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Patricia Lai-yung Chang

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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

by

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Department of Biochemistry

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Canada.
March 1971

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TO

MY PARENTS

&

GODFREY
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GLOSSARY

DAE : 3,3'-diaminobenzidine
EDTA : ethylenediaminetetraacetate
NAD : nicotinamide adenine dinucleotide
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, 5, 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 microorganisms. 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 \textit{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 \textit{Neurospora} (Matile, Jost & Moor, 1965) and a vesicle-bound fraction of penicillinase in \textit{Bacillus licheniformis} (Sargent & Lampen, 1970). An apparent exception was \textit{Bacillus subtilis} in which none of its three exoenzymes was found in any intracellular pool (May & Elliott, 1968). 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 \textit{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 \textit{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 \textit{et al.} (1965) suggested a radically different process for protease secretion in \textit{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 & Monteneoucourt, 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 (Netzenberg, 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 facilitate 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 CO₂ 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 El120, 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 intus-susception 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, 1965a). 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 macro-molecular 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 metabolites of pigments were the moribund features associated with senescence (Zalokar, 1965).

Thus, biochemical and physiological changes during the development of an organism were generally interrelated 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 dinitrosalicylate 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.
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 exoenzymes.

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 NH₄NO₃. 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, see 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.
<table>
<thead>
<tr>
<th>Mycelial wet wt (g)</th>
<th>Total Activity*</th>
<th>Cell Wall Activity* (untreated)</th>
<th>Cell Wall Activity* (G-1 treated)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>a. Ribonuclease</td>
<td>1.72</td>
<td>1.91</td>
<td>6.56</td>
</tr>
<tr>
<td>b. Acid protease</td>
<td>2.24</td>
<td>2.43</td>
<td>15.3</td>
</tr>
<tr>
<td>c. Amylase</td>
<td>0.80</td>
<td>0.79</td>
<td>8.22</td>
</tr>
<tr>
<td>d. Aryl-β-glucosidase</td>
<td>2.77</td>
<td>2.28</td>
<td>2.17</td>
</tr>
<tr>
<td>e. Trehalase</td>
<td>1.67</td>
<td>3.02</td>
<td>0.80</td>
</tr>
<tr>
<td>f. Invertase</td>
<td>1.67</td>
<td>623</td>
<td>184</td>
</tr>
</tbody>
</table>

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 Fries 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 pKₐ 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).

<table>
<thead>
<tr>
<th>pH</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0</td>
<td>glycine - hydrochloric acid</td>
</tr>
<tr>
<td>4.0</td>
<td>sodium acetate - acetic acid</td>
</tr>
<tr>
<td>5.0</td>
<td>do</td>
</tr>
<tr>
<td>6.2</td>
<td>sodium monohydrogen phosphate -</td>
</tr>
<tr>
<td></td>
<td>sodium dihydrogen phosphate</td>
</tr>
<tr>
<td>7.0</td>
<td>do</td>
</tr>
<tr>
<td>8.0</td>
<td>do</td>
</tr>
<tr>
<td>9.0</td>
<td>glycine - sodium hydroxide</td>
</tr>
<tr>
<td>10.0</td>
<td>do</td>
</tr>
</tbody>
</table>
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:—Succ 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 CaCl₂.

(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:

<table>
<thead>
<tr>
<th>Enzyme Activity Reported</th>
<th>$G_1$</th>
<th>$G_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta$-1,6-glucanase</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>$\beta$-1,3-glucanase</td>
<td>+</td>
<td>++++</td>
</tr>
</tbody>
</table>

Tanaka & Phaff (1965)

**Enzyme Activity Found**

1. **$\beta$-1,3-Glucanase**
   - paper chromatography: +++
   - enzyme assay: 0.44 U/mg protein, 4.80 U/mg protein

2. **$\beta$-1,6-Glucanase**
   - paper chromatography: +
   - enzyme assay: 0.042 U/mg protein

3. **Chitinase**
   - 0.23 $\mu$M/mg protein
   - (17 hr, 37 C)

*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

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

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 (EBL, 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)

CTR

CTR

CTR

CTR
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

TIME (HOURS)
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.
Release of Invertase and Trehalase from Cell Walls by Chitinase

Conditions of the experiment and notations are described in the legend of Figure 2.
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
Cln
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
FIGURE 8

Release of Trehalase from Cell Walls by Various Hydrolytic Enzymes

(SGJ) = Snail Gut Juice
(G) = β-1,3-Glucanase
(Ch) = Chitinase
(Ox) = Cellulase
(T) = Trypsin
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.
of the $^{14}$C-radioactivity in cell walls was solubilize
by cellulase. Therefore, the association of exoenzymes
in Neurospora cell walls probably does not depend on $\beta$-
linkages as found in cellulose, although such polymers
seem to be present, as shown by the considerable amount
of radioactivity released by cellulase. This agrees
with Galsworthy's data (1968) on the release of glucose from
Neurospora cell walls by cellulase. However, the cell
preparation used in this experiment appeared contami-
nated with sucrose- and trehalose-cleaving activities. The
contribution, if any, towards the release of radioactive
or exoenzymes from the cell wall polymers is not
known.

Fig. 4: Trypsin released only 19% of invertase into the supernatant, compared to almost 15% release control. Its effect on trehalase could not be assessed in this experiment since the enzyme was severely inhibited by trypsin. However, preliminary investigations showed a similar pattern of release as obtained for invertase though none of the radioactivity in the $^{14}$C-labeled invertase was released after 24 hours of incubation with trypsin.

Fig. 5: Chitinase was rather specific in lysing chitin with only a very slight hydrolytic activity towards laminarin after 24 hours of incubation. It
released 79% of invertase and 63% of trehalase to appear in the
supernatant.
supernatant fraction after 29 hours of digestion. For half of these enzymes to be released were 4.2 and 24 hours respectively. However, only ca. 16.6% of radioactivity was released from $^{14}$C-labeled cell walls at 24 hours of incubation.

Fig. 6: $\beta$-1,3-Glucanase, specific against 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 appear in the supernatant fraction; half of them were released 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 the efficiency of the various hydrolytic enzymes to release iron from cell walls. They can be listed in the order of potency as: cellulase, trypsin, chitinase, snail gut and $\beta$-1,3-glucanase. The same order also applied to trehalase release (Fig. 8). Furthermore, it has been revealed that the amounts of exoenzymes released were 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 been to the study of fungal ultrastructure, primarily because the rather low resolution (max. 20,000 X in the Camb Mark II model), compared to the conventional transmission electron microscopy which can generally offer 10- to fold higher magnification. The chief advantage of the technique is in its simple requirements. The sample only has to be dry and is ready for observation after of conducting material has been applied, hence circumventing the many chances of introducing artificial modifications intrinsic to the techniques of transmission electron microscopy, perhaps with the exception of the method of etching (Haggis, 1966).

Conventional types of transmission electron microscopy, e.g. sectioning of tissue with subsequent 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 been applied to the study of *Neurospora crassa* cell walls culminating in the present state of knowledge on...
the structure and composition of Neurospora cell walls. The purposes of this experiment were not to duplicate original and more meticulously pursued findings but to (i) an independent approach to monitor any gross morph changes in cell walls resulting from enzymatic hydrolysis and (ii) a means of correlating the biochemical charge with the ultrastructural composition of Neurospora ce:
as known from the literature.

Cell wall fraction digested by cellulase lost ca. 10% of $^{14}$C-radioactivity. However, the cell wall morphology was not visibly altered under the same conditions (Fig. 9·5 cf. control in Fig. 9·1). In contrast, tryptic digested cell walls appeared more granular although most $^{14}$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 the cell wall matrix in the interstices of the fibrillar structure. This probably accounted for the rougher texture of the cell wall surface observed here.

Potgieter & Alexander (1965) showed that chitin alone released only ca. 5% of Neurospora cell wall in form of N-acetylglucosamine. However, if the cell wall had been previously digested by $\beta$-1,3-glucanase, twice as much N-acetylglucosamine was released by chitinase.
Therefore, they suggested that part of the chitin was accessible to enzymatic hydrolysis only after a mask layer of glucan had been removed, such as by β-1,3-glucanase. The residues in Fig. 9·4 may represent the cell wall the easily accessible chitin was removed. If so, the substance seen would be made up of a glucan mask the inaccessible underlying chitin microfibrils.

