml of the mixed reagents at 37 C
for 15 min.

Stop the reaction by heating in boili
water bath for 2 min.

Measure glucose produced with the glu
oxidase method (e).

(ii) Histochemical stain with DAB:-

Principle : Similar to (i) except the oxidized c
is an insoluble dye deposit.

Reagents : 5.5 ml DAB solution (5 mg DAB in 1
phosphate buffer at pH 7, C
prepared just before use
1.0 ml sucrose (2 M)
1.0 ml peroxidase (1 mg/ml aq)
0.1 ml glucose oxidase (1 mg/ml ac

Procedure : Incubate the cut tissue sections wit
mixed reagents at 37 C until suitabl
intensity has developed.

Fix in formalin-saline (0.9% NaCl in
formalin) for 10 min.

Rinse in DDW and wash in 15% alcoho

Mount in glycerine or immersion oil.

(iii) Staining after disc-gel electrophoresis:-

Principle : Similar to (ii) except the dye dep
is a reduced tetrazolium salt.

Reagent : 10 ml sucrose (0.1 M)
10 ml sodium acetate buffer (1.0
6 ml phenazine methosulfate (1 :
10 ml Nitro-Blue Tetrazolium (1 :

Procedure : Immerse each gel, initially polyme
with 25 µl of glucose oxidase (0.1
per ml of gel solution, into the f
prepared reagent in a test tube.

Let stand in the dark for 2-4 hr a

Incubate at 37 C for ca. 20 min in

Destain with 7% acetic acid.

h. Assay of β-1,3-Glucanase

Principle : Measure production of reducing group
soluble laminarin at pH 5.8.

Reagents : 0.25 ml laminarin solution (2.5% aq
0.25 ml succinate buffer (0.2 M, pH

Procedure : Incubate 0.50 ml of enzyme solution w
the mixed reagents at 37 C for 30 min

Stop the reaction by heating in boili
bath for 5 min.

Assay for reducing group with 3,5-din
salicylic acid as in Appendix 3-c.

i. Assay of β-1,6-Glucanase

Principle : Measure production of reducing group
soluble pustulan at pH 6.5.

Reagents : 0.25 ml pustulan (0.5% aq)
0.25 ml phosphate buffer (0.1M, pH

Procedure : Same as for β-1,3-glucanase in (h) al

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