STUDIES

ON

FUNGAL CELL WALLS

by

Mohammad Abu Obaidah

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ABSTRACT

An investigation of the chemical structure and enzymic degradation of the cell walls of Fusicoccum amygdali has been carried out.

Mechanically isolated cell walls were found to consist mainly of polysaccharides with small amounts of protein and lipid. The main carbohydrate constituent was D-glucose; smaller amounts of D-glucosamine, D-galactose, D-mannose, L-rhamnose, xylose and arabinose were also identified.

Fractionation of cell walls with a range of different solvents and degradation with a number of enzymes indicated the presence of several different polysaccharides. These were :

(a) Chitin, which accounted for most of the cell wall glucosamine and which was isolated from cell walls in undegraded form by an enzymic method, superior to conventional chemical methods. Chitosan was not detected, but traces of glucosamine were found in other fractions;

(b) Galactomannan heteropolymers, which probably contained phosphodiester linkages as evidenced by the detection of galactose-6-phosphate and mannose-6-phosphate in acid hydrolysates and which may be linked, in part, to the cell wall by peptide bridges. Extracellular heteropolymers of similar nature produced by Fusicoccum amygdali were probably formed by the action of lytic enzymes on the cell wall;

(c) An α-glucan, which was shown to consist of linear chains of $\alpha 1 \rightarrow 3$ glucopyranose units linked together by short blocks of $\alpha 1 \rightarrow 4$ glucopyranose units;

(d) A linear $\alpha 1 \rightarrow 4$ glucan. Evidence was obtained to suggest that this amylaceous glucan was linked to a branched $\beta 1 \rightarrow 3$, $\beta 1 \rightarrow 6$ glucan via a galactomannan heteropolymer;

(e) Additional β-glucan, probably $\beta 1 \rightarrow 3$, $\beta 1 \rightarrow 6$ linked; cellulose could not be detected.

An estimate of the cell wall polysaccharide linkage composition was obtained by sequential enzymic degradation.

New modifications of methods for the purification of Basidiomycete QM806 exo-β1$\longrightarrow$3 glucanase, Helix pomatia endo-β1$\longrightarrow$3 glucanase and pullulanase were devised.

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LAYOUT OF THE THESIS

The Table of Contents is followed by the Introduction, Results, Discussion and Experimental Section. The Experimental Section includes full details of all the experiments and also the General Methods. Finally the Appendices, an index to the experiments and a list of references are given.

Cell wall fractions were designated by the Experiment Number, followed by a letter e.g. Fraction 14FJ = Fraction J in Expt. 14F.

Figures and Tables, which are included in the text, were numbered as follows :

Introduction	Table I.2 = Table 2 in Introduction.
Results Section	Fig.III.1 = Fig.1 in Section III of the Results.
	Table VIII.3 = Table 3 in Section VIII of the Results.
Discussion	Fig.D.2 = Fig.2 in Discussion.
General Methods	Fig.G.M.24 = Fig. in General Method 24.
	Table G.M.23 = Table in General Method 23.
Appendices	The tables and figures are given in numerical order.

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INTRODUCTION

During the last decade extensive studies have been carried out on the chemistry, morphology and biosynthesis of bacterial cell walls, particularly in relation to the mode of action of antibiotics, such as penicillin. In comparison relatively little has been done on the cell walls of fungi, although investigations started as early as 1811, when Braconnot (1811) isolated an alkali insoluble, nitrogenous residue from a number of mushroom species; this substance, which had a nitrogen content less than that of protein, he named "fungine"; Gilson (1895) showed that the product was similar to chitin (Gr. Chitōn meaning mantle or tunic), isolated by Odier (1823) from integuments of insects. Much of the earlier work on fungal cell walls was concerned with attempts to detect chitin and cellulose by cytochemical techniques and later by X-ray analysis (Foster and Webber, 1960; Fuller and Barshed, 1960; Aronson and Preston, 1960b). In many investigations extraction procedures have been used either to isolate specific cell wall components (Thomas, 1928, 1930, 1942, 1943; Garzuly - Janke, 1940a, 1940b) or to prepare chemically a basic cell-wall residue, which was then subjected to chemical analysis (Norman and Peterson, 1932), X-ray analysis (Frey, 1950; Blank, 1953; Winkler, Douglas and Pramer, 1960), electron microscopy (Aronson and Preston, 1960a) or infrared spectroscopy (Michell and Scurfield, 1967).

The earlier work on fungal cell walls thus suffered from the lack of availability of methods to prepare clean, isolated cell walls free from cytoplasmic inclusions. Although these problems were overcome about two decades ago when mechanical methods for isolating cell walls were developed, progress has been comparatively slow, probably due to concentration of interest on the occurrence of chitin and cellulose in various taxa (Kreger, 1954; Manocha and Colvin, 1968), and perhaps also the field has lacked the impetus of antibiotics, which has contributed greatly to interest in bacterial cell walls. Only a few thorough examinations of cell walls of filamentous fungi have been

carried out (Johnston, 1965; Zevenhuizen and Bartnicki- Garcia, 1969). Although yeast cell walls have been studied intensely by numerous workers (Phaff, 1963), much of the work involved drastic chemical treatments and may require re-interpretation in the light of present knowledge (Eddy and Woodhead, 1968; Bacon, Farmer, Jones and Taylor, 1969; Houwink and Kreger, 1953). To gain more reliable information, hydrolysates of mechanically isolated cell walls have been prepared and examined by several authors, e.g. from yeasts (Phaff, 1963, Bacon, Framer, Jones and Taylor, 1969) and from filamentous fungi (Cummins and Harris, 1958; Aronson and Machlis, 1959; Crook and Johnston, 1962; Johnston, 1965; Bartnicki - Garcia and Nickerson, 1962; Applegarth and Bozoian, 1968).

Chemically the mechanically isolated fungal cell wall consists of 80 to 90% polysaccharides with most of the remainder consisting of protein and lipid. Wide variations from the above values are rare, e.g. the cell wall of the yeast Saccharomycopsis guttulata, which contains 40% protein. Wide variations of cell wall constituents also occur at different stages in the life cycle of a single organism (Bartnicki-Garcia, 1968) (see later). The presence of nucleic acid as a true cell wall component has never been conclusively demonstrated. Although there is little doubt that lipid and protein are genuine cell wall components (Dyke, 1964; Manocha and Colvin, 1967) unusually high value of proteins and lipids should always be viewed with caution and preparations carefully checked for the presence of cytoplasmic membranes or contaminating cytoplasm. Substantial amounts of melanin, polyphosphates and inorganic matters have also been detected in certain fungal cell walls (Bartnicki - Garcia and Reyes, 1964; Aronson and Machlis, 1959).

The chemistry of the main cell wall components will now be reviewed.

Polysaccharides

At least 12 monosaccharide units (glucose, mannose, galactose, rhamnose, fucose, glucuronic acid, galacturonic acid, glucosamine, galactosamine, xylose, arabinose and ribose) have been reported to

occur, in the form of polymers, in fungal cell walls. Some of them occur in both D & L forms e.g. D- and L- galactose in Aspergillus niger, (Johnston, 1965). Glucosamine may occur both as the free amine or as the N-acetylated form (Kreger, 1954; Bartnicki - Garcia and Nickerson, 1962). The relative proportions of the monosaccharides vary widely among different fungi, but only three (glucose, mannose and glucosamine) are found consistently in most fungi. Some monosaccharides may be characteristic for certain groups of fungi e.g. D - galactose and D - galactosamine (Ascomycetes), D - glucosamine (Mucorales), L - fucose (Mucorales and Basidiomycetes), Xylose (Basidiomycetes) and D - galacturonic acid [Allomyces sporangia (Skucas, 1966)] .

A. Glucans

D - Glucose occurs in the fungal cell walls in a variety of polymeric forms, and is found in both α-linked and β-linked polymers

1. α - Glucans

(a) α - (1$\rightarrow$3) Glucans : This type of glucan was first isolated from Polyporus betulinus by Winterstein in 1895 using alkali extraction and was identified tentatively as an α1$\rightarrow$3 glucan by Duff (1952); more recently it has also been found in Polyporus tumulosus (Ralph and Bender, 1965). It was first recognised as a cell wall component by Johnston (1965), who isolated an alkali soluble glucan from cell walls of Aspergillus niger and showed it to contain mainly α1$\rightarrow$3 glucopyranose units with small numbers of α1$\rightarrow$4 glucopyranose units.

Alkali soluble glucans found by Kreger (1954) in the cell walls of several fungi (Schizosaccharomyces, Endomyces, Penicillium, Agaricus) and by Wessels (1965) in Schizophyllum commune ("S" glucan) were later recognised by Bacon, Jones, Farmer and Webley (1968) to be α1$\rightarrow$3 glucans. Their infrared spectra and X-ray diffraction patterns were almost identical with those of the α1$\rightarrow$3 glucans from A.niger and P.betulinus. α1$\rightarrow$3 glucans have also been detected in the cell walls of Paracoccidioides brasiliensis (Kanetsuna, Carbonell, Moreno and Rodriguez, 1969), Polyporus sulphureus, Boletus edulis, Schizosaccharomyces versatilis, S. pombe, Cryptococcus albidus and

Cryptococcus terreus (Bacon, Jones, Farmer & Webley, 1968 and references cited therein).

(b) α-(1→4)-Glucans : Although polymers of the starch-glycogen type are found as energy reserves in all fungi, α1→4 linked glucans have not been conclusively identified as structural components of fungal cell walls. A polymer of the glycogen type was isolated from the spore walls of Dictyostelium discoidium (Ward and Wright, 1965); a polymer assumed to be glycogen, was also isolated from the mycrocyst walls of Polysphondylium pallidum (Toama and Raper, 1967), but was not unequivocally identified. There is evidence for the presence of glycogen granules in yeast cells as storage material bounded and attached to the cell wall by fine membrances (Northcote, 1963). However although glycogen was frequently reported in yeast cell walls prepared by chemical extraction, it was not found in mechanically isolated cell walls (Northcote and Horne, 1952). Harrison (1964) noted amyloid particles along some hyphae in the stipitate Hydnaceae adhereing to the wall and Smith and Zeller (1966) found these granules to be a prominent feature in some species of Rhizopogon. They do not, however, appear to be a structural feature of the cell wall.

(c) α(1→3), α(1→4) - Glucans : Nigeran, an α-glucan having alternating α1→3, and α1→4 glucopyranose linkages has been isolated by Dox and Neidig (1914) from Penicillium expansum and from a strain of A.niger by Dox (1915). The structure of nigeran from a stain of A.niger was later determined by Barker et.al (1952, 1953 and 1957). It occurs widely in a number of Aspergillus and Penicillium species. It apparently is found in both as an intracellular and extracellular polysaccharide and as a structural component of the cell wall (Johnston, 1965; Reese and Mandels, 1964; Tung and Nordin 1967).

2. β-Glucans

(a) β(1→4)-Glucans (Cellulose) : Cellulose is a linear polysaccharide, composed of β1→4 linked glucopyranose residues. It is an important constituent of the cell walls of higher plants where it may achieve a molecular weight of up to 1.5×10^6 (Northcote, 1958).

The chains are hydrogen bonded together to form crystalline regions (Northcote, 1958; Marchessault and Sarko, 1967; Northcote, 1969), which give characteristic X-ray diffraction patterns. The unit cell of the crystalline regions of cellulose I is monoclinic, and contains four glucose residues (Preston, 1952). Treatment of cellulose I with alkali or reprecipitation of cellulose from its solution gives cellulose II with different crystalline properties. In 1898 van Wisselingh reported the presence of cellulose in the cell walls of a number of fungi. He used a microchemical test employing I_2-KI-70% H_2SO_4, which usually leads to a blue colour in cellulosic walls. The reliability of the method has been questioned and sometimes other wall constituents may mask the cellulose that is present (Preston, 1952). Later workers have found the X-ray diffraction method or hydrolysis with specific cellulases more reliable (Bartnicki-Garcia, 1966; Fuller, 1960). In fungal cell walls cellulose appears to be limited to the Acrasiales, Oomycetes and Hyphochytridiomycetes. Cellulose containing fungi are usually chitin deficient, although some organisms have been found to contain both chitin and cellulose [Rhizidiomyces sp. and Ceratocystis ulmi (Fuller and Barshad, 1960; Rosinski and Campana, 1964)] .

(b) β (1→3), β (1→6) - Glucans : Glucans containing β1→3 and β1→6 glycopyranose linkages in varying proportions are very common components of fungal cell walls and sometimes account for as much as 90% of the cell wall. They are found in both alkali soluble and alkali insoluble cell wall fractions. However very little is known of the detailed structure of the glucans. Callose, a β1→3 glucan, which occurs widely throughout the plant kingdom, has received considerable attention as a fungal cell wall constituent, but it has been extracted from whole cells and its relation to the cell wall is uncertain (Cochrane, 1958). The alkali insoluble glucan of yeast has received the most attention. Yeast glucan is weakly crystalline, but its crystallinity is enhanced by treatment with dilute acid (Houwink and Kreger, 1953) which converts it to "hydroglucan" which forms aggregates of micro -

fibrils. Presumably this conversion is accompanied by considerable hydrolysis. Although a great deal of work has been done on the structure of yeast glucan, because of the drastic conditions often employed to isolate the glucan (Houwink and Kreger, 1953; Misaki, Johnson, Kirkwood, Scaletti and Smith, 1968) and the recent demonstration that yeast glucan is composed of two distinct polymers (Bacon, Farmer, Jones, Taylor, 1969; Manners and Masson, 1969), much of the early work may require reinterpretation. It is now evident that yeast glucan contains two polymers : (a) a predominantly $\beta 1 \rightarrow 6$ glucan with only a few $\beta 1 \rightarrow 3$ branches ($\bar{DP} \sim 140$) and (b) a predominantly $\beta 1 \rightarrow 3$ glucan with some $\beta 1 \rightarrow 6$ branching ($\bar{DP} \sim 1450$, ca. 1 branch ($1 \rightarrow 6$) for every 30 glucose residues). The fine structure of these glucans or the way in which they are bound into the cell wall is not known. Bacon et. al. (Bacon, Farmer, Jones and Taylor, 1969) have suggested that the alkali insolubility of yeast glucan is due to the fact that the cell wall contains a surrounding membrane, consisting of glucan and chitin, which is semipermeable and on normal alkali extraction, allows only mannan (M.wt $\sim$50,000) to pass through, but not glycogen (M.wt. $> 2 \times 10^6$) or the glucan. Breaking the walls ballistically or mild acid treatment was postulated to damage this membrane and allow extraction of glycogen and cell wall glucan.

Zevenhuizen & Bartnicki-Garcia (1969) have examined an alkali insoluble glucan from Phytophthora cinnamoni and showed it to contain (after extraction of cellulose with Schweitzer's reagent) 15 - 20% non-reducing end groups, 45 - 50% $1 \rightarrow 3$ links 10% $1 \rightarrow 4$ links and 20% 1, 3, 6 branch points. Structural studies showed that it contained a highly branched core of $\beta 1 \rightarrow 3$ and $\beta 1 \rightarrow 6$ linked glucopyranose residues, to which were attached, long chains of $\beta 1 \rightarrow 3$ glucopyranose units.

(c) $\beta(1 \rightarrow 2)$ - Glucans : The occurrance of this type of glucan has been reported by Mitchell and Sabar (1966) in the cell walls of Pythium butleri. Identification was based mainly on partial similarity in X-ray reflections to that obtained from the Crown gall polysaccharide of Agrobactor tumefaciens. However Novaes-Ledieu (1967) and Aronson et al.