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

Snail gut juice digestion removed all the fibrillar elements from the cell walls (Fig. 9·7). Streiblová (1958) observed a thin cell wall layer in closely adposed to the plasma membrane of the cell resistant to snail gut juice digestion. Galsworthy and other workers also observed fragments of Neurospora cell walls remaining after snail gut juice digestion. Hence, the flaky residues in Fig. 9·2 possibly corr
to these structures seen in yeast and Neurospora. The residues were neither digested by phospholipase A (kindly donated by Dr. W. Magee, University of Western Ontario, London), nor dissolved by acetone (personal observation). Therefore, it was unlikely that they represented residual contaminating cytoplasmic membranes (although such contamination was improbable after the Triton wash preparation of the cell wall fraction). Because snail gut juice contained all the enzymes necessary for di-gest the three major components of the Neurospora cell wall (glucan matrix, proteinaceous matrix and chitinous fibrils), these residual smooth flakes are tentative designated as a fourth structural component.

The effects of the various specific enzymes on the ultrastructure of Neurospora cell walls have been documented. Since the scanning electron micrographs of the cell walls appeared compatible with the descriptive literature, it is possible to interpret the ultrastructural changes involved in the present biochemical investigations with greater confidence. Thus, the release of wall-bound exoenzymes was coincident with destruction part of the cell wall structure. However, the amount of the released enzyme neither corresponded to the extent
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
ewall 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 NEUROSPORA 
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 from to active metabolism.

After this logarithmic phase, the station sets in when the growth rate drops abruptly, reflect less abundance of substrates and increased populati density. The hyphae can be seen to branch freely a join together to form an amorphous three-dimension ramifying mass, which is collectively called a myce According to Zalokar (1959a), the mycelium growing contact with air differed markedly in its biochemic
cytological characteristics from that which was compl submerged in a liquid medium. The differentiation between these two populations of mycelia was probably in response to a difference of oxygen tension. In the submerged moribund features such as intense vacuolation of cytoplasm, accumulation of fat globules were prevalent.

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

In this part of the project, six morphological or cytologically distinct stages were chosen from the life cycle of Neurospora to study the distribution of enzymes in the culture medium, in the total cell mass, and in cell walls. The purpose was to correlate the occurrence of the exoenzymes in cell walls with the changing physiological states of the organism. The six stages were (i) conidial

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

A. Materials and Methods

1) Culture and Growth Medium

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

2) Culture conditions to produce Neurospora of different physiological states

(i) Conidia

The method of Zalokar (1959a) was used to produce a large quantity of conidia. A conidial suspension (2 ml, absorbance at 600 nm = 20) was inoculated into Fernbach flask containing 500 ml of standard growth medium solidified with 2% agar. After 7 days, the thick mat of 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
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 stand-
growth medium in a 250-ml Erlenmeyer flask. The ci
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 membrané filter (type HA, 0.45 µm, Millipx
Massachusetts).

(iii) **Mycelium in post-log phase**

Procedure was similar to (ii) above but the
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 sta-
ctionary phase**

The procedure was adapted from the me-
by Zalokar (1959a). A standard culture medium (50 ml
containing one drop of Tween 80 (polyoxyethylene-so-
monooleate) in a 125-ml Erlenmeyer flask was inocu-
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
through a Millipore membrane filter and washed with 50 ml of sterile water. The mycelial pad was dispersed in a flask containing 200 ml of the standard medium and 4.1% (v/v) Tween 80. This was incubated at 30 °C as a submerged culture. After 4 days, a translucent mycelium mat appeared on the surface of the liquid medium in contact with a sterile cover. This mat was designated as the aerial mycelium. When examined under the phase contrast microscope, it showed the dominance of hyphal filaments with fine granular cytoplasmic content. Beneath this mat in the Roux bottle, there were dispersed wisps of mycelium entirely submerged in the medium. These were referred to as the submerged mycelium.

Under the phase contrast microscope, it showed a predilection for extensively vacuolated filaments and signs of conidiophore formation at some hyphal ends to form conidiophores. Actual conidiophores were 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 an Erlenmeyer flask was inoculated with 0.5 ml of a conidial suspension (absorbance at 600 nm = 20). The culture was grown in a gyratory shaker bath at 30 °C and 200 rpm for 48 hours. Portions of the mycelial culture (15–20 ml) were filtered on Whatman #1 filter paper in a 10-cm diameter Büchner funnel connected to a vacuum pump. The filtrate
was carefully controlled so that the mycelial mat was moist all the time. The final moist looking mat (ca thick), together with the filter paper, was transferred to a petri dish and moistened with 0.2-0.6 ml of phosphate buffer (0.1M, pH 6.0) containing 800 U/ml of penicillin-streptomycin mixture (BBL, Div. of Bioquest, Maryland). The amount of buffer used was such that the mat was moderately moist but not dripping wet. This was critical for the successful induction of conidiophores. The mycelial mat was maintained at 1 mm thick instead of 2-3 mm as recommended so that a proportion of the mycelium was exposed to air and could develop into erect conidiophores. The mycelial mats were harvested after 8 hours at room temperature. Most of the hyphae on the surface of the mat had developed into conidiophores with constrictions where the future conidia were 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 experiment was saved. The harvested conidia or mycelia were dispersed by sonic treatment. An aliquot was set aside as the mycelial homogenate. The rest of the sample was used for cell wall preparation as described in Chapter II-D.
### Table II.

**Invertase Distribution in Neurospora crassa of Different Physiological Stages**

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.
<table>
<thead>
<tr>
<th></th>
<th>Conidia</th>
<th>Mid-Log (100 ml)</th>
<th>Post-Log (50 ml)</th>
<th>Stationary Culture - 4 days (200 ml)</th>
<th>Conidiophores &amp; Mycelium</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A) Culture Medium</strong></td>
<td></td>
<td>Average</td>
<td>Average</td>
<td>Average</td>
<td>Average</td>
</tr>
<tr>
<td>i. Total activity&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27.3</td>
<td>30.3</td>
<td>171&lt;sup&gt;d&lt;/sup&gt;</td>
<td>263&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>28.4</td>
<td>29.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ii. Specific activity&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.9</td>
<td>3.8</td>
<td>2.9&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3.5</td>
</tr>
<tr>
<td>iii. % of (B): A&lt;sub&gt;i&lt;/sub&gt; / B&lt;sub&gt;i&lt;/sub&gt; x 100</td>
<td>13.6</td>
<td>16.9</td>
<td>17.9&lt;sup&gt;d&lt;/sup&gt;</td>
<td>16.1&lt;sup&gt;e&lt;/sup&gt;</td>
<td>17.0</td>
</tr>
<tr>
<td></td>
<td>13.8</td>
<td>13.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>B) Total Cellular Activity (100%)</strong></td>
<td></td>
<td></td>
<td></td>
<td>Aerial Mycelium Av.</td>
<td>Submerged Mycelium Av.</td>
</tr>
<tr>
<td>i. Wet wt (g)</td>
<td>3.46</td>
<td>0.35</td>
<td>0.29</td>
<td>0.69&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.26&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.36</td>
<td>0.30</td>
<td>1.32&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.39&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.25</td>
</tr>
<tr>
<td>ii. Total activity&lt;sup&gt;b&lt;/sup&gt;</td>
<td>144</td>
<td>201</td>
<td>617&lt;sup&gt;d&lt;/sup&gt;</td>
<td>429&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1450</td>
</tr>
<tr>
<td></td>
<td>206</td>
<td>179</td>
<td>963&lt;sup&gt;e&lt;/sup&gt;</td>
<td>671&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>iii. Specific activity&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.5</td>
<td>8.6</td>
<td>10.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>14.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>16.2</td>
</tr>
<tr>
<td></td>
<td>0.63</td>
<td>6.6</td>
<td>8.9</td>
<td>11.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>14.5&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>C) Cell Wall Fraction</strong></td>
<td></td>
<td></td>
<td></td>
<td>16.2 16.2</td>
<td></td>
</tr>
<tr>
<td>i. Total activity&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.7</td>
<td>38.1</td>
<td>43.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>96.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>239</td>
</tr>
<tr>
<td></td>
<td>37.2</td>
<td>38.0</td>
<td>89.6&lt;sup&gt;e&lt;/sup&gt;</td>
<td>137&lt;sup&gt;e&lt;/sup&gt;</td>
<td>289</td>
</tr>
<tr>
<td>ii. Specific activity&lt;sup&gt;c&lt;/sup&gt;</td>
<td>82.0</td>
<td>128</td>
<td>40.8&lt;sup&gt;d&lt;/sup&gt;</td>
<td>22.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>2.93</td>
<td>88.8</td>
<td>40.7</td>
<td>26.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>24.2 75 74</td>
</tr>
</tbody>
</table>
protein concentration (see Chapter II-B) and invertase activity (see Chapter III-A1, p.43) of the culture in the total mycelial homogenate and the purified cell wall fraction were determined.

B. Results and Discussion

Table II: The specific activities and percentages of invertase associated with cell wall fractions varied significantly in the different phases of the Neurospora asexual life cycle.

Conidia in the dormant stage had the lowest activity in the cell wall fraction. In young hyphae of the mid-log phase (10 hr), invertase specific activity in the total mycelial homogenate rose to 6.5 units/mg protein (ca. tenfold higher than conidial homogenate) and that in the cell wall fraction was 85.4 units/mg protein (ca. tenfold higher than the conidia cell wall fraction). The percentage of total cellular activity associated with cell wall fractions also rose from 8% in conidia to 18.5% in young hyphae. The trend of increasing specific activity in total cells, in cell wall fraction and percentage of mural activity continued to the post-log phase mycelium (18 hr), reaching 8.7 units/mg protein, 120 units/mg protein, and 30% cell wall activity, respectively.
and 23.4%, respectively; the last two data were the half of their groups in all the stages investigated. The stationary phase under the specified growth conditions lasted 12 hours (Appendix 3).

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

The lower specific activity in the cell wall fractions of the stationary cultures may be the result of decreased invertase activity and/or increased protease activity. The following comparison of post-log mycelium with the mycelia is an attempt to delineate which of these two factors had been more dominant. Data of the post-log mycelia were multiplied by four in order to normalize the culture from 50 ml to 200 ml, similar to that of the stationary cultures. Data of the stationary culture were pooled with those of the aerial and submerged mycelia.
<table>
<thead>
<tr>
<th></th>
<th>Post-log (P)</th>
<th></th>
<th>Stationary (S)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt. I</td>
<td>S/P</td>
<td>Expt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mycelial wet wt</td>
<td>1.18 g</td>
<td>0.95 g</td>
<td>0.8</td>
<td>1.7:</td>
<td></td>
</tr>
<tr>
<td>Cellular activity</td>
<td>720 U</td>
<td>1046 U</td>
<td>1.4</td>
<td>164:</td>
<td></td>
</tr>
<tr>
<td>Cell Wall activity</td>
<td>156 U</td>
<td>140 U</td>
<td>0.8</td>
<td>22:</td>
<td></td>
</tr>
</tbody>
</table>

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

The effects of differentiation were demonstrated by the two populations of mycelia of the same age, i.e., the aerial mycelium and the submerged mycelium in the day-stationary culture. As in the case of aging, specific 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 interesting to compare the data of these two mycelial populations before in order to surmise what factor might have caused the lower cell wall specific activity.
Expt. I

<table>
<thead>
<tr>
<th></th>
<th>Aerial (A)</th>
<th>Submerged (S)</th>
<th>S/A</th>
<th>(A)</th>
<th>(S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycelial wet wt</td>
<td>0.69g</td>
<td>0.26g</td>
<td>0.38</td>
<td>1.32g</td>
<td>0.7</td>
</tr>
<tr>
<td>Cellular activity</td>
<td>617 U</td>
<td>429 U</td>
<td>0.69</td>
<td>963 U</td>
<td>67</td>
</tr>
<tr>
<td>Cell Wall activity</td>
<td>44 U</td>
<td>96 U</td>
<td>2.20</td>
<td>90 U</td>
<td>13</td>
</tr>
</tbody>
</table>

The ratios of cell wall activity between the submerged and aerial mycelia exceeded both their mycelial wet weight and total cellular activity ratios. Therefore, proportionally higher invertase activity must be in the cell wall fraction of the submerged mycelium than that of the aerial mycelia. However, in spite of the increased mural activity, the specific activity of this cell wall fraction was only half of that of the aerial mycelium. Hence, this could be the result of a lower mural invertase activity but likely a marked increase of non-invertase protein in the submerged cell wall fraction.

Some reservations about the data obtained from the conidiophore induction experiment must be made. The normal life cycle, conidiophores should be chronologically older than the vegetative mycelium. In the experiments, the conidiophores were induced according to the method of Stine & Clark (1967) from two-day-old mycelia whereas the vegetative mycelium harvested in stationary 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 to the reverse order of age difference and the heterogeneous condition of the sample, it was difficult to make an accurate biochemical comparison between conidiophores and the vegetative phase of the Neurospora life cycle represented by the four-day-old culture. The only decision made was that the conidiophores, together with a substantial amount of 56-hour-mycelium, had the highest intracellular specific activity of invertase (16.2 U/mg protein). In contrast, the specific activity of the cell wall fractions of conidiophores and associated mycelium was intermediate between that of the younger log phase mycelia and the stationary phase mycelia. More accurate localizatiion of invertase in conidiophores by a cytochemical technique will be reported in the next chapter.

In conclusion, significant changes of invertase activity were observed during the Neurospora life cycle. Transition from dormancy to active growth was accompanied by increased specific activity of the total cellular invertase as well as the cell wall fractions. In contrast, older mycelia, either from aging or differentiation, showed decreased activity of the total cellular invertase as well as the younger mycelia but their cell wall fractions did not show a concomitant increase. An interpretation of these findings will be presented in the General Discussion, Chapter...
CHAPTER VI. HISTOCHEMICAL LOCALIZATION OF INVERTASE 
DURING ITS ASEXUAL LIFE CYCLE

Results from experiments in the last chapter showed that the Neurospora cell walls from different stages of the sexual life cycle differed significantly in their carbohydrate content and specific activity of invertase.

The cytochemical aspect of the same problem was also investigated. Histochemical stains specific for invertase were used to localize the enzyme in the cell wall (i) confirming the association of invertase with cell wall carbohydrates, and (ii) comparing the cytological distribution of invertase throughout its asexual life cycle with the known biochemical data, and (iii) elucidating finer details of enzymatic distribution that had evaded biochemical approaches. Two methods were used: (a) a histochemical stain with diaminobenzidine (DAB) as the chromogen, and (b) an immunofluorescent staining technique. In the first method, glucose produced by invertase activity was detected using a second method, invertase in mycelial sections reacted with rabbit anti-invertase serum. The antibody-antigen complex so formed reacted further with a fluorescein-conjugated goat-anti-rabbit serum. Invertase occurrence was
visible as green fluorescence under a microscope equipped with a UV light source. This method was used to study the same six different stages in the Neurospora asexual cycle as in the last chapter.

A. Materials and Methods

1) Histochemical Staining with 3,3'-diaminobenzidine

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

\[
\text{Sucrose} \xrightarrow{\text{Invertase}} \text{Fructose} + \text{Glc}
\]

\[
\text{Glucose} + \text{O}_2 \xrightarrow{\text{Glucose Oxidase}} \text{Gluconic acid} + \text{H}_2\text{O}
\]

\[
\text{H}_2\text{O}_2 + \text{DAB} \xrightarrow{\text{Peroxidase}} \text{H}_2\text{O} + \text{oxidize (brown deposit)}
\]
The stained sections were observed under oil immersion 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 the following modifications:

(i) An inoculum (4 l) of 40 hours old mycelium from culture was injected into a fermentor containing 80 liters sterilized growth medium.

(ii) Mycelium was harvested with a press filter, washed with about 10 gallons of water and wrung dry in cheese cloth to give a wet weight of 2.78 Kg. (The yield 100% more than that from 8-1 carboy cultures).

(iii) The final purification step with carboxymethyl cellulose column chromatography, which caused 50% of invertase activity, was now replaced by Sephadex column chromatography. A 3 x 100 cm column of G-200 Sephadex was equilibrated with buffer B (sodium succinate buffer, 0.005 M, pH 5.0, containing 0.001M EDTA) for over 24 hr. The column was charged with ca. 100 mg protein dissolved in 1.5 ml of buffer A (sodium succinate buffer, 0.1M, pH 5.0, containing 0.001M EDTA) at a specific activity of ca. 290 units of invertase activity per mg of protein. Elution with buffer B proceeds
a flow rate of 0.2 ml/min and 3-ml fractions were collected. The whole operation was performed below 4°C.