(Aronson, Cooper and Fuller, 1967; Cooper and Aronson, 1967) have demonstrated convincingly that the hyphal walls of Pythium butleri and P. debaryanum contain mainly β1→4, β1→3 and β1→6 linked glucans. Although the absence of a small number of β1→2 linkages could not be ruled out, more rigorous proof is needed to substantiate their presence in the wall glucans of these fungi.

B. Mannans

Small amounts of mannose have been found in many fungal cell walls. Mannans are major components of the cell walls of the yeast forms of the Ascomycetes and Deuteromycetes, and the pink yeasts of the genera, Rhodotorula and Sporobolomyces. Mannans of the last two named are of interest because they do not form insoluble copper complexes (Bartnicki - Garcia, 1968).

Mannans of the pathogen, Candida albicans, the causative agent of thrush, are of immunological interest, since they are the major antigens of the cell wall. They are branched structures containing α1→2 and α1→6 pyranose linkages and also α(1→2) and α(1→3) furanose linkages (Yu, Bishop, Cooper, Hasenclever and Blank, 1967; Bishop, Blank and Gardner, 1960). Much structural information on the mannans of yeasts has been obtained from N.M.R. spectroscopy (Gorin, Spencer and Eveleigh, 1969; Gorin, Spencer and Bhattacharjee, 1969). A mannan containing β1→3 and β1→4 linkages was isolated from the urediospores of Puccinia graminis tritici (Prentice, Cuendet, Geddes and Smith, 1959).

The mannan of Saccharomyces cerevisiae is the most studied mannan of fungi and constitutes 31 - 43% of the cell wall (Bacon, Farmer, Jones and Taylor, 1969). It is usually prepared by extraction of whole cells or cell walls with hot alkali. The mannan is then precipitated from the neutralised solution as its copper complex. This type of preparation usually contains traces of amino acids, glucosamine and phosphates and has the structure shown in Fig. 1 (according to Peat, Turvey and Doyle, 1961).

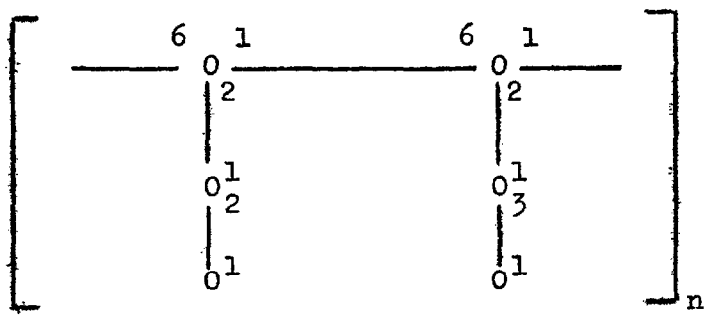


Fig.1. Structure of Saccharomyces cerevisiae mannan

O = represents α-mannopyranosyl residues

Sentandreu and Northcote (1968) isolated a mannose containing glycopeptide by extracting yeast cell walls with ethylenediamine. Structural studies indicated that the glycopeptide contained two types of peptide carbohydrate linkages. One of these connects a number of small monosaccharide or oligosaccharide units to the peptide by glycosyl bonds involving the hydroxyl groups of serine and threonine, and the other connects the high molecular weight, highly branched mannan to the peptide, and is thought to involve a nitrogen glycosyl bond between N-acetylglucosamine and aspartamide. Cawley and Letters (1968) found that when defatted yeast cell walls were treated with pronase, about 54% of the total carbohydrate and 38% of the total phosphorus was released. They isolated a soluble phosphoglycopeptide from the supernatant and suggested, on the basis of periodate oxidation and alkali degradation that highly branched mannan polymers were linked together by 1→6 phosphodiester bridges as in the phosphomannan of Hansenula holstii (Jeanes and Watson, 1962). Lampen (1968) suggested a structure for the yeast cell wall consisting of (a) an outer layer of phosphomannan, (b) an adjacent layer of mannan, glucan and protein and (c) a rigid inner layer of glucan. It is clear that further studies are needed before the way in which the various polymers are linked in the cell wall can be delineated.

C. Galactans

Polysaccharides containing galactose are produced by a wide range of fungi (Martin and Adams, 1956). Bound galactose as a cell wall constituent occurs in many fungi including a number of Penicillium species, Verticillium alboatrum and Mucor rouxii, but it is absent in Phytophthora cactorum, a number of Saprolegniaceae, several yeasts and a number of other fungi (Parker, Preston and Fogg, 1963; Cummins and Harris, 1958; Crook and Johnston, 1962). Very little is known concerning the structure or architectural role of these cell wall galactans.

D. Chitin

Chitin, a linear polysaccharide composed of $\beta 1 \rightarrow 4$ linked N-acetyl-D-glucosamine residues was first prepared from fungal cell walls by Braconnot (1811). The widespread occurrence of chitin in fungal cell walls was recognised by van Wisselingh (1898), who devised a cytochemical test, which involved conversion of chitin to chitosan with alkali. The test gave positive results with over one hundred genera of fungi, but the reliability has been challenged (Aronson, 1965). The use of X-ray diffraction patterns for chitin detection is generally regarded as a more reliable method. The linear chitin chains bind together to form crystalline regions. The unit cell is orthorhombic and has two chains, one "up" and the other "down". Chains having the same polarity are hydrogen bonded through planes of $>C = O \cdots H - N<$ running perpendicular to the fibre axis. Anti parallel chains are joined by hydrophobic bonds by contact of the methyl groups of the acetamido groups or cross chain hydrogen bonds involving hydroxymethyl groups (Foster and Webber, 1960; Marchessault and Sarko,1967). Confirmatory evidence for the presence of chitin is often obtained using specific chitinases e.g. Streptomyces chitinase (Skujins, Potgieter and Alexander, 1965). Chitin occurs in most taxa (see Aronson 1965) but it is generally not found in cell walls which contain cellulose (with the exception of Hyphochytridiomycetes). However the proportion of chitin varies enormously from, for example,

60% in Allomyces macrogynus (Aronson and Machlis, 1959) to 2-5% in Schizophyllum commune (Wessels, 1965).

E. Chitosan

Chitosan is the name given to a family of polymers, derived from chitin, in which the nitrogen atoms are totally or partially deacetylated (Foster and Webber, 1960). Its occurrence in fungal cell walls was not suspected until Kreger (1954) reported its presence in the cell walls of the mycelium and sporangiophores of Phycomyces. Later its presence in the cell walls of Mucor was reported by Bartnicki-Garcia and Nickerson (1962), who used identification based on X-ray diffraction, solubility in acids, the van Wisselingh microchemical method and the chitosan sulphate test. Its presence appears to be characteristic of the Zygomycetes, whose walls are composed mainly of chitin and chitosan and contain very little glucose.

F. Polysaccharides containing 2-amino-2-deoxy-D-Galactose.

Galactosamine is known to occur in the cell walls of a number of Ascomycetes e.g. Aspergillus niger (Johnston, 1965); Neurospora crassa (Mahadevan and Tatum, 1965). It is a major constituent of the cell walls of Amoebidium parasiticum, the only Trichomycete so far examined. It is of interest that, unlike the walls of other fungi, the thallus walls of A.parasiticum are completely soluble in alkali (Trotter and Whistler, 1965).

The structural role of cell wall galactosamine polymers is unknown, but it is of interest that Buck, Chain and Darbyshire (1969) found that a virus containing strain of Penicillium stoloniferum contained a much higher cell wall galactosamine level than those of five non-infected strains of the same species. It was suggested that the galactosamine might be part of a virus receptorsite.

G. Glucuronic acid

Until comparatively recently there was little conclusive evidence for the presence of uronic acids in fungal walls (Aronson, 1965). However they have now been clearly demonstrated in Dactylium dendroides,

and Alternaria, Fusarium, Penicillium and Aspergillus species (Gancedo, Gancedo and Asensio, 1966). A heteroglycan composed of mannose, galactose, glucose and glucuronic acid was isolated from the cell walls of Pullularia pullulans (Brown and Lindberg, 1967) and Bartnicki-Garcia and Reyes (1968) found two types of glucuronic acid containing polymer in the sporangiophore walls of Mucor rouxii. One was an alkali soluble polymer ("mucoran"), containing 50% D-glucuronic acid with some fucose, mannose and galactose, while the other was an insoluble polyuronide, thought to be a homopolymer of D-glucuronic acid. Kreger (1970) isolated polyuronides [probably as polyglucuronic acid as has been isolated from Mucor rouxii by Bartnicki - Garcia and Rayes (1968)] from the mycelial and sporangiophore forms of Mucor rouxii and from other Phycomycetes and Geaster by warm dilute alkali. Their infrared spectra and X-ray diagrams were identical with each other and similar, but not identical with those of algal polyuronides. They are resistant to 72% sulphuric acid hydrolysis, soluble in alkali and in hot glycerol. The crystalline form remained unaltered on precipitation from solution.

Protein

Evidence for the presence of protein in the fungal cell wall was asked to be taken with caution by Aronson (1965). Although contamination of cell wall preparations with protein from the cytoplasm and/or enzymes is possible, it has been shown by several workers that even drastic chemical and enzymic treatment often fails to remove it completely (Bartnicki - Garcia, 1966, Ruiz-Herrera, 1967). The presence of a particular amino acid in a high proportion e.g. serine or threonine in cell wall fractions of Saccharomyces cerevisiae (Sentandreu and Northcote, 1968) and aspartic and glutamic acids which accounted for 30 to 40% of the protein in cell wall fraction of Saccharomyces cerevisiae, (Kessler and Nickerson, 1959), and the presence of hydroxy proline, which is only found in cellulosic cell walls, (Bartnicki - Garcia, 1968 and reference cited therein) indicates that protein (or peptide) is really a part of the cell walls in these

cases. Clear evidence is provided by the cell wall of yeast where it has been shown by Cawley and Letters (1968) that 54 % of the total cell wall carbohydrate becomes soluble by simple pronase treatment, indicating that polysaccharide components were held together by peptide bonds. Further evidence was provided by the work of Sentandreu and Northcote (1968) who showed that alkali treatment of a glycopeptide, isolated from yeast cell walls, resulted in the loss of 64% of the threonine and 42% of the serine and liberation of mannose and other diffusible mannose oligosaccharides. Further alkali treatment of the residue gave a mannan, still containing small amounts of amino acids and having a glucosamine : aspartic acid ratio 1:1. Nickerson (1963) found that the integrity of the cell wall of Saccharomyces cerevisiae depended largely on polysaccharide - protein complexes, which could be degraded with consequent lysis by purified proteases from Streptomyces fradiae. It thus appears probable that the role of peptides in fungal cell walls is in joining together certain of the polysaccharide components.

Lipid

Small amounts of lipid are found in most fungal cell walls and are regarded as genuine cell wall components and not merely an artifacts arising from the cytoplasmic membrane and/or cytoplasm. Dyke (1964) demonstrated clearly that the lipid found in the cell wall of Nadsonia elongeta was a bona fide component and not a cytoplasmic contaminant; he showed that the cell wall lipid was mainly saturated and lacked palmitoleic acid whereas cytoplasmic lipids were mainly unsaturated and contained palmitoleic acid. Ruiz-Herrera (1967) suggested that cell wall lipids may form somekind of complex with wall polysaccharides and/or protein, and Hurst (1952) suggested that in the case of Saccharomyces cerevisiae it contributes stiffness to the cell wall. In the sporangiophore wall of Phycomyces lipoidal material accounts for more than 25% of the dry weight (Kreger, 1954) and the presence of lipid in the spores and sporangiophores was suggested by Bartnicki - Garcia (1968) to confer the hydrophobic

Table I.1 Cell Wall Chemistry and Taxonomy of Fungi

		Taxonomic Group	Chemical Category	Distinctive Features
		Acrasiales	I. Cellulose-glycogen	Pseudoplasmodia
		Trichomycetes	VIII. Galactosamine-galactose polymers	Heterogeneous group of arthropod parasites
PHYCOMYCETES		Zygomycetes	IV. Chitosan-chitin	Zygospores
		Oomycetes	II. Cellulose- glucan	Biflagellate zoospores
		Hyphochytridiomycetes	III. Cellulose-chitin	Anteriorly uniflagellate zoospores
		Chytridiomycetes	V. Chitin- glucan	Posteriorly uniflagellate zoospores
HIGHER FUNGI	MYCELIAL	Ascomycetes[a]	V. Chitin- glucan	Septate hyphae, ascospores
		Basidiomycetes[b]	V. Chitin- glucan	Septate hyphae, basidiospores
		Deuteromycetes[c]	V. Chitin- glucan	Septate hyphae
	YEASTS	Cryptococcaceae	VI. Mannan- glucan	Yeast cells
		Rhodotorulaceae	VII. Mannan-chitin	Yeasts (carotenoid)
		Saccharomycetaceae	VI. Mannan- glucan	Yeast cells, ascospores
		Sporobolomycetaceae	VII. Mannan-chitin	Yeast (carotenoid), ballistospores

[a]Except Saccharomycetaceae

[b]Except Sporobolomycetaceae

[c]Except Crytococcaceae

properties to these structures.

Cell Wall Composition and Structure as an Aid to Fungal Taxonomy.

One of the main procedures for classification of any organism is based on the morphology during its whole life cycle. It is generally agreed that the shape of a fungal cell depends to a large degree on its cell wall structure. This is evident because (a) dissolution of the cell wall, e.g. by enzymes, results in the formation of proloplasts, which are spherical and (b) chemical removal of the cytoplasm often leaves a residual cell wall structure bearing the original shapes of the fungus. Wettstein (1921) was the first investigator to utilize cell wall composition as a criterion for assessing relationships among the major taxa. Wettstein employed cytochemical methods for distinguishing different polymers (cellulose, chitin) for ascertaining relationships within the Phycomycetes and the more recent X-ray work of Frey , Aronson, Fuller and coworkers (Aronson, 1965) has reaffirmed the validity of equating the presence of chitin and cellulose with taxonomic positions within the Phycomycetes. Nabel (1939) reported positive cytochemical tests for both chitin and cellulose in the cell walls of Rhizidiomyces bivellatus, which was described as a new species of Chytridiales, and proposed that, in the possession of both polysaccharides it established phylogenetic continuity between the Chytridiales and Blastocladiales on the one hand and the cellulosic Oomycetes on the other.

As more information regarding structure and composition of fungal cell walls has accumulated it has become increasingly evident that the entire spectrum of fungi may be subdivided into various categories according to the chemical nature of their walls, and that these categories closely parallel conventional taxonomic boundaries. Bartnicki - Garcia (1968 and 1969) established eight cell wall categories based on the principle components of the vegetative walls and these are given in TableI. It is clear that this represents a very preliminary classification and a more sophisticated analysis will be possible when

more structural details of fungal cell walls are known.

Ultrastructure of the Cell Wall

The cell walls of plants and fungi are regarded as two-phase systems : one phase consisting of the microfibrils, which are embedded within the other phase, referred to as the amorphous matrix (Aronson, 1965 and references cited therein). The fibrous nature of fungal walls was observed for the first time by Frey - Wyssling and Mühlethaler (1950) in the sporangiophore of Phycomyces. The existence of such fibrous structures, however, had been deduced much earlier through the application of polarization optics (Oort and Roelofsen, 1932) and X-ray diffraction (van Iterson, 1936). Later Houwink and Kreger (1953) and Kreger (1954) demonstrated a fibrous texture in the chemically treated walls of yeasts and fungi.