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

b. Preparation of Anti-invertase Rabbit Serum

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

Conidia or mycelia harvested in the various physiological stages were embedded in Tissue-Tek (American Optical 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 slides which had been soaked in PBS overnight. They were fixed in 90% alcohol and stained with the "sandwich technique" according to the method of Nairn (1969). Rabbit anti-invertase serum in 1:15 dilution was applied to the section. The mycelial invertase reacted with the rabbit immunoglobulins to form an antigen-antibody complex. After the unreacted rabbit serum was rinsed off, goat anti-rabbit serum conjugated with fluorescein (Hyland Lab, Los Angeles, California) was applied in 1:10 dilution. In order to reduce nonspecific staining from the fluorescein conjugate, the goat serum had been previously adsorbed with acetone-dried liver powder, either prepared according to the method of Holborow & Johnson (1967) or purchased from Difco. Excess goat anti-rabbit serum was washed away and sites of invertase occurrence in the sample were revealed as green fluorescence under a fluorescent microscope (Carl Zeiss Standard Universal Microscope, excitation filter II, barrier filter 50/44).
FIGURE 10A

Disc-gel Electrophoresis of Purified Invertase

About 30 μg of protein from a representative fraction of the first peak after G-200 Sephadex Chromatography was stained with Amido-Black and the single prominent band coincided with invertase activity.

FIGURE 10B

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

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

r: anti-invertase rabbit serum.

n: normal serum from the same rabbit before immunization.

The four unlabeled wells on the left contained sera of two other immunized rabbits which have not developed a strong immunologic reaction against this preparative of invertase. The peripheral wells on the right were filled with the normal sera from the corresponding rabbit. Procedures were according to Campbell et al., 1963.
Purification of Invertase by Sephadex G-200 Chromatography

Conditions of the experiment are described in Chapter.

Protein concentration = \(\frac{\text{Absorbance at } 280 \text{ nm}}{\varepsilon_{0.1\%}^{1 \text{ cm}}\varepsilon_{1 \text{ cm}}}\)

\(\varepsilon_{0.1\%}^{1 \text{ cm}} = \text{extinction coefficient of invertase} = 1.86\)

Invertase activity = \(\text{(Absorbance at } 420 \text{ nm} \times \text{C.F.})\)

C.F. = Conversion Factor

= 1,333
Neurospora Hypha (2-μm section) in Post-log Phase

Stained for Invertase with DAB

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

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

Incubation lasted for 90 min. x 4,2
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 substituting for sucrose in the reagent, to test for chromogenic reaction due to endogenous glucose in the tissue. Incubation lasted for 90 min.

x 2,000.
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 activity and marked activity in the cell wall of the germ tube.
(c) Young hypha in early log phase, showing decreased cytoplasmic invertase but very strong activity associated with the cell wall, x 2,1

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 2,1
Neurospora Hyphae (Stained for Invertase)

(a) Aerial hypha with intensely than left with its s
(b) Submerged hypha

Neur
Stained for Invertase

Strong invertase ac
the cell walls.
c: newly detached c
x: unstained portio
The control sections were: (i) immunization against anti-invertase serum was adsorbed with rabbit serum was used conjugate was applied.

B. Results and Discussion

1) Histochemical

Fig. 12A. Sepharose phase were stained: first 10-20 min of light brown patch occurred. By 30 min, a generally darkly stained phase was observed under microscope. Incubation to permit the cell wall than the cytoplasmic material. Suitable for photographs, the slides were changed in the course of incubation, the occurrence of in
walls was confirmed. In addition, budding points (showed strong activity in the cytoplasm and especially 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 reagent that had fully developed the chromogenic deposit (i.e., replacing the sucrose in the incubation medium with showing no significant staining as compared to the experimental section over a period of 30 min of incubation (Fig. Therefore, nonspecific adsorption of the dye deposit the tissue section was not apparent. The control sections supplied with water instead of sucrose also showed significant staining as compared to the experimental section over a period of 30 min of incubation. However, if the incubation was extended to 90 min as in Fig. slight nonspecific staining appeared in the cytoplasmic plasma membrane, but to a much lower intensity than in the experimental sections and such nonspecific staining occurred on the cell walls. Hence, artifacts due to endogenous glucose in the tissue were negligible.

Nitro-blue tetrazolium salts (NBT) was a natural 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 was
inferior to the DAB reagent, both in sensitivity and
specificity. Reaction color began to appear after 3
min of incubation with DAB, as compared with more to
an hour in the case of NBT. With DAB, 90 min was suffi-
for development of an intense dark-brown stain when
the NBT stain required at least 3 hr to develop an
intense but detectable color. The definition and
of localization demonstrated by NBT stain were poor
defined whereas those by DAB were comparable to the
immunofluorescent staining.

An invaluable asset of this staining method
DAB is in the electron opaqueess of the reaction so that the localization of enzymes can be readily
to the electron microscopic level. Furthermore, the
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 histotechnique 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 c
reaction, as indicated by fluorescence, were more e
detected against a dark background than histochemic staining reactions.

2) Immunofluorescent Staining

The specificity of this indirect immunofluorescent staining depended on three primary factors: the puri
the antigen; the specificity of the rabbit anti-serum 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 prepar
can provoke independent immune responses and most a
that meet the laboratory criteria of purity are cont
by small amounts of immunologically unrelated antiga
(Davis et al., 1967). A sensitive method to detect
specificity of anti-serum against an impure antigen gel diffusion precipitin reactions. The double diffusion technique is extremely sensitive and valuable in detecting the number of antigen-antibody systems, which was in turn determined by the number of precipitin lines between the antigen and the antibody well. Demonstration of a single precipitin line is adequate for most immunohistochemical purposes and is sufficient to define the homogeneity of the immune system (Hopsu- & Ekfors, 1969). Immunelectrophoresis is a more powerful technique. The antigen in a small well is first allowed to migrate across an electric field before the double diffusion precipitin reaction occurs with the antiserum, which was placed in a long trough parallel to the axis of the electric field after the electrophoresis. This technique is usually used for antigens of complex composition, e.g. human unfractionated serum (Davis et al., 1967), tissue extract (Raunio, 1968).

In the present study, the antigen invertase was demonstrated by polyacryl gel electrophoresis (Fig. 10A), but slight contamination by immunologically unrelated substances was possible. Therefore, the double diffusion technique was applied to test for the specificity of this immune system. According to the results from the Ochterlony's test, only a single precipitin line was obtained between the antigen and
antibody (Fig. 10B). The absence of additional pre-
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 dilute sera and adsorption with acetone-dried tissue powder, two methods often capable of reducing such NSS. These tactics were applied to the fluorescein-labeled goat antiserum, which was previously adsorbed with acetone liver powders and used at 1:10 dilution. As shown in Fig. 17D, the goat anti-serum was not adsorbed to tissue sections when the middle layer of rabbit immune globulin was absent, thus showing the absence of NSE in the fluorescein-conjugated goat antiserum.

The above considerations and controls indicate the high degree of specificity obtainable with the present indirect immunofluorescent method in that specific staining arising from impure antigen, including specificity of rabbit antibodies, or random adsorption antiserum did not contribute significantly to the staining pattern in the experimental tissue sections.

Fig. 13. The localization of invertase was noted through germination. The conidium showed enzyme activity on the periphery as an intense fluorescing ring and throughout the cytoplasm as a weaker diffuse fluorescence. During germination, invertase fluorescence was found in the germ tube cytoplasm and markedly increased in the c
cytoplasm. Only the cell wall at the germ tube showed fluorescence; the rest of the conidial cell wall had the discrete peripheral stain (Fig. 13b). In the log phase, the young hyphal cell wall became strongly fluorescent and the hyphal cytoplasm was weakly so (Fig. 13c). In the meantime, the residual conidium seemed to be quite depleted of activity.

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

Fig. 15. The immunofluorescent staining of representative hyphae from two mycelial populations of the same stationary culture of four days are shown. The aerial hypha of Fig. 15a, the cell wall was much fluorescent than that of the post-log phase hyphae. This was also reflected in the lower specific activity.
well as the total activity per unit wet weight of the mycelium in the cell wall fraction. Again, relatively stronger fluorescent activity was observed in the initial at the lower left portion of the hypha. This type of localized activity was not confined to the log phase. In the submerged hypha of the cell vacuoles, a prominent feature in this kind of submerged mycelium, were devoid of activity. This especially remarkable as the total and specific act of the mycelial homogenate were relatively higher than 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 localization 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" were relevant to the conidiophores per se. The newly deconidia in this Figure 16 differed from the conidia in 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 differences 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 conidium aged. The subsequent shrinkage of the coat cause it to fit more snugly outside the conidial cytoplasm. On the other hand, it could be the residual cell wall of the conidiophore and the septa that used to demarcate the conidium from the next in the differentiating conidia.

In conclusion, two methods have been success 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 immunofluorescent method not only confirmed the biochemical localization of invertase in Neurospora cell walls of different physiological states; they also indicated more subtle feat such as activities in budding points, conidiophores, their absence, in vacuoles, of any activity.
CHAPTER VII. GENERAL DISCUSSION

Several features about the relationship between exoenzymes and Neurospora cell walls have been revealed in the foregoing chapters. The different exoenzymes were associated with cell walls to various degrees; covalent bonds had to be disrupted when the bound enzymes were released, and the extent of invertase association varied with the age of the mycelium. Most of the questions raised in the beginning of the investigation can be satisfactorily answered now, but a few still defy clear-cut solutions. The first part of this discussion is devoted to the more positive and direct answers with an interpretation of their biological significance. The second part deals with the equivocal answers and an interpretation of them based on 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 significant portions of aryl-β-glucosidase, invertase and trehalase (15%, 26% & 29%, respectively) were cell-wall-bound (Table 1). These different percentages indicated that enzymes were associated with the cell wall fractions to various extents. Furthermore, the nature of their substrates appeared to correlate with the extent of enzyme association. Exoenzymes that acted on macromolecules, e.g. ribonuclease (on polynucleotides), protease (on polypeptides), and amylase (on polysaccharides) were in small amounts in the cell wall fractions. Enzymes that acted on small molecules, e.g. aryl-β-glucosidase (on substituted monosaccharides), invertase (on disaccharides), and trehalase (on disaccharides), were quite extensively associated. From this, it is postulated that when the substrates are small enough to diffuse into the cell it would be more economical for part of the corresponding exoenzymes to remain cell-wall-bound. The enzyme itself 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 the
enzymes in a mural location. An appreciable 9% of the
was in the cell walls and yet appeared inaccessible
its macromolecular substrate starch. According to the
above postulate, this 9% might be responsible for by
the smaller oligosaccharides, e.g. amylo-dextrins, which
are hydrolytic intermediates of starch and are probably
small enough to penetrate the cell walls.

**Distribution of Invertase in the Asexual Life Cycle**

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

The lowest invertase activity (total and specific)
was in the conidial wall preparation. A number of
enzyme systems in Neurospora (Zalokar, 1959a) also
the lowest activity in conidia, which was in accord with the lower state of metabolic activity in dormant structures. Histochimically, invertase was found primarily at the conidial periphery. This was perhaps the best strategic position to attack substrates during sequent germination. Metzenberg (1963b) studied the accessibility and enzyme inhibition of invertase and came to similar conclusions. In his work, no distinction could be made whether the enzyme was in the cytoplasmic membrane, periplasmic space, or cell wall. However, at 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 metabolic state often entails an elevation of many enzyme activities. In Neurospora, a spectrum of enzymes involved in biosynthetic pathways, e.g. NAD-, NADP-dependent glutamic dehydrogenase (Stine, 1968), in respiratory activities, e.g. succin dehydrogenase (Zalokar, 1959a), cytochrome oxidase (1963), and in various hydrolytic reactions, e.g. trehalase (Hill & Sussman, 1964), aryl-sulfatase (Schram & Metzenberg, 1970), increased markedly during these initial stages of the Neurospora life cycle. Therefore, the increasing specific activities of the total cellular...
invertase from the conidial stage to the mid-log phase and then to the post-log phase (Table II) were compared with the above general pattern of heightened activity. In addition to a marked increase of cellular activity, cyto-
lar invertase activity and specific activity of the enzyme fractions also rose during the logarithmic phase, probably indicating active synthesis as well as secretion of the enzyme (although the possibility that higher specific activity due to lower amount of Folien-positive material cannot be ruled out). The function of invertase is to supply the early metabolized glucose from sucrose, which otherwise cannot be transported into the intracellular milieu (Marzluf & Good 1967). The increased synthesis and secretion of such an enzyme are the logical answer to the need of an actively growing organism.

However, during the later stages of the life cycle when senescent cytological features and slow growth rates were brought about either by aging or di-
tation, the specific activities of the total cellular invertase also increased. A number of inducible enzymes, e.g. β-galactosidase (Zalokar, 1959a), aryl-β-glucosidase (Eberhart, 1961), and de-repressible enzymes, e.g. in-
trehalase (Netzenberg, 1962) in Neurospora has been shown to increase in aging cultures, or in a medium with poor sugar substrates, or on the depletion of exogenous sugar. The increased cytoplasmic activities of these enzymes
been attributed to the accumulation of intracellular glucose (Zalokar, 1959a), or the absence of repressor, such as the production of trehalase (Hanks & Swanson, 1969b). Therefore, the increased cytoplasmic specific activity of Neurospora invertase associated with senescence might be due to a depletion of accessible exogenous supply and a reduced metabolic rate, with subsequent relief from intracellular catabolite repression, possibly by glucose. The mycelial mats that had been starved for eight hours to induce conidiophore formation also showed very high specific activity in the total cellular extract (Table II). This is also explicable on the basis above proposition.

In spite of the increased cytoplasmic specific activity that accompanied senescence in Neurospora cell wall fractions showed a lower specific activity than their younger counterparts. In both cases, i.e., due to aging or differentiation, the decrease was to be the result of a lower mural activity relative to that of the younger mycelia. Furthermore, a difference noted here between senescence caused by aging and caused by differentiation. In aging, the ratio (invertase activity/mycelium wet weight) was quite high in both the mycelia of post-log phase and those of older stationary phase (p.100, Chapter V). In di
the ratio (mural invertase activity/mycelial wet \textsuperscript{v}
was much higher in the submerged mycelium than in
aerial mycelium (Chapter V, p.101). A preferential
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 conidi-
strong invertase activity occurred in both the cyt
and the cell walls of these newly differentiated s
(Fig. 16). Similar accumulation of NAD glycohydro-
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 subtr
source was encountered. Several other exoenzymes
similar functions were also found on the conidial
besides invertase, e.g. aryl-$\beta$-glucosidase, malta
ceIlobiase (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 of proteases, ribonuclease, amino-peptidase and esterase in the yeast vacuole (Matile & Wiemken, 1967). The di was readily rationalized by considering their function. The lysosomal enzymes in the yeast vacuole had the role to degrade vital cytoplasmic components, such as protein, ribonucleic acids, nascent peptides etc. It was on the segregation of these enzymes that the rest of the contents of the vacule could be protected from uncontrolled and premature destruction. A similar precaution would be unnecessary for invertase, whose function is mainly to cleave sucrose into glucose and fructose, which are good sources of metabolic energy. However, recently, invertase was found in yeast vacuoles (Beteta & Gascon, 1971). The enzyme characteristics of these vacuoles have not been very well studied to show that they were identical to those studied by Matile & Wiemken (1967). Beteta & Gascon (1971) also suggested that the vacuoles containing invertase served a similar function as the zymogen granules in mammalian pancreas studied by Palade et al., 1964). If so, the yeast vacuoles would correspond to "secretory vesicles" in Neurospora hyphae, or the lysosomal vacuoles in animal cells (Matile & Wiemken, 1967).
A recurrent phenomenon observed in hyphae of different physiological stages was the concentration of invertase activity at incipient branching points (Fig. 12A, 14 & 15). This was not an artifact arising from intense staining of any dome-shaped structures, such as branch initials, that were not cut in a median section. Primary growth tips had similar geometrical constructions, but such intense localization of activity was never observed, whereas branch initials were frequently observed to have this type of localization. It may be a local response to the greater need of energy supply at sites of cell wall synthesis (Zalokar, 1965). Initiation of new growing fronts requires additional energy for the partial digestion of old cell wall and deposition of new wall material. Aggregation of invertase is able to furnish the necessary energy supply by generating fermentable monosaccharides.