Electron microscopic analysis have shown that the cell wall is multilaminate e.g. in the Phycomyces sporangiophore (Frey-Wyssling and Mühlethaler, 1950), where the primary wall of the growth zone has been resolved into two lamellae which differ in their microfibrillar orientations. Distinct lamellae have also been observed in the hyphal walls of Allomyces (Aronson and Preston, 1960a), and the cell walls of Saccharomyces cerevisiae revealed a multilaminate structure when viewed in the electron microscope (Agar and Douglas, 1955, Mundkur, 1960; Vitols, North and Linnane, 1961).

Chemical Differentiation of the Cell Wall and Morphogenisis

The morphological changes in fungi in different environmental conditions, which occur during the life cycle of the organism, have been studied mainly from the aspect of physical changes, but little is known about the biochemical bases of morphogenesis in fungi via a better-understanding of cell wall properties and behaviour, an approach which was suggested by Nickerson (1959).

At present the subject is limited to looking at overall chemical differences in cell wall composition of different morphological forms;

Table1.2 Chemical differentiation of the cell wall in the life cycle of Mucor rouxii (Bartnicki - Garcia, 1968) [a]

Wall component	Yeast	Hyphae	Sporangiophores	Spores
Chitin	8.4	9.4	18.0	2.1[b]
Chitosan	27.9	32.7	20.6	9.5[b]
Mannose	8.9	1.6	0.9	4.8
Fucose	3.2	3.8	2.1	0.0
Galactose	1.1	1.6	0.8	0.0
Glucuronic Acid	12.2	11.8	25.0	1.9
Glucose	0.0	0.0	0.1	42.6
Protein	10.3	6.3	9.2	16.1
Lipid	5.7	7.8	4.8	9.8
Phosphate	22.1	23.3	0.8	2.6
Melanin	0.0	0.0	0.0	10.3

a = Values are per cent dry wt. of the cell wall.

b = Not confirmed by X-ray. Value of spore chitin represents N-acetylated glucosamine; chitosan is nonacetylated glucosamine.

frequently gross chemical changes occur from one form to another in fungi, e.g. Mucor rouxii (Bartnicki - Garcia, 1968) (Table1.2), in which the high value of glucose content of the spore walls contrasts with its virtual absence in the other three forms. Another striking feature is that the yeast walls have 5 - 6 times as much mannose as the hyphal walls of aerobically grown mycelium. On the other hand the yeast form of Histoplasma capsulatum had only a fifth of the mannose content

of the mycelial form (Domer, Hamilton and Harkin, 1967). However since the structure or architectural role of these two mannans is not known, no deductions can be made at present.

For a better understanding it is necessary to investigate the role which the individual chemical components play during the different processes and also the enzyme systems involved. On the basis of such an investigation melanin in the spores of Mucor rouxii (but not in the hyphal or yeast forms or sporangiophores) was shown to be a shield against detrimental radiation and also to confer resistance to enzymic lysis. The change in wall chemistry thus equips the fungus with a coat of greater survival value. The occurrence of lipids in the sporangiophore and spore probably confer hydrophobic properties (Bartnicki - Garcia, 1968). Lytic as well as synthetic enzymes are of importance in cell wall development. The role of an R-glucanase in the development of Schizophyllum commune has been studied by Wessels (1965).

Biosynthesis of Cell Wall Components

Little is known about the cell wall biosynthesis in fungi, except limited data concerning the precursors of a few individual polysaccharides. They are formed by transfer of the appropriate hexose from a nucleotide sugar to a suitable acceptor (primer). So far particulate enzyme preparations have been obtained associated with membrane fractions (20,000 g, 10 min. pellets). Some of the sugar nucleotide donors identified are uridine-diphosphate-glucose (UDPG) which was reported to be the natural precursor for the synthesis of noncellulosic glucan(s) in Phytophthora cinnamomi; both 1, 3 and 1, 6 bonds were formed (Wang and Bartnicki - Garcia, 1966); it is also the glycosyl donor for the synthesis of glycogen-like polymer of Dictyostelium discoideum spore coats (Ward and Wright, 1965); uridine-diphospho-N-acetyl-glucosamine serves as the glycosyl donor for chitin system in Neurospora crassa (Glaser and Brown, 1957) and many other fungal chitin systems; guanosine-diphosphate-mannose serves as the glycosyl donor for the synthesis of mannan in Saccharomyces carlsbergensis (Algranati

and Carminatti & Cabib, 1963) and cell free extracts of Mucor rouxii catalyzed the synthesis of wall polyuronides from uridine-diphospho-glucuronic acid (Bartnicki - Garcia, 1968). However very little is known concerning (1) where the cell wall polymers are synthesised i.e. are they polymerised in some intracellular site and then transported to cell wall or does the synthesis take place in situ ? (2) how cell wall growth is controlled.

Enzymic Digestion of Cell Walls and Formation of Fungal Protoplasts

The fungal protoplast can be considered to be a spherical unit of mycelial protoplasm, containing the normal cell organelles, bounded by a cytoplasmic membrane (plasma membrane) but devoid of a cell wall, thereby rendering it osmotically sensitive. Protoplasts, which are not completely free from cell wall material are known as spheroplasts. Fungal protoplasts in nature are very rare and are only found during the brief amoeboid or flagellate stages of a minority of fungi (Bartnicki - Garcia, 1968).

Fungal protoplasts may be prepared by a number of methods, e.g. (1) By a controlled autolytic process as described by Necas (1956) for Saccharomyces cerevisiae; (2) by direct digestion of the cell wall constituents by enzymes (a) from the digestive juice of snails (Rodriguez Aguirre and Villanueva, 1962) (b) from microorganisms (Rodriguez Aguirre, Garcia Acha and Villanueva, 1964) or (c) from commercial sources (Garcia Acha, Rodriguez, López-Betmonte and Villanueva, 1966); (3) by metabolic disturbance in the presence of sorbose or other sugars which causes inhibition of cell-wall formation (Hamilton and Calvet, 1964); (4) by cultivation of the fungi on thickened serum as described by Meinecke (1960); (5) by physical means (Necas, 1956).

In forming protoplasts by enzymic methods it may not be necessary for all the cell wall to be solubilised before the protoplast can emerge. Holter and Ottolenghi (1960) and Svihla, Schlenk and Dainko

(1961) using snail enzymes reported the emergence of protoplasts through a hole in the cell wall of the yeast cells. Frequently however more than one enzyme is required. For example, Skujins, Potgieter and Alexander (1965) found that hyphal walls of Aspergillus oryzae or Fusarium solani were solubilised by the co-operative action of chitinase and $\beta 1 \rightarrow 3$ glucanase, neither of which was active by itself.

The role of cell wall digestion in the ecology of micro-organisms has been considered by a number of authors (Mitchell and Alexander, 1963; Lloyd, Noveroske and Lockwood, 1965; Skujins, Potgieter and Alexander, 1965; Potgieter and Alexander, 1966; Bartnicki-Garcia and Lippman, 1967). In a number of cases it was shown that one organism produces extracellular enzymes able to digest the cell walls of another organism in the same habitat. For example Bartnicki-Garcia and Lippman (1967) showed that a soil Streptomycete produced enzymes readily able to digest the mycelial walls of the plant pathogen Phytopthora cinnamoni, although the organism did not necessarily die as a result. Although a subject of great complexity the role of cell wall structure in the ecology of fungi is deserving of further investigation.

Scope of the Present Work

Fusicoccum amygdali Del., the pathogen of almond (Prunus amygdalus st.) and peach [Prunus persica (L) st.] canker, is widely spread in Europe and in both North and South America (Graniti, 1964). The organism was described for the first time in 1905, following the observation of the French mycologist Delacroix on the almonds of Provence (Delacroix, 1905).

The wilting action caused by Fusicoccum was shown to be due to a toxin, named Fusicoccin, synthesised by the fungus. Fusicoccin contains a number of components, one of which, Fusicoccin A, has been crystallised and its structure determined (Ballio, Chain, De Leo, Erlanger, Mauri and Tonolo, 1964; Ballio, Brufani, Cassinovi, Cerrini, Fedeli, Pellicciari, Santurbano and Vaciago, 1968; Barrow and Chain, 1969). It was shown that Fusicoccin A caused the same symptom of

wilting when introduced into the xylem, as was produced by the pathogen itself (Graniti, 1964). The toxin was shown to act by opening the stoma of leaves with changes in cell wall permeability, thus increasing dehydration with an increase in water uptake, increased rate of K^+, Ca^+ and Mg^+ release, increase in oxygen uptake and metabolism of glucose and other reserved energy sources (Graniti, 1964; Turner and Graniti, 1969).

The present problem arose out of work, which was initiated at the Istituto Superiore Di Sanita, Rome, and continued at the Biochemistry Department, Imperial College with a view to isolating sufficient Fusicoccin for determination of its chemical structure. Since the wild type Fusicoccum produced very little Fusicoccin when grown in submerged culture, it was necessary to devise strain selection procedures with a view to increasing the yield. Since under submerged culture conditions the organism does not produce spores (the organism in nature produces asexual spores), selections of strains were then attempted by way of protoplast formation. It was found that protoplasts could be produced using Helicase, a preparation of enzymes from the gut of the Snail, Helix pomatia, but the yield was low and the results were not reproducible (unpublished results of G.T. Banks). It was decided to examine the chemical structure of the Fusicoccum cell wall and to ascertain which components were removed by the Helix pomatia enzymes with a view to suggesting more efficient methods of protoplast formation. In addition to the above application protoplast formation is of interest as a useful method of isolating cellular enzyme systems and also in investigating in detail cell wall regeneration and the enzyme systems involved.

This thesis describes the isolation and purification of cell walls from Fusicoccum amygdali, examines methods for fractionation of the different components and provides evidence for the structures of the main cell wall polysaccharides. New methods of purification of enzymes for use in structural studies were devised and the action of number of enzymes on the cell walls and cell wall fractions is described. Finally the relationship of the cell wall of Fusicoccum amygdali to the cell walls of other fungi is considered.

RESULTS

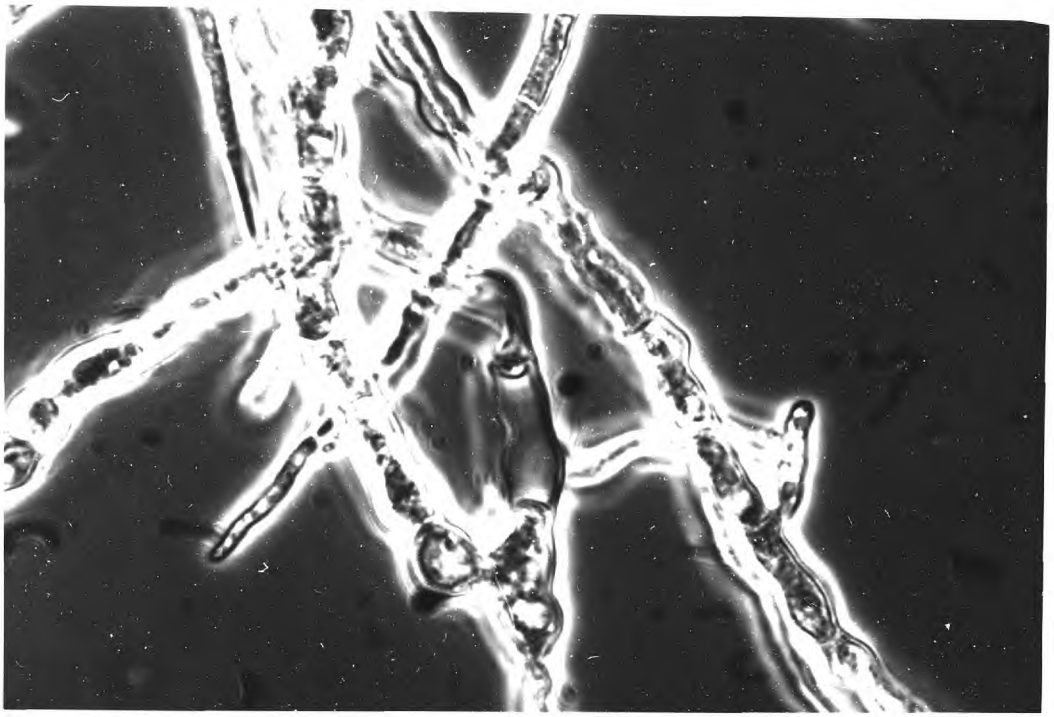


Fig. I.1(a). Photomicrograph of Fusicoccum amygdali, Del., intact mycelium.

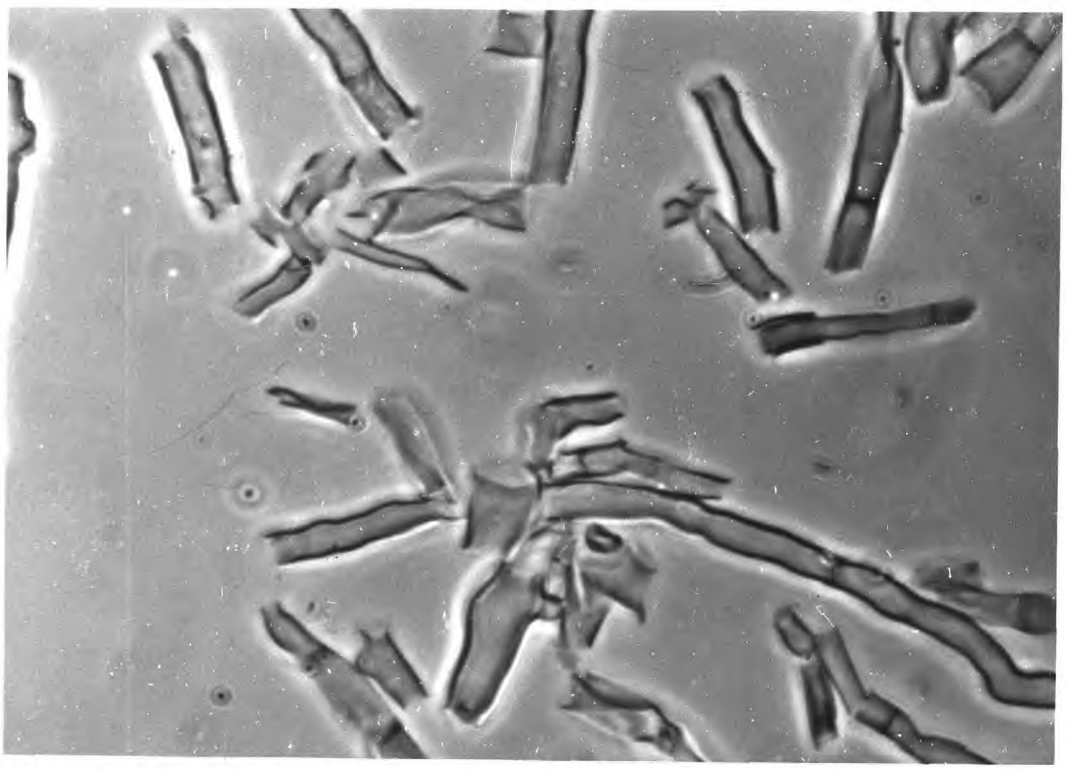


Fig. I.1(b). Photomicrograph of Fusicoccum amygdali, Del., isolated cell walls. (Expt. 1B).