Aside from this probable physiological significance, the cause of the accumulation is not known but will be discussed in the second part of this chapter in conjunction with the hypothesis of cell wall structure.

Remarks

The greatest satisfaction during this project was derived from the revelation of the high degree of efficiency and organization in living organisms. Even
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 accessibility 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 concerned results that are less amenable to an interpretation purpose or design. However, an attempt to account for them, although speculative in nature, became even more 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 molecular to regulate a two-way transit of enzyme and substrate molecules, and as an anchorage for some of the exoenzymes. However, the experimental findings thus far did not provide any direct solution to the question: how did exoenzymes traverse the cell walls that seemed too nonporous for 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 rationalize the phenomenon of exoenzyme association with cell wall. Furthermore, it offers a theoretical reconciliation of the various conflicting aspects of the molecular sieving 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.
\( \alpha \): non-extensible zone

\( \beta \): region of maximal intussusception

\( \gamma \): region of maximal extensibility

\( \delta \): region of rigidification
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 been repeatedly reaffirmed and extended in later experiment: on hyphal tips. The accumulated data led to a propo: 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 reqi 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 by many physiological experiments on fungal tip behavior, apical region had a cell wall whose structure and characteristics are more flexible and less thickened than that in the rest of the hypha (i.e. α, β, γ against δ).

Exoenzymes are now suggested to pass through the cell wall, or rather the incomplete cell wall, in the apical region. According to this hypothesis, the process of diffusion is visualized as follows. The cell wall of this apical dome can be regarded initially as a very loose sieve so that passage of macromolecules is unimpeded by extension growth in the apex, what has been observed. As the elastic and porous apex (zones α, β, γ) gradually pass through this region, a zone where cell wall thickening by microfibril addition occurs. When more microfibrils are added, the open spaces of the sieve become proportionally smaller. In effect, the pores of the molecular sieve are gradually being closed. At the end of the rigidification process, the pores are stabilized and have become too small for the passage of most macromolecules such as exoenzymes.
Interpretation of Data from Literature

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

As mentioned in the historical review in Chapter 6, Neurospora cell walls did exert a fractionation effect on the passage of the smaller invertase units into the culture medium (Trevithick & Metzenberg, 1966). This is also accountable according to the present scheme because the cell walls are still acting as a sieve, a continuously closing one. Smaller molecules would stand a better chance of passing through. In the osm6 mutant, the proportion of light invertase in the medium decreased. Hence, more heavy invertase had been able to pass the wall. This decrease of fractionation effect correlated with changes in the cell wall composition. One of the changes noted was the increased ratio of galactose...
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**

<table>
<thead>
<tr>
<th>Molecular Weights(^a) and Per Cent Activities(^b) of Exoenzymes in Neurospora Cell Wall</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a): approximate mol wt from literature</td>
</tr>
<tr>
<td>(b): data from Chapter III</td>
</tr>
<tr>
<td>(c): % in parentheses = % activity detectable after degradation of cell wall fraction by lytic enzymes G-1</td>
</tr>
<tr>
<td>(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.</td>
</tr>
<tr>
<td>(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.</td>
</tr>
<tr>
<td>Exoenzymes</td>
</tr>
<tr>
<td>--------------------</td>
</tr>
<tr>
<td><strong>Group 1:</strong></td>
</tr>
<tr>
<td>Ribonuclease</td>
</tr>
<tr>
<td>Acid proteases</td>
</tr>
<tr>
<td>Amylase</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Group 2:</strong></td>
</tr>
<tr>
<td>Aryl-β-glucosidase</td>
</tr>
<tr>
<td>Invertase (heavy)</td>
</tr>
<tr>
<td>(light)</td>
</tr>
<tr>
<td>Trehalase</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
Interpretation of Present Findings

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

1. Exoenzymes and Their Molecular Weights:

The amounts of various exoenzymes associated with Neurospora cell walls differed significantly from each other (see Table 1). If cell walls are molecular sieves, the molecular weights, and hence the sizes, of the exoenzymes should determine the ease with which they pass through the sieve. However, if the cell walls responsible for diffusion are a continuously closing sieve, some exoenzymes may be trapped during their transit. During the process of rigidification, the cell walls whose pores are initially large enough to admit the macromolecules from the periphery become too small to let them out on the exterior side. This portion of trapped exoenzyme is postulated to correspond to that found in the cell wall. On the other hand, the portion of exoenzymes that manages to 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 trap
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

The molecular weights of the second group were present in greater amounts, i
d-aril-β-glucosidase, invertase, trehalase. In the case of invertase, both heavy and light forms were found in the cell wall fraction (Fig. 19). The exact percentage activity in the cell wall fraction were not: critical; they varied with the physiological state of the organ.

Inasmuch as the molecular weights of the enzymes were approximate and unconfirmed by independent approaches, it is not determined directly on the enzymes from the cell wall fractions (except invertase), any conclusions drawn therefrom must be tentative. However, the direct correlation between molecular weights and of mural activity of these exoenzymes seemed to be persistent than mere coincidence and was quite compat
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 the exoenzymes are related to the cell walls merely by pH juxtaposition and not by any chemical bonds between them. As will be shown below, no direct verification of this premise could be obtained from the studies on the relation of exoenzymes from cell walls.

The failure to release cell-wall-bound invertase 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 release the cell-wall-bound invertase and trehalase indicated covalent bonds were involved.

β-1,3-Glucanase released more than 90% of invertase and trehalase. Chitinase released ca. 80% of invertase and 60% of trehalase. Trypsin released only a few percent of invertase. Cellulase had no significant effect. The
deductions could be made. (i) At least a few per cent of invertase was released after hydrolysis of either a β-1,4-glycosidic linkage or a β-1,3-glycosidic linkage in glucan. (ii) A substantial amount of invertase was released after each of a β-1,4-glycosidic linkage in chitin or a β-1,3-glycosidic linkage in glucan was hydrolysed. (iii) Trehalase appeared 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. The possibilities exist to account for their release. Firstly, the exoenzymes were only physically confined within the matrix of the cell wall. The various hydrolytic enzymes that degraded the cell wall polymers caused a breakdown of its structural integrity. The loosened "molecular suture" became permeable to the macromolecules once more. Secondly, the exoenzymes were chemically bonded to one or more of the cell wall polymers. The hydrolytic enzymes not only disrupted the cell wall integrity but also "dissected" out fragments of the cell wall polymers that were chemically bonded to the invertase molecule. The loosened enzymatic molecules, together with the attached cell wall fragments, were able to diffuse through the partially degraded cell walls.
At this point, it is impossible to make a decision between these two possibilities. However, in the model proposed by Kidby & Davis (1970b), invertase was also found to be within the yeast cell wall, which was impermeable to invertase because of disulfide and/or phosphodiester linkages. This permeability barrier was broken, free diffusion of invertase became possible. Although no disulfide or phosphodiester bridges have been found in Neurospora cell walls, the permeability barrier can be readily conferred by \( \beta-1,4 \) linkages or even peptide bonds in glucan, chitin, or protein, respectively.

Another observation also favored the first possibility. During routine storage of purified cell walls, a cycle of freeze-thawing released from a few to about 50 per cent of the cell-wall-bound invertase into a supernatant fraction. Hence, mere mechanical force was sufficient to liberate some invertase from its mural association. No covalent bonds could have been involved, at least in a fraction of enzyme that was so released.

Furthermore, if cell wall fragments were still attached to the liberated mural enzymes, as indicated by the second possibility, these attached enzymes would be larger than the pure enzymes and migrate slower in deoxycholate electrophoresis. However, no such difference was detected (Fig. 19).
FIGURE 19

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

a: Purified invertase (heavy) containing about 0.2 U activity.
b: Purified invertase after dissociation into subunits containing about 2.5 U activity, by incubation at 50°C for 30 min (Metzenberg, 1962).
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 digested 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 invertase activity was applied.
e: Supernatant fraction of a cell wall preparation after a cycle of freeze-thawing. About 0.04 U of invertase activity was applied.