I. PREPARATION AND CHEMICAL COMPOSITION OF FUSICOCCUM CELL WALL

A. Preparation of Fusicoccum amygdali Cell Walls

Cultures of Fusicoccum amygdali were harvested after $3\frac{1}{2}$ days (maximum growth). Cell walls were prepared by disrupting mycelium either with the Edebo press or with the Manton-Gaulin homogeniser, followed by differential centrifugation (Expt. 1).

The following batches were prepared.

Cell Wall Batch	Culture Conditions	Method of Preparation
1 st	Medium IIa; $3\frac{1}{2}$ days; 400 l. fermenter	Edebo press
2 nd	Medium IIb; $3\frac{1}{2}$ days; 400 l. fermenter	Edebo press
3 rd	Medium IIb; $3\frac{1}{2}$ days; 400 l. fermenter	Edebo press
4 th	Medium IIa; $3\frac{1}{2}$ days; 40 l. fermenter	Manton-Gaulin homogeniser

1st Batch : The cell wall preparation from the 1st Batch was used for total chemical and enzymic analysis and comparison of polysaccharide composition with the 4th Batch.

2nd and 3rd Batches : The cell wall preparations from the 2nd and 3rd Batches were used for preliminary fractionation studies previous to final fractionation of the 4th Batch for structural studies.

4th Batch : The cell wall preparation from the 4th Batch was used exclusively for fractionation, structure determination of polysaccharide fractions, and comparison with the 1st Batch.

All the preparations were white and fluffy in appearance, and consisted of fragments of cell walls of various sizes as revealed by phase contrast microscopy (Fig.I.1). A photomicrograph of intact

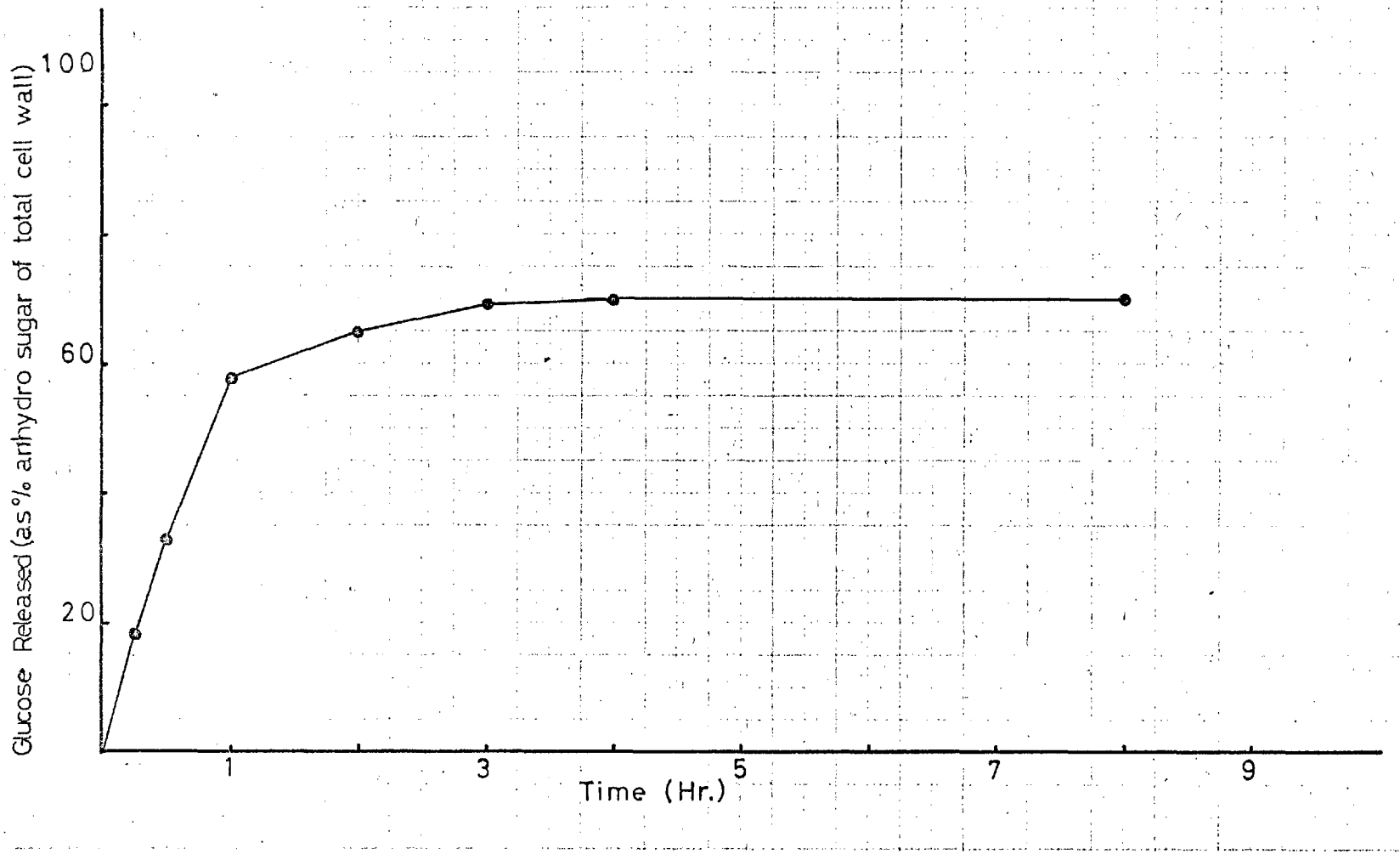


Fig. I.2. Hydrolysis of Cell Walls with N-Sulphuric Acid.
Cell walls were hydrolysed with sulphuric acid as described in G.M.15A; at suitable time intervals aliquots were withdrawn for determination of free glucose (G.M.3).

mycelium is included for comparison. Cell walls prepared using the Manton-Gaulin homogeniser were three to four times as long as cell walls prepared using the Edebo press.

The purity of cell wall preparations was assessed by light microscopy after staining with either cotton blue or dilute iodine solution. Cell walls were not stained by cotton blue, whereas intact cells and cytoplasmic debris (protein and nucleic acid) were stained blue. With dilute iodine solution cell walls were stained dark blue to black, whereas cytoplasmic debris gave a bright - yellow colour and intact cells were stained reddish brown. The amount of cytoplasmic contamination in all the cell wall preparations was estimated to be well below one percent.

B. Chemical Composition of Cell Walls

The composition of cell walls, prepared by the X-press method (1st Batch), are given in Table I.1, together with some data obtained from other batches for comparison.

Neutral Sugars : Neutral sugars were determined on N - sulphuric acid hydrolysates of cell wall preparations (G.M.15A). In order to determine the optimum conditions a time course for the release of glucose was obtained and is given in Fig.I.2. Maximum glucose release was obtained after 4 hr. and this hydrolysis time was used for the assay of all the neutral sugars. The values are not corrected for destruction of sugars during acid hydrolysis (Francois, Marshall and Neuberger, 1962; Neuberger and Marshall, 1966 and references cited therein) or for interaction of neutral sugars and aminoacids during hydrolysis (Gottschalk, 1966 and references cited therein); the magnitude of such corrections would be difficult to determine for the hydrolysis of a complex mixture. β - D - galactose dehydrogenase was the preferred reagent for galactose determination; parallel determinations were made using Dactylium dendroides D - galactose oxidase and the Worthington Galactostat Kit, but results were variable both with standards and cell wall hydrolysates.

Neutral sugars were isolated from cell wall hydrolysates by

Table I.1 Hyphal wall composition of Fusicoccum amygdali.

Component	Method	Weight % of Cell Wall		
		1st Batch	2nd & 3rd Batches	4th Batch
Anhydro total hexose	Phenol/sulphuric acid (G.M.1)	79.2	81.0	80.0
Anhydro-D-Glucose	D-Glucose oxidase (G.M.3)	69.2	69.9	69.0
Anhydro-D-Galactose	D-Galactose dehydrogenase (G.M.4a)	3.8	4.0	3.5
Anhydro-D-Mannose	Paper chromatography Solvent c (G.M.7)	2.8	2.9	
Anhydro-L-Rhamnose	"	1.3	1.0	
Anhydro- Xylose	"	1.0	0.8	
Anhydro-Arabinose	"	0.5		
Anhydro-D-Glucosamine	Elson-Morgan (G.M.6)	4.5		
Protein	Amino-acid analysis (G.M.8c)	3.7		
	Ninhydrin (G.M.8a)	6.1		
	(Total N-Amino sugar N) X 6.25	6.0		
Lipids (Free)	Solvent extraction (G.M.9)	2.2		
Lipids (Bound)	Acid hydrolysis, Solvent extraction (G.M.9)	2.7		
Total Nitrogen	Micro-Kjeldahl	1.34		
Total Phosphorus	Bartlett (G.M.10)	0.103	0.047	0.044
Ash	Incineration (G.M.12)	1.2		

preparative paper chromatography and identified as follows (Expt.2) :-
D - Glucose was identified by paper chromatography in Solvents a, b and c (G.M.22), paper electrophoresis in 0.05M - sodium tetraborate, pH 9.3, and 0.05M - sodium germanate, pH 10.7, buffers (G.M.23) and by reaction with glucose oxidase to give a product chromatographically identical to that obtained from authentic D - glucose; it was further characterised by acetylation with sodium acetate and acetic anhydride to give in high yield, the crystalline penta-acetate, identical with an authentic sample (Expt. 2B).
D - Galactose was identified by paper chromatography in solvents, a, b and c (G.M.22) and by reaction with D - galactose oxidase to give a product chromatographically identical to that obtained from authentic D - galactose [$R_{Galactose}$ = 0.33 in Solvent c (Expt.2C)] , and by reaction with D - galactose dehydrogenase (G.M.4a). The absence of a significant amount of L - galactose was indicated by the optical rotation ($[\alpha]_D + 80^\circ$) of the isolated sugars (Expt. 2C). It was further characterised by preparation in good yield of the crystalline methylphenylhydrazone, identical with an authentic sample (Expt. 2C).
D - Mannose was identified by paper chromatography in Solvents b, c and d (G.M.22) and paper electrophoresis in 0.05M - sodium tetraborate, pH 9.3, and 0.05M - sodium germanate, pH 10.7, buffers (G.M.23), and shown to be of the D - configuration from the optical rotation ($[\alpha]_D + 14.0^\circ$) of the isolated sugar (Expt.2E).
L - Rhamnose was identified by paper chromatography in Solvents b,c and d (G.M.22) and paper electrophoresis in 0.05M - sodium tetraborate, pH 9.3, and 0.05M - sodium germanate, pH 10.7, buffers (G.M.23), and shown to be of the L - configuration from the optical rotation ($[\alpha]_D + 8.2^\circ$) of the isolated sugar (Expt.2D)
Xylose and Arabinose were identified by paper chromatography in Solvents b, c and d(G.M.22) and paper electrophoresis in 0.05M - sodium tetraborate, pH 9.3, and 0.05M - sodium germanate, pH 10.7, buffers (G.M.23), in which they were readily distinguishable from hexoses and ribose, lyxose, and 2 - deoxy ribose. With the aniline hydrogen

phthalate spray (G.M.22b) they gave the cherry red colour, characteristic of pentoses.

Unidentified Pentose : Paper chromatography indicated a small amount of a sugar, which gave the cherry red colour of pentose with the aniline hydrogen phthalate spray (G.M.22b) and had an R_G value of 2.3 in solvent b, higher than rhamnose and different from ribose, arabinose, xylose and lyxose. This sugar was not identified further.

Amino Sugars : Amino sugars were determined with the Elson - Morgan reagent after hydrolysis of the cell walls with 5.7 N - hydrochloric acid for 18 hr., conditions optimal for chitin hydrolysis (Hackman, 1962). Interference caused by the reaction of neutral sugars with amino acids in the Elson - Morgan assay was overcome using the modification of Boas (1953), which involves separation of the neutral and amino sugars on columns of Amberlite 1R-120 (H^+) (G.M.6). The value obtained (4.5% anhydro hexosamine, equivalent to 5.7% of anhydro N - acetyl - hexosamine) was not corrected for destruction of hexosamine during the acid hydrolysis, and is therefore a minimum value.

D - Glucosamine : The only hexosamine detected was D - glucosamine, which was identified by paper chromatography in Solvents a and b (G.M.22). D - Glucosamine was isolated from cell wall hydrolysates by preparative paper chromatography and characterised as the crystalline hydrochloride, which was shown to be of the D - configuration from its optical rotation ($[\alpha]_D$ + 73°). It was further characterised by preparation in good yield of the N-benzyloxy-carbonyl derivative, identical with an authentic specimen (Expt.2A).

Total protein was determined by three methods :-

Method I was based on the ninhydrin-hydrindantin colouration (G.M.8a), after acid hydrolysis and destruction of hexosamine at pH 10.6, using a bovine serum albumin hydrolysate as standard (G.M.8a). This method gave a value of 6.1%.

Method II involved addition of the amounts of individual amino acids obtained from amino acid analysis (G.M.8c), but does not include values for cystine (or cysteine), methionine or proline, which were not determined. This method gave a value of 3.7%

Table I.2 Amino acid composition of Fusicoccum cell walls (1st Batch)

Amino acid	Wt.% Anhydro amino acid	Molar % of Total amino acids
Alanine	0.25	10.62
Arginine	0.70	13.52
Aspartic acid	0.38	9.93
Glutamic acid	0.33	7.70
Glycine	0.19	10.05
Histidine	0.12	2.71
Isoleucine	0.14	3.74
Leucine	0.24	6.40
Lysine	0.11	2.71
Phenylalanine	0.27	5.52
Serine	0.28	9.69
Threonine	0.20	6.03
Tyrosine	0.20	3.68
Valine	0.25	7.68

Method III was based on the approximation. Total protein = 6.25 X (Total Nitrogen - Hexosamine Nitrogen) and gave a value of 6.0%. Amino Acid Composition was determined after hydrolysis with 6N - hydrochloric acid hydrolysis (G.M.15B) using an automated analyser (G.M.8c). Values obtained for three hydrolysis times are given in Table 1 (Appendix). Where values were decreasing due to destruction of amino acid during acid hydrolysis they were extrapolated to zero time. In cases where the values were increasing with time the highest value was taken; in other cases an average value was calculated. Table I.2 shows the corrected amino acid composition.

Methionine, cysteine and cystine (which are very susceptible to acidic destruction) were not determined. It would be possible to determine them by preoxidation with performic acid followed by hydrolysis as usual (Hirs, 1956). The amino acid analyser used was not able to determine proline and hydroxyproline.

Indications from two dimensional thin layer chromatography on silica gel G using chloroform: methanol: 17% ammonia (2:2:1, 1st direction) and phenol: water (75:25 W/W, 2nd direction) showed the presence of proline, and the amino acid analysis of cell wall chitin showed the presence of methionine (Table IV. 3).

Lipids : (1) Readily extractable Lipids (2.2%) were those extractable by an ethanol: diethylether mixture and chloroform (G.M.9). (2) Bound lipids (2.7%) were those extractable by an ethanol:diethyl-ether mixture and chloroform after incubation with hydrochloric acid acidified ethanol:diethylether mixture at 50° (G.M.9).