All samples were applied on the cathodic end at the top and stained for invertase activity after electrophoresis as indicated in Chapter VI-A2, p. 106.
If there were indeed no direct binding between the cell wall and the exoenzymes, the hypothesis regarding the origin of the mural enzyme as being trapped in the wall molecular sieve could be viewed more favourably. It was interesting to note that Kidby & Davis (1970b) 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 cell wall. "There is evidence to suggest that the *S. frag* invertase escapes (from the cell walls) more readily than the *S. cerevisiae* because the enzyme molecule is large. These features from the yeast model are quite compatible with the present proposal that accounts for diffusion of macromolecules through cell walls.

Although the proposed scheme did not receive overwhelming confirmation from the above experimental work, it was able to explain the following situation most satisfactorily.

3. Exoenzyme and Branch Initials:-

In the histochemical studies, branch initials showed very strong localized invertase activity (see 12A,14 & 15). It was indicated in the first part of discussion to be teleologically important because gro
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 permitting macromolecular diffusion. Similar trar of cell wall structure is not expected to arise at the primary hyphal apex. Therefore, no accumulation of activity occurred. Hence, the presence of intense in 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 accom macromolecular diffusion through cell walls can neith confirmed nor refuted. However, previous data and p findings appeared quite compatible with the interpret based on it. The obvious merits of this mechanism at its being the first proposal to explain satisfactorily the diffusion of macromolecules through cell walls, its accommodation of another model for mural location enzymes derived from completely different lines of emental evidences. Unfortunately, several other obse portend difficulties in accepting this hypothesis pe. They are as follows:--
a. The proposal was based exclusively on the characteristic apical growth of filamentous hyphae, chiefly found in fungi. Many unicellular organisms, as yeasts and bacteria, do not grow by apical extension although they have well developed cell walls and may secrete exoenzymes. A possible modification to accommodate the various growth forms is that diffusion of macromolecules may occur through any region where cell walls are broken, i.e. regions of maximum elasticity and porosity.

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

c. This hypothesis implies that no macromolecules whose molecular weights exceeded the porosity size limit of cell walls could diffuse through them. However, in 1967 showed that Neurospora acid proteases (molecular weight 22,000) did penetrate cell walls. The enzyme activ...
eluted from a column of cell walls plus glass beads later than the solvent front \( (V_e/V_0 = 3.5) \). The molecular 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 the present proposed mechanism, or the apparent contradiction arose from a difference of cell wall porosity in the heterotroph strain of Neurospora that Matile used for experiments.


d. According to the hypothesis, exoenzymes are bound to cell walls in a rather non-specific manner. However, some experimental observations indicated that there were specific binding sites in cell walls for peptidases. In the same report by Matile (1967), a fraction of a peptidase (mol wt ca. 85,000) was regularly eluted faster than the solvent front although the bulk of the enzyme was excluded from a column of Neurospora cell walls plus glass beads. This enzyme in vivo was located outside the cell membrane (fully accessible to substrates). Nevertheless, it was not secreted into the medium so that penetration of cell walls was expected (Matile, 1968). Therefore, its partial retention in the column could be from a reversible binding to cell walls. Specific binding sites are thus implied. The implication becomes even more evident in a gram-positive bacteria Staphylococcus...
Part of its penicillinase, an exoenzyme, was bound to cell wall surface but the binding could be instantaneous displaced by certain inorganic anions or polyanions (Gross, 1967). This portion of penicillinase appeared to be bound to the cell walls at specific ionic binding. The above cases only stress the complexity of relation between enzymes and cell walls so that their association may well be the results of a variety of mechanisms.

e. If macromolecules such as exoenzymes could 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 filament. However, Girardt (1969) and other workers (McClure & 1968; Grove et al., 1970) observed numerous vesicles in the apical region and some even fused with the cell membrane. He suggested that these vesicles also transport exoenzymes to the tip where they were discharged into the periplasmic space. Therefore, the hyphal tip did seem have some role in secretion.
f. So far, no zonation of cell wall structure at the apical region has been evident. This is consistent from examination of thin section electron micrographs of germinated hyphae in Neurospora (Manocha, 1968), in \textit{rouxii} (Bartnicki-Garcia, Nelson & Cota-Robles, 1966) and in somatic hyphae of many fungi (McClure \textit{et al.}, Girbardt, 1969). However, in most cases, the type of method and the resolution of the pictures did not permit unequivocal identification of detailed cell wall structure. Girbardt (1969) proposed a three-dimensional model for the apical region of a filamentous fungus Polystictus versicolor. No specific division of the cell wall into zones was mentioned but in the model diagram (Abb. 1) the thickness of the cell wall was definitely decreased to a minimum at the apex, a region called the "Spitz" containing numerous vesicles. Therefore, the requirement of thinner cell wall for extrusion of secretory material at the tip was arrived at in a more intuitive manner.

During the final preparation of this manuscript, two groups of workers have independently discovered structural differences in the apical cell walls of fungi. Grove \textit{et al.} (1970) showed that in the Oomycete \textit{P. i}. the lateral hyphal wall was about 60 μm thick but the apical wall could be as thin as 10-20 μm. Studying pentatimates from the fungal classes of Oomycete, Asc
and Basidiomycete, Hunsley & Burnett (1970) also concurred that the microfibrils in the apical wall had smaller diameters than those in the rest of the hyphal wall. Further, the Ascomycete representative Neurospora, the hyphal wall as revealed by shadow-casting technique, had only a granular layer. The thick strands of the reticulum and the outer amorphous layer of glucan characteristic of lateral walls were absent. These are the first instances in which ultrastructural differences have been demonstrated in the apical wall of a variety of fungi. The thinner dimensions and simpler organization in this region are compatible with the hypothesis that they may offer egress passages for the secreted macromolecules into the media.
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.
## APPENDIX 2-a

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

<table>
<thead>
<tr>
<th>Enzyme pH</th>
<th>Sample</th>
<th>Activity units/ml cell wall</th>
<th>% Activity released</th>
<th>Net % Activity released</th>
<th>% Activity recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>7.0</td>
<td>Residue</td>
<td>4.53</td>
<td>8.15</td>
<td>10.5</td>
<td>4.5</td>
</tr>
<tr>
<td>3.0</td>
<td>Supernatant</td>
<td>0.53</td>
<td>0.38</td>
<td>11.0</td>
<td>3.3</td>
</tr>
<tr>
<td>4.0</td>
<td>R</td>
<td>4.60</td>
<td>7.32</td>
<td>11.0</td>
<td>3.3</td>
</tr>
<tr>
<td>5.0</td>
<td>S</td>
<td>0.56</td>
<td>0.24</td>
<td>12.5</td>
<td>6.0</td>
</tr>
<tr>
<td>6.2</td>
<td>R</td>
<td>4.08</td>
<td>7.55</td>
<td>12.5</td>
<td>6.0</td>
</tr>
<tr>
<td>8.0</td>
<td>S</td>
<td>0.56</td>
<td>0.33</td>
<td>12.1</td>
<td>4.2</td>
</tr>
<tr>
<td>9.0</td>
<td>R</td>
<td>4.16</td>
<td>7.92</td>
<td>11.7</td>
<td>4.3</td>
</tr>
<tr>
<td>10.0</td>
<td>S</td>
<td>0.73</td>
<td>0.68</td>
<td>14.3</td>
<td>8.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pH</th>
<th>(Control)</th>
<th>Activity units/ml cell wall</th>
<th>% Activity released</th>
<th>Net % Activity released</th>
<th>% Activity recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>0.088</td>
<td>0.260</td>
<td>11.1</td>
<td>3.7</td>
</tr>
<tr>
<td>3.0</td>
<td>S</td>
<td>0.011</td>
<td>0.010</td>
<td>5.6</td>
<td>2.7</td>
</tr>
<tr>
<td>4.0</td>
<td>R</td>
<td>0.084</td>
<td>0.292</td>
<td>10.4</td>
<td>4.1</td>
</tr>
</tbody>
</table>
APPENDIX 2-b

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

<table>
<thead>
<tr>
<th>Duration of Treatment (in min)</th>
<th>Activity units/ml cell wall</th>
<th>% Activity in Supernatant</th>
<th>% Activity recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Residue</td>
<td>Supernatant</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>6.00</td>
<td></td>
<td>(4.6)*</td>
</tr>
<tr>
<td>5</td>
<td>5.54</td>
<td>0.53</td>
<td>8.7</td>
</tr>
<tr>
<td>30</td>
<td>5.34</td>
<td>0.53</td>
<td>9.0</td>
</tr>
<tr>
<td>120</td>
<td>5.50</td>
<td>0.46</td>
<td>7.7</td>
</tr>
</tbody>
</table>