Phosphorus : The cell wall phosphorus was 0.103% (1st Batch), 0.047% (2nd Batch and 3rd Batch) and 0.044% (4th Batch), equivalent to 0.340%, 0.154% and 0.145% of phosphate expressed as $NaPO_3$. respectively, determined according to G.M.10.

In the case of the 4th Batch approximately 48% of the total cell wall phosphorus was labile to dilute acid (N - hydrochloric acid, 15 min. at 100°, G.M.11).

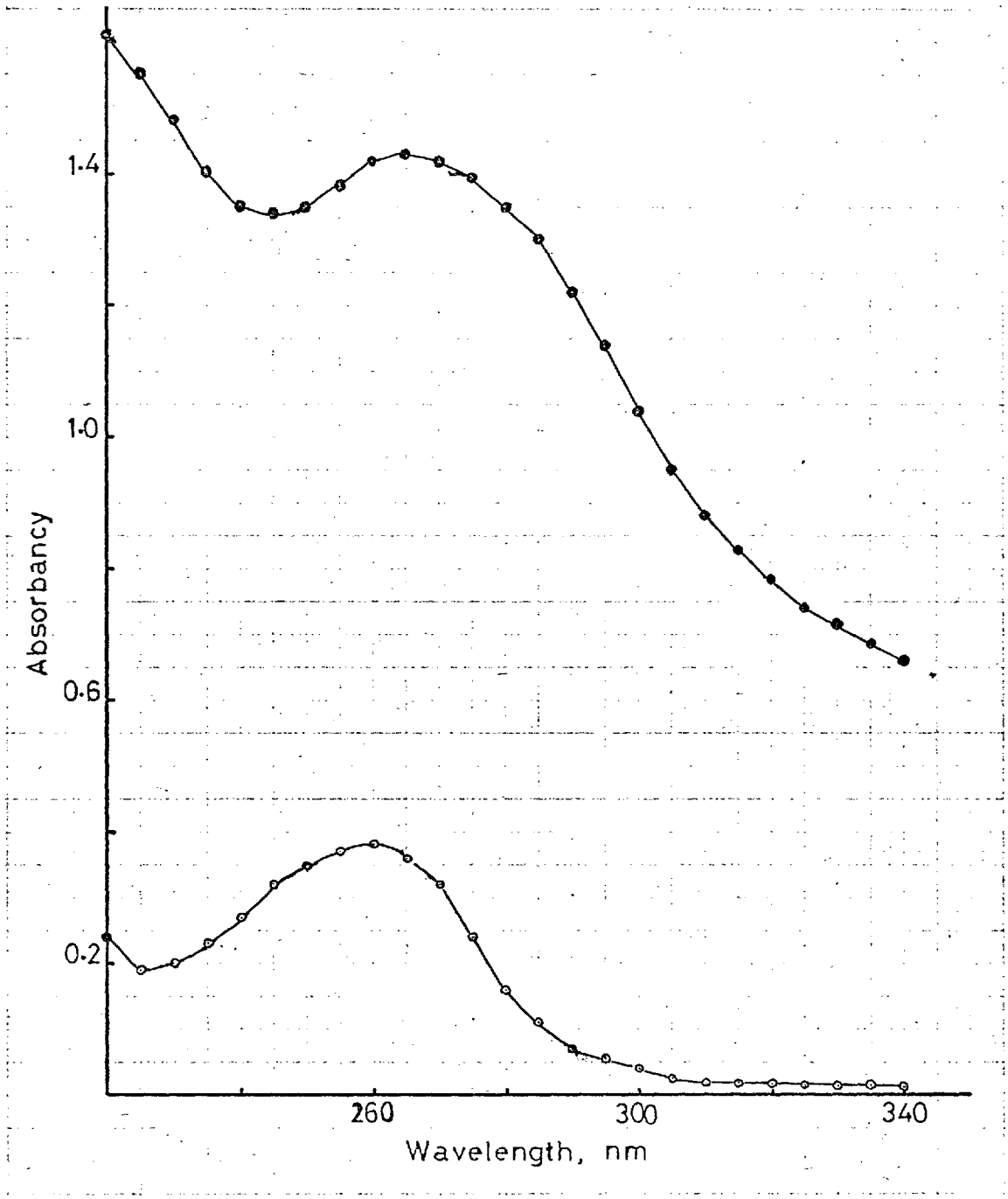


Fig. I.3. U.V. Spectra.

o , Cell walls (13.5mg.) were hydrolysed with 6N-HCl (2ml.) as described in G.M.15B, for 7hr. and then diluted 1:10 with 6N-HCl; o , Equimolar mixture of adenine, guanine, thymine, cytosine and uracil (0.00527mg./ml.) in 6N-HCl.

Ash : The amount of ash yielded on incineration of cell walls was 1.2%. The composition of ash was not determined, but phosphate would account for 0.34% (calculated as $NaPO_3$).

Nucleic Acids : Ultraviolet absorption spectra of 6N - hydrochloric acid hydrolysates of cell walls (λ_{max} 265 nm., Fig I.3) suggested the possibility of small amounts of nucleic acids in the cell walls. However the high absorption at 320 nm. indicated the presence of a large amounts of interfering substances, and this was confirmed by paper chromatography of 70% perchloric acid hydrolysates of cell walls, which revealed a continuous streak of ultraviolet absorbing material from the origin to the solvent front, under conditions where the five common nucleic acid bases are well separated (G.M.22c). By connecting the segments of the spectra of 6N - hydrochloric acid hydrolysates above and below 240 nm. with smooth curves, the ordinate value of these curves at 260 nm. may be approximated as background absorption as described by Bartnicki-Garcia and Nickerson (1962). The corrected optical density at 260 nm. may then be used to estimate nucleic acid base concentration using an extinction coefficient based on an equimolar mixture of adenine, guanine, cytosine and uracil. The value obtained (0.4%) must represent a maximum value due to the uncertainty of assessing the contributions of the interfering substances.

II. ACTION OF ENZYMES ON THE TOTAL CELL WALLS AND MYCELIUM

In order to study the chemical nature of the components of the cell walls the action of a number of enzymes was investigated.

A. Enzymes of the Snail, Helix pomatia (Expt.10)

The digestive enzymes of the snail, Helix pomatia, contain sulphatase, phospho-mono- and di-esterase, lipase, little or no protease and a wide range of carbohydrases (Margaret, Holden and Tracey, 1950; Anderson and Millbank, 1966), which include amylase, $\beta 1 \rightarrow 3$ glucanases and chitinase. Snail enzyme preparations are known to hydrolyse the β- glucan and chitin components of a large number of fungal cell walls (Chattaway, Holmes and Barlow, 1966; Mendoza and

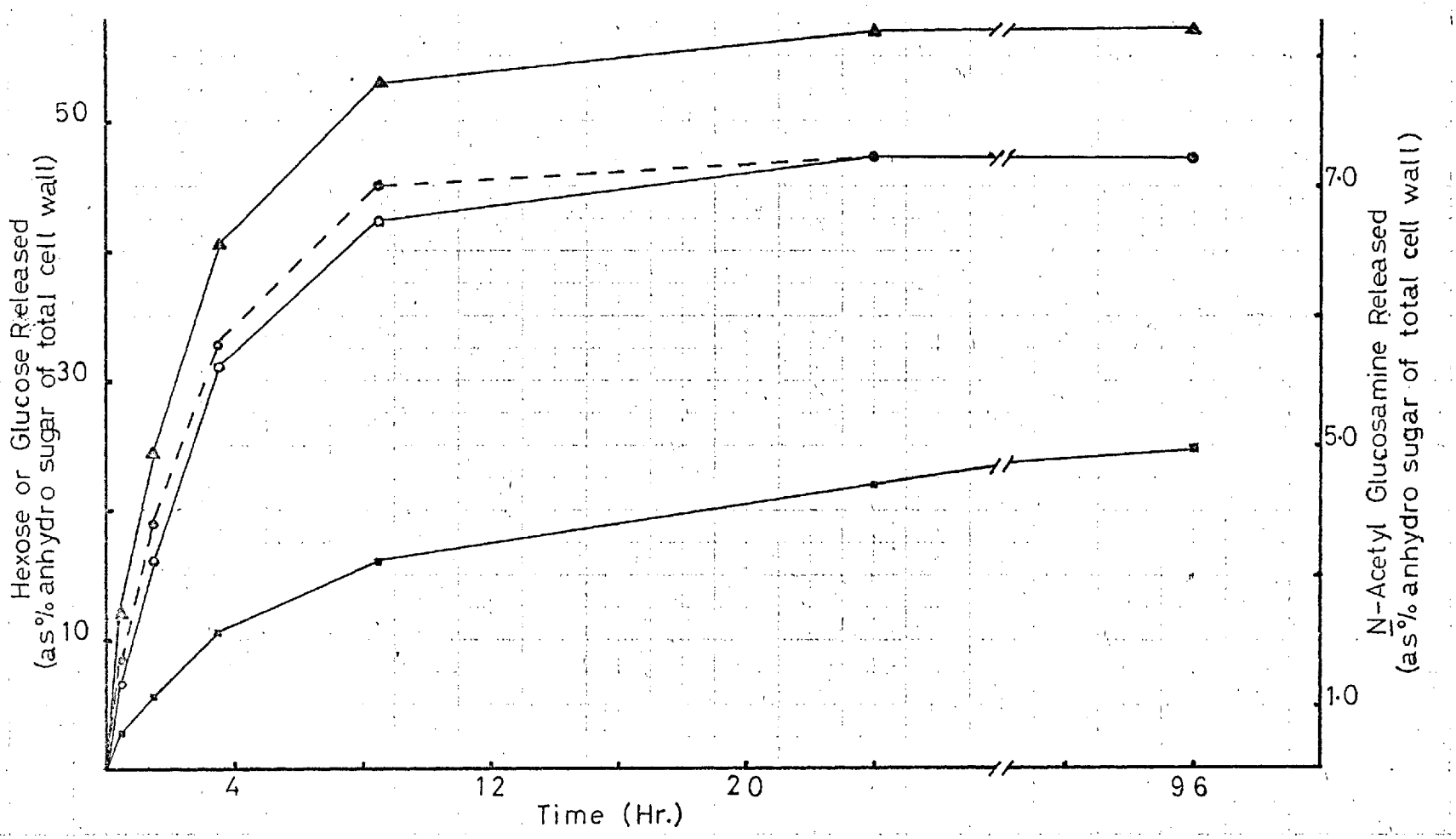


Fig. II.1. Hydrolysis of Cell Walls by Snail Enzyme Preparation.

Cell walls were incubated with snail enzymes and the soluble sugars released at different interval of times were determined as described in Expt. 10. ▲ ,Total Hexose in the supernatant; ● , Glucose after N-sulphuric acid hydrolysis of the supernatant; o , Glucose before N-sulphuric acid hydrolysis; ■ , N-Acetyl Glucosamine

Ledieu, 1968) and have been used widely in the preparation of fungal protoplasts (Aguirre and Villanueva, 1962; Mendoza and Ledieu, 1968). Since it has been found that protoplasts of Fusicoccum amygdali could be prepared by the action of snail digestive enzymes (G.T. Banks and A.G. Dickerson, unpublished results), it was of interest to know which components of the cell wall were attacked by the snail enzymes. The snail enzyme preparation used in this experiment contained inter alia, $\beta 1 \rightarrow 3$ glucanase activity 0.3 units/mg. (G.M.19), chitinase activity and amylase activities (Expt.4).

Cell walls were incubated with snail enzyme preparation as described in Expt.10 for 96 hr. and the amounts of total hexose, glucose (before and after N - sulphuric acid hydrolysis) and N - acetyl hexosamine liberated into the supernatant were determined and are shown in Fig.II.1 and Table II.1. Paper chromatography (Solvents b and d) of the supernatant after 24 hr. revealed only glucose and N - acetyl - D - glucosamine.

Table II.1. Rate of hydrolysis of cell walls by snail enzyme preparation.

Duration of incubation	Sugars released (as % anhydro sugar of total cell wall)			
	Total Hexose (G.M.1)	Glucose (G.M.3) before and after N-Sulphuric acid hydrolysis		N-Acetyl Hexosamine (G.M.5)
		Before	After	
30min.	12.0	6.5	8.3	0.56
1hr.30min.	24.5	16.2	18.9	1.15
3hr.30min.	40.5	31.0	32.8	2.12
8hr.30min.	53.1	42.3	45.0	3.20
24hr.	57.1	47.3	47.3	4.40
96hr.	57.1	47.3	47.3	4.97

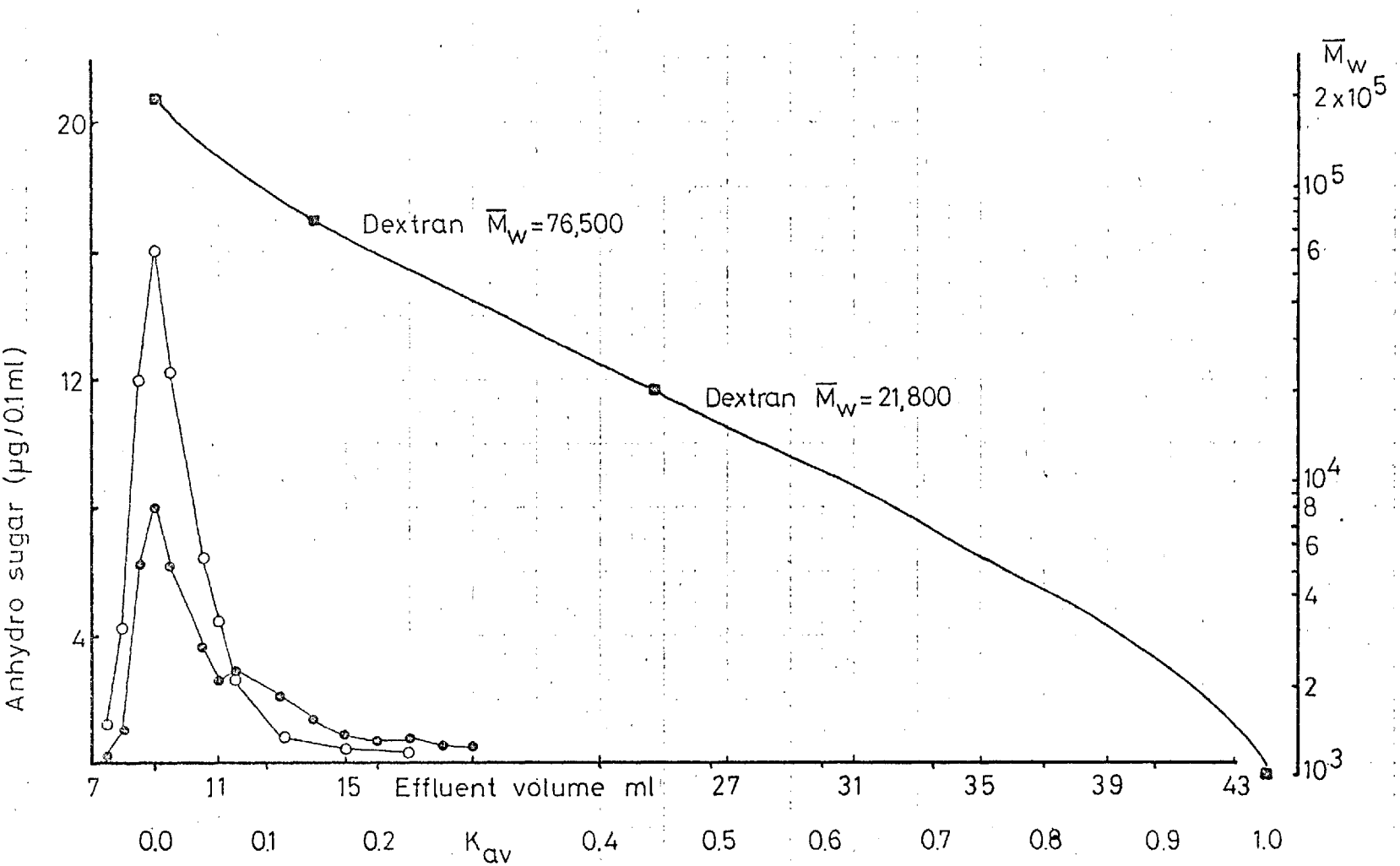


Fig. II.2. Elution Profiles and Molecular Weight Distribution Analysis on Sephadex G-200. ▪ , Molecular weight calibration curve; ○ , Blue dextran 2000; ● ,Non-dialysable heteroglycan released by Helix pomatia enzymes from total cell walls.

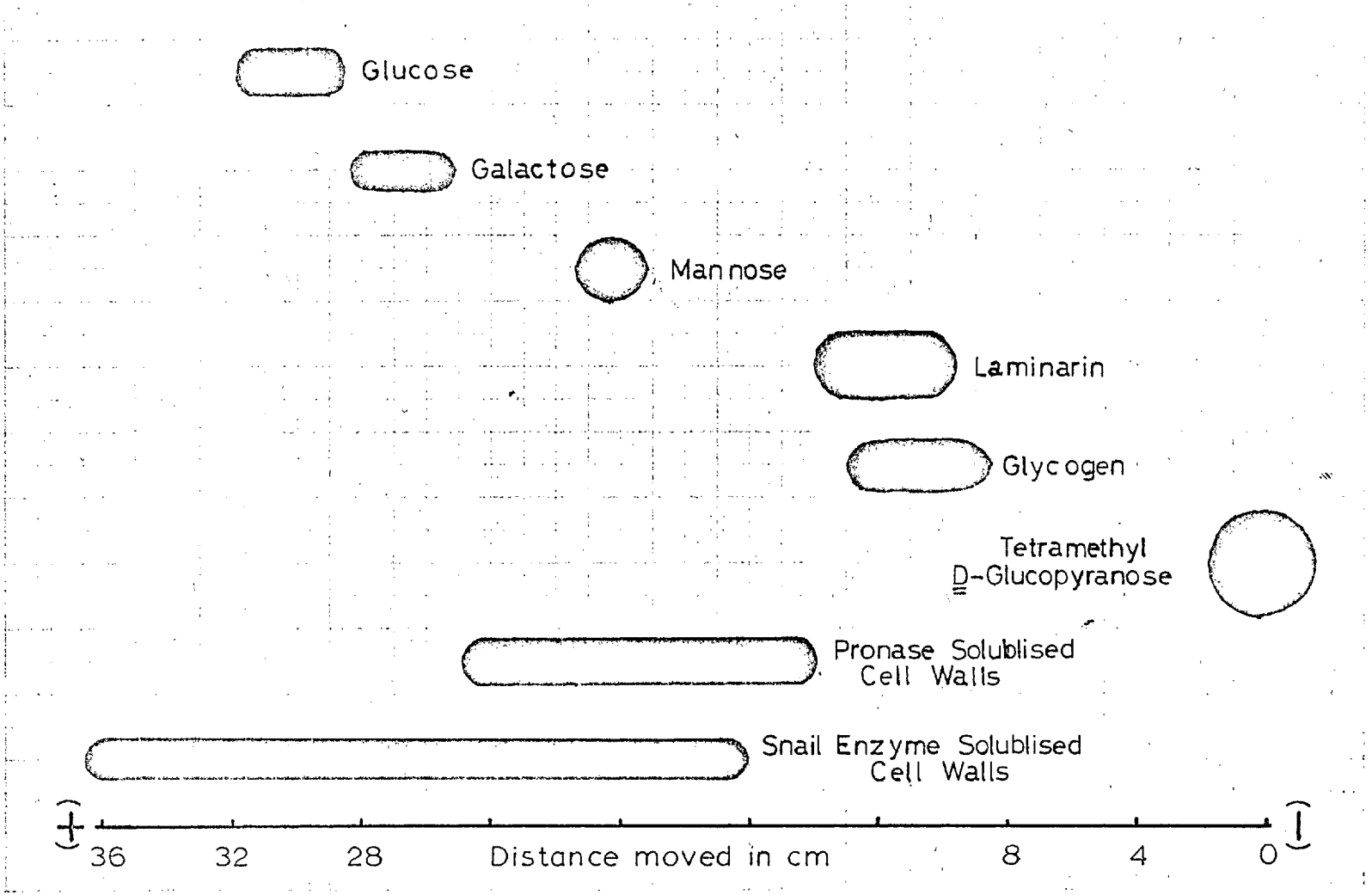


Fig. II.3. Glass Paper Electrophoretic Separation of Monosaccharides and Polysaccharides from Different sources.

Electrophoresis in 0.05M- sodium borate buffer was carried out at 58 volts/cm. for 35min. as described in G.M.23.

The higher value of total hexose compared with that of glucose suggested the presence of polymeric carbohydrate in the supernatant. Hence the supernatant was dialysed to remove glucose and N-acetyl glucosamine; the non-dialysable portion was found to contain polymeric material comprising 8% of the cell wall, composed of glucose, galactose, mannose, rhamnose and xylose. (Table II.2). Examination of N-sulphuric acid hydrolysates of the dialysable supernatant by paper chromatography

Table II.2. Percentage composition of sugars in the non-dialysable supernatant, as obtained from incubation of total cell walls with snail enzymes for 96 hr. (Expt.10)

Sugar	Per cent (expressed as anhydro sugar)	
	of the recovered material	of the total cell walls
Total Hexose (G.M.1)	70	5.60
Galactose (G.M.8)	39.2	3.14
Mannose (G.M.8)	26.5	2.12
Rhamnose (G.M.8)	14.6	1.17
Glucose (G.M.3)	5.6	0.45
Xylose (G.M.8)	Traces.	

(Solvents b and c) revealed in addition to glucose, and glucosamine found also before hydrolysis, traces of xylose and arabinose.

The elution profile of the non-dialysable heteropolysaccharide on Sephadex G-200 (Fig II.2) shows that the polymers were mainly excluded from the gel (M.wt. $>$ 200,000), although there was a small amount of lower molecular weight material.

Glass paper electrophoresis in 0.05M - sodium borate, pH 9.3, (Fig II.3) suggested that the material was heterogeneous, as the

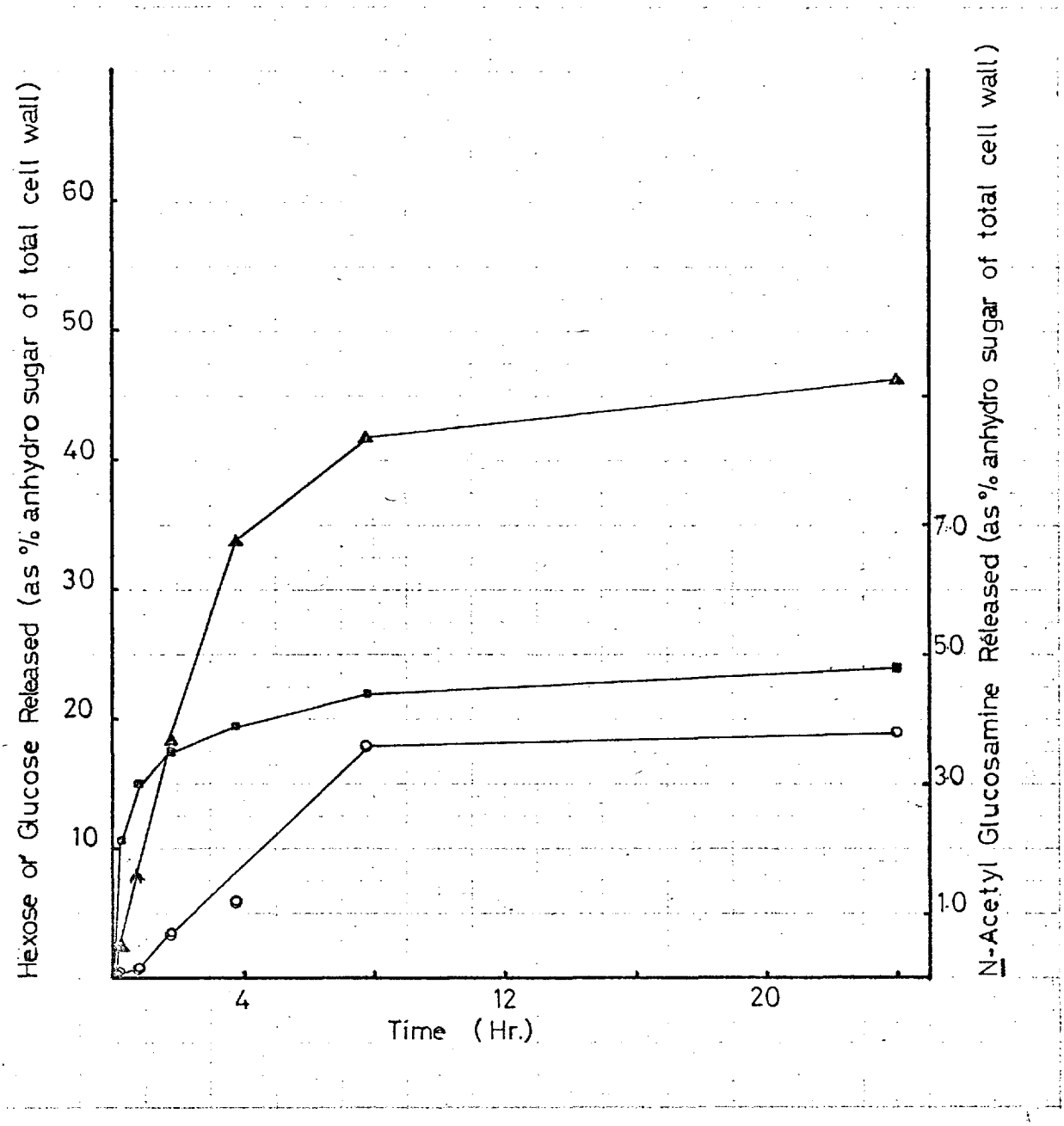


Fig. II.4. Hydrolysis of Cell Walls by Streptomyces Chitinase.
Cell walls were incubated with Streptomyces chitinase and the soluble sugars released at different interval of times were determined as described in Expt.11.
▲ ,Total Hexose; ■ , Glucose; o , N-Acetyl Glucosamine.

mobility was not discrete.

The water insoluble residual cell wall, which was resistant to further action of the snail enzymes, (30% of the total cell wall) contained 69.1% anhydroglucose and 72.4 % anhydro hexose; paper chromatography (Solvents b and c) of acid hydrolysates (G.M.15A and C) revealed in addition to glucose (main component), small amounts of galactose, mannose, xylose, arabinose and rhamnose and traces of glucosamine.

80% of the residue was soluble in N- sodium hydroxide. This soluble glucan had $[\alpha]_D$ + 212°, stained blue-black with iodine (but much less intensely than the original cell wall) and had an infrared spectrum almost identical to an Aspergillus niger cell wall $\alpha 1 \rightarrow 3$ - glucan, $[\alpha]_D$ + 260°, kindly given by Dr. I.R. Johnston (Fraction IV R, Johnston, 1965). The Aspergillus glucan did not however stain with iodine.

B. Chitinase, (Expt.11)

An inducible enzyme system obtained from culture filtrates of Streptomyces griseus (Reynolds, 1954) contains at least three types of chitinase activity, as had been shown by Berger and Reynolds (1958) using a fractionation method on starch bed zone electrophoresis followed by bauxite column separation. Two of the enzymes had activity on chitin and chitodextrins upto trisaccharides producing oligomers and the third had activity only towards chitobiose and chitotriose. The unfractionated enzyme system produced only N-acetyl-D-glucosamine from chitin.

Fusicoccum cell walls were incubated with Streptomyces griseus chitinase preparation as described in Expt. 11 and the amounts of total hexose, glucose and N-acetyl-D-glucosamine liberated in the supernatant at different intervals of time are shown in Fig II.4 and Table II.3.

Paper chromatography (Solvents b and c) of the dialysable supernatant after 24 hr. incubation revealed the presence of laminaribiose, gentiobiose, glucose and N-acetyl-D-glucosamine. The non-dialysable

Table II.3. Rate of hydrolysis of Fusicoccum cell walls by Streptomyces griseus chitinase. (Expt. 11)

Duration of incubation	Sugars released (as % anhydro sugar of total cell walls		
	Toal Hexose (G.M.1)	Glucose (G.M.3)	N-acetyl Glucos -amine (G.M.5)
15min.	2.5	0.4	2.1
45min.	8.0	0.7	3.0
1hr.45min.	18.5	3.6	3.5
3hr.45min.	33.8	6.0	3.9
7hr.45min.	41.9	18.0	4.4
24hr.	46.5	18.9	4.8

supernatant contained polymeric material (14% $^{w}/_{w}$ of original cell walls), which did not stain with iodine and which comprised glucose, galactose and mannose in the ratio 30:40:30, with a trace of arabinose and xylose.

The residual cell wall (47.5% $^{w}/_{w}$ of original cell wall), which was resistant to further attack by chitinase, still stained blue-black with iodine and contained 63.5% anhydro glucose and 69.2% anhydro hexose. Paper chromatography (Solvents b and c) of acid hydrolysates (G.M.15A and C) of this residue indicated the presence of glucose (main component), with small amounts of galactose, mannose and rhamnose, and traces of glucosamine, xylose and arabinose.