*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**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Activity units/ml cell wall</th>
<th>% Activity in Supernatant</th>
<th>Net % Activity in Supernatant</th>
<th>% Activity recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Residue</td>
<td>Supernatant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water (Control)</td>
<td>8.80</td>
<td>0.73</td>
<td>7.7</td>
<td>0.0</td>
</tr>
<tr>
<td>KCl (1 M)</td>
<td>8.02</td>
<td>1.28</td>
<td>13.8</td>
<td>6.1</td>
</tr>
<tr>
<td>β-Mercaptoethanol (19 mM)</td>
<td>7.01</td>
<td>1.14</td>
<td>14.0</td>
<td>6.3</td>
</tr>
<tr>
<td>EDTA (0.5 mM)</td>
<td>7.85</td>
<td>1.23</td>
<td>13.5</td>
<td>5.8</td>
</tr>
</tbody>
</table>
### APPENDIX 2-d

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th># hr</th>
<th>Sample</th>
<th>Activity units/ ml cell wall</th>
<th>% Activity released</th>
<th>Net % Activity released</th>
<th>% Activity recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>INV</td>
<td>TRE</td>
<td>INV</td>
<td>TRE</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>Residue Supernatant</td>
<td>9.00</td>
<td>0.0635</td>
<td>6.4</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>7.80</td>
<td>0.0530</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>0.96</td>
<td>0.0100</td>
<td>10.9</td>
<td>15.9</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>R</td>
<td>7.80</td>
<td>0.0530</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>1.19</td>
<td>0.0124</td>
<td>13.2</td>
<td>18.9</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>R</td>
<td>7.62</td>
<td>0.0510</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>1.26</td>
<td>0.0155</td>
<td>14.2</td>
<td>23.3</td>
</tr>
<tr>
<td>7½</td>
<td>7½</td>
<td>R</td>
<td>7.57</td>
<td>0.0500</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>1.42</td>
<td>0.0185</td>
<td>15.8</td>
<td>27.0</td>
</tr>
<tr>
<td>11¼</td>
<td>11¼</td>
<td>R</td>
<td>6.85</td>
<td>0.0475</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>1.68</td>
<td>0.0225</td>
<td>19.7</td>
<td>32.1</td>
</tr>
<tr>
<td>24</td>
<td>24</td>
<td>R</td>
<td>3.36</td>
<td>0.0354</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>6.26</td>
<td>0.0266</td>
<td>65.0</td>
<td>42.9</td>
</tr>
<tr>
<td>EXP</td>
<td></td>
<td>R</td>
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<td>0.0202</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>S</td>
<td>7.94</td>
<td>0.0355</td>
<td>82.2</td>
<td>63.4</td>
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<tr>
<td>1</td>
<td>1</td>
<td>R</td>
<td>1.12</td>
<td>0.0120</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>8.25</td>
<td>0.0380</td>
<td>88.0</td>
<td>76.0</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>R</td>
<td>0.61</td>
<td>0.0050</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>8.60</td>
<td>0.0390</td>
<td>93.3</td>
<td>88.6</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>R</td>
<td>0.41</td>
<td>0.0025</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>8.70</td>
<td>0.0370</td>
<td>95.5</td>
<td>93.7</td>
</tr>
<tr>
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<td>7½</td>
<td>R</td>
<td>0.36</td>
<td>0.0023</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*EXPERIMENTAL*
## APPENDIX 2-e

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th># hr</th>
<th>Sample</th>
<th>Activity units/ml cell wall</th>
<th>% Activity released</th>
<th>% Activity recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
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<td>TRE</td>
<td>INV</td>
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## APPENDIX 2-f

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

<table>
<thead>
<tr>
<th>Treatment</th>
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<th>Sample</th>
<th>Activity units/ml cell wall</th>
<th>% Activity released</th>
<th>% Activity recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>INV</td>
<td>TRE</td>
<td>INV</td>
</tr>
<tr>
<td>CONTROL</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td>Residue</td>
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<td>0.0000</td>
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## APPENDIX 2-g

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

<table>
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<th>Treatment (no chitinase)</th>
<th>Sample</th>
<th>Activity units/ml cell wall</th>
<th>% Activity released</th>
<th>% Activity recovered</th>
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<td>13</td>
<td>S</td>
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<td>4.13</td>
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### APPENDIX 2-h

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th># hr</th>
<th>Sample</th>
<th>Activity units/ml cell wall</th>
<th>% Activity released</th>
<th>% Activity recovered</th>
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<tbody>
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<td></td>
<td></td>
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<td>TRE</td>
<td>INV</td>
</tr>
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<td>CONTROL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td></td>
<td>Residue</td>
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<td>0.0530</td>
<td>0.7</td>
</tr>
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<td>63.7</td>
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**EXPERIMENTAL**
**APPENDIX 2-i**

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

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

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

Logarithmic Growth of Neurospora crassa

In each experiment, a 1-liter Erlenmeyer flask containing 500 ml of Fries' Minimal Medium supplemented with 2.7% galactose at pH 5.2 was inoculated with a cell suspension (5.4 ml, absorbance at 600 nm = 20) and incubated in a gyratory shaker bath at 30°C and 200 rpm. Duplicate aliquots (5-10 ml) were withdrawn aseptically at time intervals, washed, filtered and dried in an oven at 80°C for over 48 hr. The average dry weights were calculated per 100 ml of culture.

Each point in the graph represents the mean logarithmic values from two experiments.
APPENDIX 4

a. Assay of Ribonuclease

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

Reagents:
- 0.10 ml enzyme solution
- 0.25 ml sodium phosphate buffer (pH 7.5)
- 0.40 ml Double Distilled Water (DDW)
- 0.25 ml RNA (3 mg/ml aq) prepared just before use

Procedure: Incubate the mixed reagents at 37 C for 30 min.

Stop the reaction with 0.25 ml of uranyl acetate (0.75% in 25% HClO₄).

Centrifuge. Supernatant (0.2 ml) + 5 ml of 1 M nitric acid containing 0.05% NaN₃ (made just before use).

Read absorbance at 260 nm.

b. Assay of Acid Protease

Principle: Measure acid-soluble tyrosine in digests of proteins from gelatin.

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

Reagent b: 2.6 M nitric acid containing 0.05% NaN₃ (made just before use)

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

Stop the reaction with 1 ml perchloric acid.

After 10 min at room temperature, centrifugate.

Heat (0.5 ml supernatant + 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 emulsion.

Measure absorbance of upper phase at 412 nm.
e. Assay for glucose with Glucose Oxidase

**Glucose**

**Principle:** glucose + O₂ + H₂O₂ → Glucose Oxidase → H₂O₂ + oxidized chromogen

**H₂O₂ + reduced chromogen** → oxidized chromogen

**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 the mixed reagents at 37°C for 30 min.

Stop the reaction with 2 drops of 6N HCl.
Read absorbance at 420 nm.

f. Assay of Trehalase

**Principle:** Measure the production of glucose from trehalose

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

**Procedure:**
Incubate 0.5 ml of enzyme solution with the mixed reagents at 37°C for 60 min.

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

Assay for glucose with the glucose oxidase method (e).
g. Assay of Invertase

(i) Biochemical assay:

Principle: Measure production of glucose from sucrose.

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 with 0.95 ml of the mixed reagents at 37°C for 15 min.

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

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

(ii) Histochemical stain with DAB:

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

Reagents: 5.5 ml DAB solution (5 mg DAB in 1 M phosphate buffer at pH 7, 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 aq)

Procedure: Incubate the cut tissue sections with mixed reagents at 37°C until suitable intensity has developed.

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

Rinse in DDW and wash in 15% alcohol.

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 polymerized, with 25 μl of glucose oxidase (0.1 ml per ml of gel solution, into the following reagent in a test tube.

Let stand in the dark for 2–4 hr at

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 0.5 ml of enzyme solution with the mixed reagents at 37°C for 30 min.

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

Assay for reducing group with 3,5-dinitrosalicylic 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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cycle of Neurospora crassa. II. NAD- & NADP-de-
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cycle of Neurospora crassa. III. Nicotinamide
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