C. Basidiomycete QM806 Exo - β - D - (1→3) - Glucanase (Expt.12)

The action of this enzyme was studied in order to determine if the cell walls contain β1→3 D-glucopyranose linkages. β1→3 D-glucopyranose linked glucans are very widely occuring in fungal cell walls (Bartnicki-Garcia, 1968 and references cited therein). The enzyme used for this preliminary experiment was an acetone precipitate of

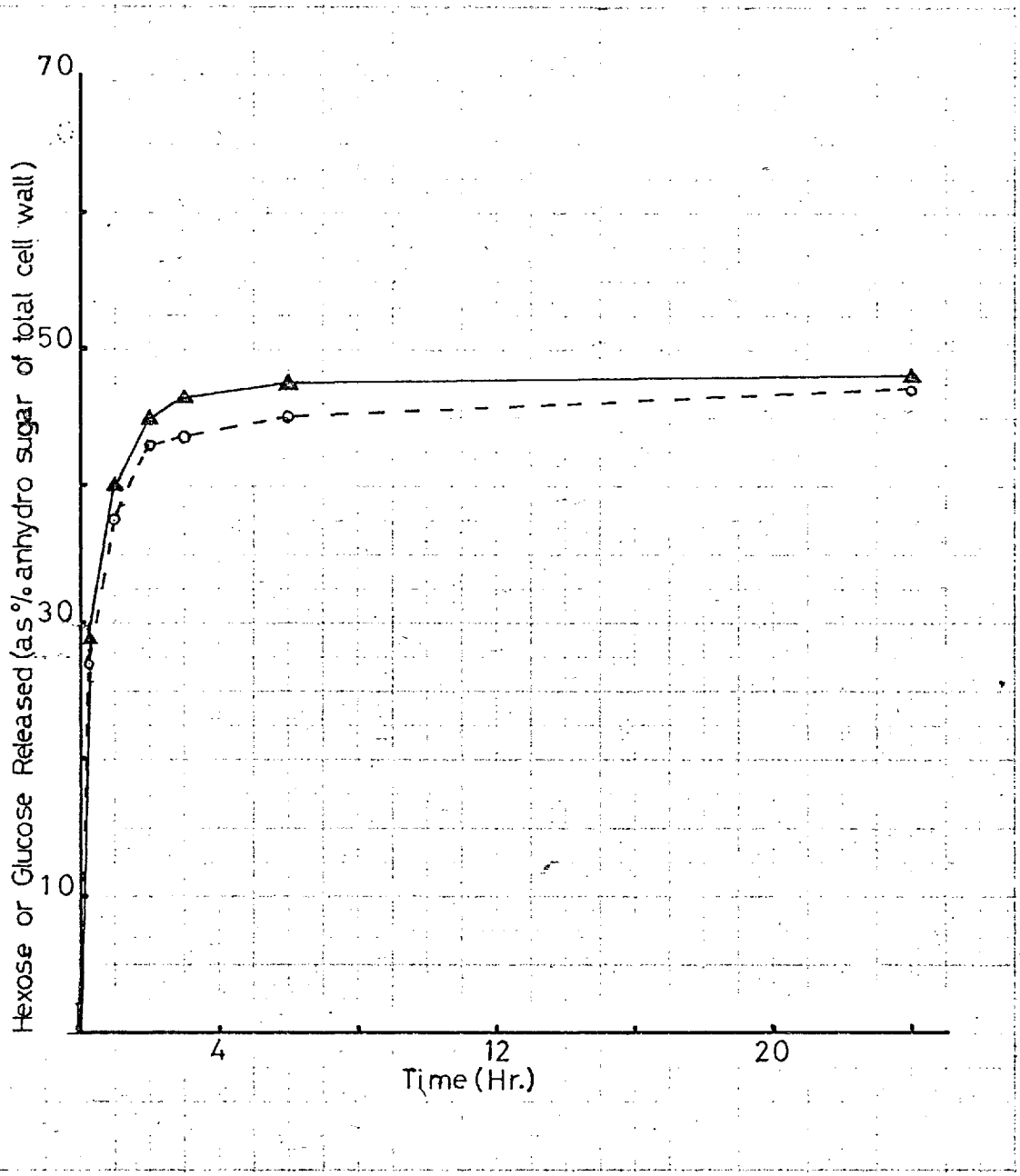


Fig. II.5. Hydrolysis of Cell Walls by Basidiomycete QM 806 Exo-β-D-(1→3)-glucanase.

Cell walls were incubated with Basidiomycete QM 806 exo β-D-(1→3)-glucanase and the soluble sugars released at different interval of times were determined as described in Expt.12.

▲ , Total Hexose; o , Glucose.

Basidiomycete QM806 culture filtrate (Expt.6), kindly given by Dr. E.T. Reese. Reese and Mandels (1959) had shown that the Basidiomycete QM806 β-D-(1→3)-glucanase enzyme was an exo enzyme and the crude acetone powder also contained traces of cellulase activity. The preparation was found to have activity towards starch and laminarin.

Cell walls were incubated with exo-β-D-(1→3)-glucanase for 24hr. as described in Expt. 12. The amounts of total hexose and glucose liberated at different intervals of time are given in Fig.II.5 and Table II.4. Liberation of soluble sugars by the enzyme was found to be almost complete within 6 hr. Paper chromatography (Solvents b and c) confirmed that glucose was the main sugar released; traces of gentiobiose and laminaribiose were also detected.

Table II.4 Rate of hydrolysis of cell walls by exo-β-D-(1→3)-glucanase

Duration of incubation	Sugars released (as % anhydro sugar of total cell wall)	
	Total Hexose (G.M.1)	Glucose (G.M.3)
15min.	29.0	27.0
1hr.	40.0	37.5
2hr.	45.0	43.0
3hr.	46.5	43.5
6hr.	47.5	45.0
24hr.	48.0	47.0

The residue after 24hr.'s incubation (40.5% $^{w}/_{w}$ of the original cell wall) contained 78% anhydro hexose, 57.6% anhydroglucose, 10.5% anhydroglucosamine and 4% protein (G.M.8a). Paper chromatography (Solvent b and c) of acid hydrolysates (G.M.15A and C) revealed the presence of glucose (main component) with amounts of galactose,

mannose, rhamnose, xylose and of arabinose in excess of those found in the original cell walls.

The residual cell wall still stained with iodine, and reincubation with fresh enzyme did not liberate any sugar or change the property of iodine staining.

D. ∝- Amylase (Expt.39)

It was of interest to determine if the characteristic iodine staining properly of cell walls was due to an amylose-like component. It had been shown that microcyst walls of Polysphondylium pallidum prepared by sonic treatment contain equal amounts of two glucans : cellulose and an alkali soluble glucan assumed to be glycogen (Toama and Raper, 1967). Glycogen like polysaccharides were also found in spore walls of Dictyostelium discoideum prepared by alkali extraction (Ward and Wright, 1965). The foregoing evidence of cell wall glycogen was equivocal and its occurrance may be explained in the same way as has been shown by Northcote (1953 and 1963), who demonstrated the occurrance of glycogen storage granules bounded by fine membranes to the yeast cell wall.

Cell walls were incubated with hog pancreatic ∝-amylase (free from cellulase, β 1→3 glucanase, dextranase, β 1→6 glucanase and β - glucosidase) for 24 hr. as described in Expt. 39. Examination of the supernatant by paper chromatography (Solvents b, f and g) indicated the presence of glucose maltose, maltotriose and traces of other oligomers. After $\underline{\underline{N}}$ - sulphuric acid hydrolysis (G.M.15A), in addition to glucose (main component), traces of galactose and mannose were detected. The supernatant was analysed for total hexose, glucose and reducing sugars and the results are given in Table II.5.

The cell wall residue, which was resistant to further attack by hog pancreatic ∝- amylase, did not stain blue black with iodine and had lost the rigidity as well as the apparent thickness of the original cell wall as observed under the microscope (Fig X.2p.123).

Table II.5 Action of hog pancreatic α - amylase on total cell walls

Cell wall preparation	Wt. of Cell walls taken (mg)	Sugars released (as anhydro glucose in mg.)			Residue (mg.)	Per cent recovery
		Total Hexose G.M.1	Reducing Sugar G.M.2	Glucose G.M.3		
1st Batch	500	42.0	24.3	13.0	420	92.4
4th Batch	500	48.6	28.0	15.0	430	95.7

Average DP of oligosacharides in the supernatant

$$= \frac{\text{Total Hexose-Glucose}}{\text{Reducing sugar-Glucose}} \quad \text{(all as anhydro)}$$

= 2.57 (1st Batch)

= 2.58 (4th Batch)

E. Proteases

The action of proteases on Fusicoccum cell walls was studied in order to determine if polysaccharide components were linked by peptide bonds (Eddy,1958; Cawley and Letters, 1968) and to aid in the fractionation of cell wall polysaccharides in order that the structure of individual components may be investigated.

(a) Pronase (expt.13A) : Pronase, a complex of proteolylic enzymes isolated from Streptomyces griseus, contains a wide range of proteases and also esterase activity (Nomoto and Narahashi, 1959). The enzyme mixture released 54% of the total carbohydrate and 38% of the total phosphorus from yeast cell walls as phosphoglycopeptide, where a large amount of the phosphorus is in the form of phosphodi-ester linkages (Cawley and Letters, 1968). It has also been used for the purification and fractionation of a glycopeptide in the ethylenediamine extract of yeast cell walls (Sentandreu and Northcote, 1968)

Exhaustive treatment of Fusicoccum cell walls with pronase (Expt.13A) solubilised a small portion of the original cell wall

(recovery of residue 87.2% of the original cell wall). The supernatant contained 5% of the original cell walls' total hexose (G.M.1) of which 0.4% was free glucose (G.M.3).The chemical composition of the non-dialysable fraction of the supernatant (4% of the original cell wall) is given in Table II.6. and that of the pronase resistant residue (87.2% of the original cell wall) in Table II.7. Paper chromatography of acid hydrolysates (G.M.15A) of the non-dialysable fraction of the supernatant in Solvent b revealed the presence of galactose, mannose and rhamnose with small amounts of glucose and xylose.

Table II.6. Composition of the non-dialysable fraction, as obtained from incubation of total cell walls with pronase (Expt. 13A)

Component	Method	Per cent
Total Hexose (anhydro)	G.M.1	50
Glucose (anhydro)	G.M.3	2.3
Total Phosphate (as $NaPO_3$)	G.M.10	0.66
Acid labile phosphate (as $NaPO_3$)	G.M.11	0.16

Table II.7. Composition of the residue after incubation of Fusicoccum cell walls with pronase (Expt. 13A).

Component	Method	Per cent
Total Hexose (anhydro)	G.M.1	83.3
Glucose (anhydro)	G.M.3	75.4
Protein	G.M.8a	2.0
Nitrogen	Micro-Kjeldahl	0.8
Total Phosphate (as $NaPO_3$)	G.M.10	0.11
Acid labile phosphate (as $NaPO_3$)	G.M.11	0.00

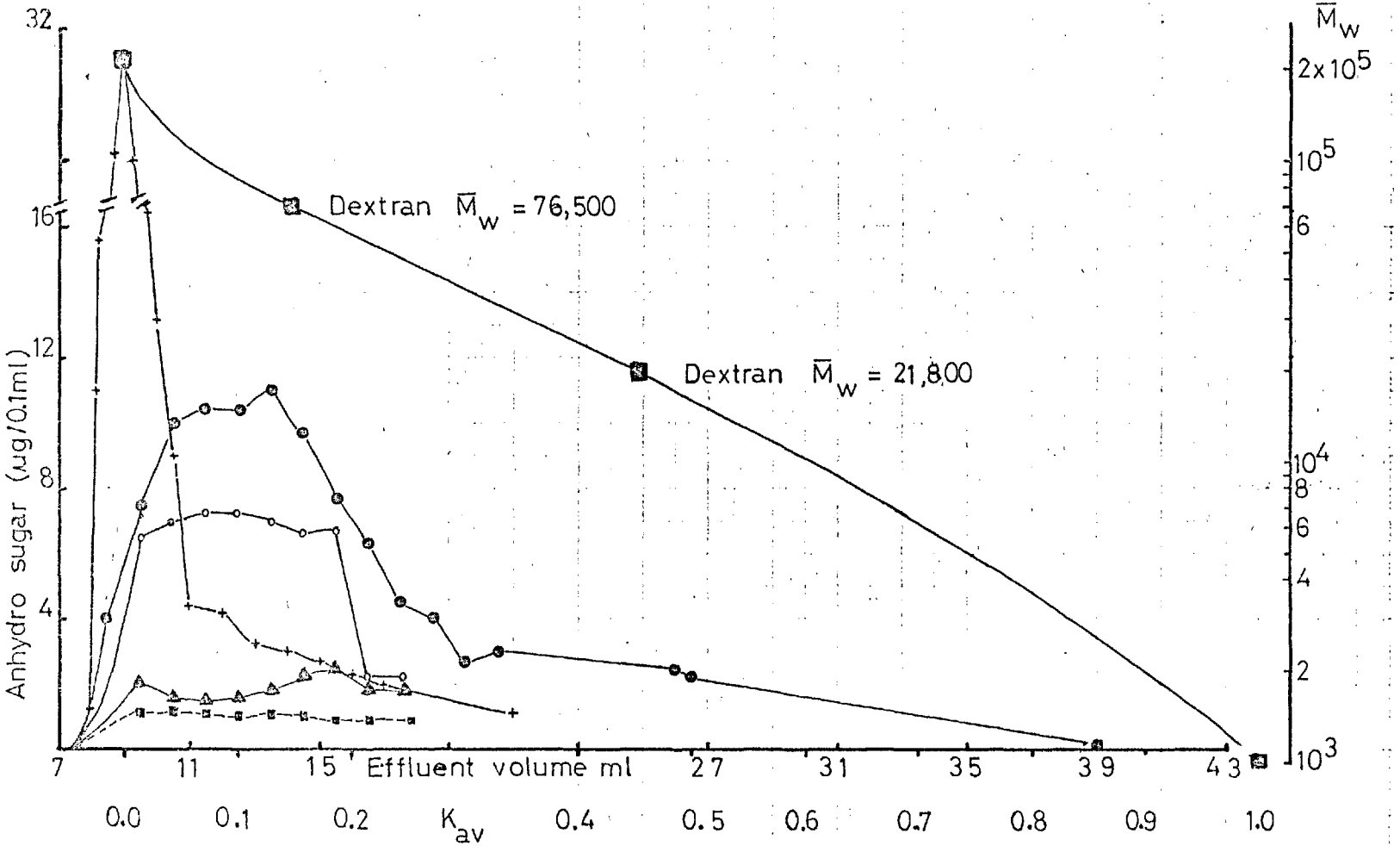


Fig. II.6. Elution Profiles and Molecular Weight Distribution Analysis on Sephadex G-200. ▣ , Molecular weight calibration curve; + , Blue dextran 2000; ● , Non-dialysable hetroglycan released by pronase enzyme;(o , Galactans of the hetroglycan; ▲ , Mannans of the hetroglycan; ■ , Rhamnans of the heteroglycan).

Table II.8. Percentage composition of sugars in different fractions of the non-dialysable water soluble polymer(s) liberated from Fusicoccum cell walls by pronase and fractionated on Sephadex G - 200.

Bed dimensions :- 1.4 x 28 cm. Eluant :- Water
Flow rate :- 4.5 ml./hr. Operating pressure :- 15 cm. water.
Sample volume :- 0.4 ml.(0.4% solution)

Carbohydrate composition was determined after N-sulphuric acid hydrolysis (G.M.15A) by paper chromatography in Solvent b (G.M.7).

Fraction No.	Volume of Eluant (ml.)		Total Hexose G.M.l	Approx.Percent composition of Sugar			
	Fraction	Cumulative		Galactose	Mannose	Rhamnose	Glucose
1	6.5	6.5	0.0	0.0	0.0	0.0	nil
2	1.5	8.0	9.0	-	-	-	-
3	0.5	8.5	21.0	-	-	-	-
4	1.0	9.5	75.0	65.5	21.2	12.3	tr.
5	1.0	10.5	100.0	70.1	17.3	12.6	tr.
6	1.0	11.5	105.0	73.0	15.6	11.4	tr.
7	1.0	12.5	105.0	73.5	16.3	10.2	tr.
8	1.0	13.5	110.0	70.1	18.2	11.7	tr.
9	1.0	14.5	97.5	66.9	22.6	10.5	nil
10	1.0	15.5	77.5	66.9	24.5	8.6	nil
11	1.0	16.5	62.5	43.9	37.0	19.1	tr.
12	1.0	17.5	45.0				
13	1.0	18.5	40.0	59.6	28.5	11.9	tr.
14	1.0	19.5	27.5				
15	1.0	20.5	30.0				
16	5.5	26.0	137.5				
17	0.5	26.5	11.25	-	-	-	-
18	12.0	38.5	-	-	-	-	-
19	0.5	39.0	0.5	-	-	-	-

(-) not determined

The non-dialysable fraction of the supernatant was applied to a column of Sephadex G-200 (G.M.25 and 27) and the compositions of the eluted fractions were determined and is shown in Table II.8. The elution profile is shown in Fig.II.6. This fraction was also examined by glass paper electrophoresis in 0.05M - sodium borate buffer, pH 9.3, (G.M.23) and the result is shown in Fig.II.3.

(b) ∝- Chymotrypsin (Expt.13B). ∝-chymotrypsin is one of the the major pancreatic proteolytic enzymes. Although it acts on a wide variety of peptide and ester linkages it preferentially hydrolyses bonds involving L -tyrosine or L -phenylalanine. (Desnuelle, 1960 and references cited therein).

Exhaustive treatment of Fusicoccum cell walls with ∝-chymotrypsin (Expt.13B) solubilised a small portion of the cell walls (91.1% of the original cell walls was recovered as insoluble residue). The supernatant contained 2.5% of the original cell walls' hexose of which 0.35% was free glucose. The chemical composition of the ∝-chymotrypsin resistant residue is given in Table II.9.

Table II.9. Composition of the residue after incubation of Fusicoccum cell walls with ∝-chymotrypsin (Expt.13B)

Component	Method	Per cent
Total Hexose (anhydro)	G.M.1	83.00
Glucose (anhydro)	G.M.3	73.00
Protein	G.M.8a	3.00
Nitrogen	Micro-Kjeldahl	0.85

F. Action of Enzymes on the Intact Mycelium of Fusicoccum amygdali (Expt.9)

The action of α- amylase and exo-β- D - (1→3)-glucanase on the intact mycelium was carried out in order to determine if the availability of the substrate was different to that in the isolated cell walls.

Cell walls of living mycelia stain black with iodine solution whereas the cytoplasm assumes the very light brown of the original iodine solution.

When living mycelium was treated with salivary α- amylase, after 2 hr. the cell walls no longer stained with iodine. Microscopic examination indicated that the mycelium retained its original shape.

When living mycelium was treated with Basidiomycete QM806 exo -β-D- (1→3) glucanase (which had amylase impurity), after 2 hr. the cell walls behaved as with salivary α-amylase.

Cotton blue staining in either case of enzyme treatment was the same as the untreated mycelium.

III FRACTIONATION OF CELL WALLS

The results already given indicated that the cell wall contained mainly carbohydrate (neutral sugars and hexosamine) with small amounts of protein and lipid. The enzyme experiments suggested that most of the hexosamine could be accounted for as chitin, that most of the neutral sugars other than glucose (i.e. galactose, mannose, rhamnose, xylose and arabinose) were in the form of a separate polymer(s), (which could be released by Helix pomatia enzymes) and that glucose was present as $\alpha 1 \rightarrow 4$, $\beta 1 \rightarrow 3$, $\beta 1 \rightarrow 6$ and $\alpha 1 \rightarrow 3$ glucopyranose linkages. Since glucans represented the major components of the cell wall, it was of major interest to separate the glucan components from the other cell wall components and to separate the individual glucans in order that their structures may be determined. The following fractionation experiments had these aims in view .

A. Deproteinisation with 90% Phenol (Expt. 13C)

90% Phenol is an excellent protein solvent and has been used for the preparation of bacterial Lipopolysaccharides (Westphal and Jann, 1965). 90% Phenol has also been used for the purification of human blood-group specific mucopolysaccharides from saliva, gastic juice, ovarian-cyst fluids or tissue digests, which contain unspecific protein and fatty substances (Morgan, 1965).

Fusicoccum cell walls were extracted with 90% phenol to determine if protein could be removed simply in this way and to see if the solubilities of the polysaccharide components were affected. The extraction was carried out as described in Expt. 13C. Although a small amount of material (about 10%) went into solution, the composition of the residue (Table III.1) was very similar to that of the original cell wall.

B. Fractionation of Cell Walls with Alkali

Alkali fractionation is one of the most commonly used methods for separating mixtures of polysaccharides (Johnston, 1965; Bacon, Jones, Farmer and Webley, 1968; Kreger, 1954). Since degradation of

Table III.1 Composition of the cell wall residue after 90% phenol extraction (Expt. 13C)

Composition	Method	Per cent
Total Hexose (anhydro)	G.M.1	80.0
Glucose (anhydro)	G.M.3	69.2
Nitrogen	Micro Kjeldahl	1.2
Protein	G.M.8a	5.0

carbohydrates can occur in alkaline solution either by erosion from the reducing end group [especially 1→3 linked polymers, which unlike 1→4 and 1→6 linkages do not require any rearrangement before chain elimination can proceed in oxygen free alkaline solution, (Corbett and Kenner & Richards, 1955)] or by oxidation (Whistler and BeMiller, 1958 and references cited therein), all fractionations were carried out after reduction of the reducing end group with borohydride and in an atmosphere of oxygen free nitrogen. That a degradation of yeast glucan did occur during extraction with alkali in the presence of air, was suggested by Bacon, Farmer, Jones and Taylor (1969) who showed that dissolution was hindered by exclusion of air or preliminary reduction with borohydride.

Evidence for a fractionation of Fusicoccum cell walls was based on chemical compositions of supernatant or residues, optical rotation, infrared spectra, X-ray diffraction patterns and iodine staining properties.

(a) N - Potassium hydroxide (Expt. 14A): Cell walls were extracted repeatedly with N - potassium hydroxide as described in Expt. 14A. This method of fractionation depends on both the solubility (of individual polysaccharides) and the rate of solubilisation [which depends on the accessibility and availability of polymers (Bacon, Davidson, Jones and

Taylor, 1966; Northcote, 1953; Bacon, Farmer, Jones and Taylor, 1969) as well as the hydrolysis of alkali labile linkages e.g. esters, which may link different polymers]. Table III.2 shows the total hexose extracted in each fraction and the composition of each fraction.

Table III.2 Composition of fractions obtained by cell wall fractionation with N - potassium hydroxide (Expt. 14A)

Fraction No.	Time of extraction (Total 30hrs.) Hr.	Total Hexose extracted (anhydro hexose as per cent of original cell wall) G.M.1	Per cent (expressed as anhydro sugar)				Per cent protein
			Total Hexose G.M.1	Glucose G.M.3	N-acetyl hexosamine G.M.5	Glucose liberated by Basidiomycete QM806 β-D-(1→3) glucanase G.M.3	G.M.8a
14 AA	3	25.2	76.0	64.0	-	34.7	6.6
14 AB	6	10.6	83.1	76.6	-	44.1	3.6
14 AC	15	3.8	86.0	85.0	-	47.0	-
14 AD	30	2.2	-	-	-	-	-
14 AE (insoluble residue)			78.0	70.0	10.4	41.0	2.9

- = Not determined

Examination of acid hydrolysates (G.M.15A) of the fractions by chromatography (Solvents b and c) indicated that all the fractions contained glucose (main component) with small amounts of galactose, mannose, rhamnose, xylose and arabinose. However fraction 3 contained a much smaller proportion of the sugars other than glucose (See also Table III.2). Extraction with 5N-potassium hydroxide did not solubilise a greater fraction of the cell wall.

(b) It was later found that a single extraction for 24 hr. with N-potassium hydroxide solubilised the same amount of cell wall polymers as the combined repeated extractions. In a preparative extraction (Expt.14B) it was found that 36.0% of the cell walls was recovered as alkali insoluble residue and 56.5% from the alkali soluble supernatant (overall 92.5% recovery). The alkali soluble fraction could be fractionated further on the basis of water solubility. When the alkali extract was neutralised, 20% (by weight of the original wall) remained in solution (but non-dialysable), while 36.5% (by weight of the original cell wall) was precipitated. All the fractions stained blue-black with iodine.

The composition of the fractions is given in Table III.3.

Table III.3 Composition of fractions obtained by cell wall fractionation with N-potassium hydroxide (Expt. 14B)

Fraction	$[\alpha]_D$	Per cent (expressed as anhydro sugar)			
		Total Hexose G.M.1	Glucose G.M.3	Galactose G.M.4b	Mannose G.M.7
14BA. Alkali soluble, water soluble	+15°	68.4	46.2	10.5	6.2
14BB. Alkali soluble, water insoluble	+136°	86.0	81.2	1.7	0.8
14BC. Alkali insoluble, water insoluble		77.4	69.6	3.1	1.5

Examination of acid hydrolysates (G.M.15A) by paper chromatography (Solvents b and c) showed the presence of all the minor neutral sugars in all the fractions (Table III.3). 55% of the cell wall galactose and 44% of the cell wall mannose was found in Fraction 14BA. Glucosamine was found mainly in Fraction 14BC, but traces were also found in both alkali soluble fractions, as determined by paper chromatography of

5.7N-hydrochloric acid hydrolysates (G.M.15C) using either the Elson-Morgan or silver nitrate sprays.

The sedimentation profile of fraction 14BB on Model E Analytical Ultracentrifuge using Schlieren optics (G.M.29) was very similar to that of Fraction 14FJ (Fig.VII.1) except that the amount of the faster moving component was higher than in Fraction 14FJ. The fraction (Fraction 14BB) has a similar elution profile on Sephadex G-200 as the Fraction 14FJ (i.e. they are both excluded).

(c). Effect of heating cell walls in 0.05M-sodium acetate buffer, pH 5.0, on potassium hydroxide fractionation (Expt.14C.). Bacon, Davidson, Jones and Taylor (1966) found that when the alkali insoluble fraction of pressed yeast was heated with 0.05M-sodium acetate buffer, pH 5.0, for 3 hr. at 75° most of it became alkali soluble and only 2% of the cell wall material remained insoluble; this consisted of 50% chitin and 50% glucan. Bell and Northcote (1950) and McAnally and Smedly-MacLean (1937) found a similar effect after prior heating with 0.5N-acetic acid at 75° and cold N-hydrochloric acid. Their suggestion that the resultant solubility in alkali was due to hydrolysis of glycosidic bonds would be unlikely to be significant in the sodium acetate buffer. Bacon, Farmer, Jones and Taylor (1969) suggested the presence of an enclosing semi-permeable membrane which in the intact yeast would allow the passage of mannan but not glucan on extraction with alkali. This membrane could be damaged by mild acid treatmant or ultrasonic ballistic disintegration, thus allowing the passage of glucan.

Although the Fusicoccum cell wall fragments would not be expected to be completely enclosed by a membrane, as open ends are formed during the preparation, it was nevertheless of interest to determine if the sodium acetate treatment had an effect on the alkali solubility of the Fusicoccum walls.

Heating Fusicoccum cell walls for 3 hr. in 0.05M-sodium acetate buffer, pH 5.0, at 75° (Expt. 14O) liberated 2% $^{w}/_{w}$ total hexose as assayed with the phenol/sulphuric acid reagent (G.M.1). When the residual

cell wall was fractionated with N-potassium hydroxide as described in Expt.14C, the amounts and compositions of the fractions obtained (Table III.4)were very similar to those obtained without prior sodium acetate treatment (e.g. Table III.3). All fractions stained blue-black with iodine solution. Traces of glucosamine were again found in the alkali soluble fractions.

Table III.4 Composition of fractions obtained by cell wall fractionation with N-potassium hydroxide, after prior heating with sodium acetate buffer, pH 5.0,(Expt. 14C).

Fraction	$[\alpha]_D$	Wt.% of original cell wall	Per cent (expressed as anhydro sugar)			
			Total Hexose G.M.1	Glucose G.M.3	Galactose G.M.4b	Mannose G.M.7
14CA.Alkali soluble, water soluble	+14°	16.2	67.7	46.1	10.0	6.0
14CB.Alkali soluble, water insoluble	+138°	35.1	86.6	82.9	1.7	0.7
14CC.Alkali insoluble, water insoluble		37.0	73.8	68.5	3.0	1.5

(d) Effect of pre-incubation of cell walls with pronase (Expt.13A) on N-potassium hydroxide fractionation (Expt.14D). Cawley and Letters (1968) have shown that pronase treatment solubilised 54% of the carbohydrate from isolated cell walls of Saccharomyces cerevisiae. In the case of Fusicoccum cell walls, pronase treatment solubilised only 5% of the total cell wall carbohydrate. It was of interest to determine if prior treatment of the cell walls with pronase affected the alkali solubility of the polysaccharide components.

The residue obtained after incubation of cell walls with pronase

(Expt.13A) was fractionated with N-potassium hydroxide as described in Expt.14D; the amounts and compositions of the fractions are given in Table III.5. All the fractions stained blue-black with iodine solution. It appeared that prior treatment with pronase increased the amount of water soluble material extracted.

Table III.5 Composition of fractions obtained by cell wall fraction with N-potassium hydroxide, after prior incubation of cell walls with pronase (Expt.14D)

Fraction	Wt.Per cent of		Per cent (expressed as anhydro sugar)		
	Original cell wall	Pronase resistant residue	Total Hexose G.M.1	Glucose G.M.3	Galactose G.M.4b
14DA.Alkali soluble, water soluble	27.5	31.5	78.2	69.0	4.5
14DB.Alkali soluble, water insoluble	27.5	31.5	89.8	88.0	1.5
14DC.Alkali insoluble, water insoluble	30.5	35.0	81.4	75.1	2.4

Examination of acid hydrolysates (G.M.15A and C) by paper chromatography (Solvent b and c) showed the presence of, in addition to glucose, all the minor neutral sugars in the same proportions relative to galactose as found in the original cell walls. Glucosamine was found mainly in the residual cell walls, but traces were again found in the soluble fractions.

The infrared spectra of all the fractions obtained by N-potassium hydroxide fraction had absorption maxima at 850 cm^{-1} and 890 cm^{-1} characteristic of α and β glucans respectively (Barker, Bourne and Whiffen, 1960 and references cited therein) very similar to unfractionated cell wall (Fig.1, appendix).

(e) Effect of prior heating with 2% hydrochloric acid on N-potassium hydroxide fractionation (Expt.14E). Houwink and Kreger (1953) found that when yeast cell walls were boiled with 2% hydrochloric acid, the glucan became alkali soluble. The alkali soluble glucan, which exhibited sharp X-ray diffraction patterns, demonstrating its rather high crystallinity, was termed hydroglucan. It was of interest to compare the same treatment on Fusicoccum cell walls [See also result IV B(a)].

When Fusicoccum cell walls were boiled with 2% hydrochloric acid for 2 hr. as described in Expt. 14E, about 64% was hydrolysed forming free glucose (56%) with traces of galactose, mannose, glucosamine and oligomers of R_G values 0.68, 0.55 and 0.42 as detected by paper chromatography (Solvent b).

The residual cell wall was fractionated with N-potassium hydroxide as described in Expt. 14E, and the compositions of the fractions obtained are shown in Table III.6. None of the fractions stained with iodine solution.

Table III.6 Composition of fractions obtained by cell wall fractionation with N - potassium hydroxide, after prior boiling with 2% hydrochloric acid (Expt. 14E).

Fraction	Wt.% of original cell wall	Per cent (expressed as anhydro sugar)		
		Total Hexose G.M.1	Glucose G.M.3	Glucosamine G.M.6
14EU. Acid treated residue	36.0	76	76	9
14EV. Alkali insoluble	10.8	36	31	34
14EW. Alkali soluble	25.2	84	84	-

- = Not determined.

C. Atempted Separation of Glucan and Heteroglycan Polymers in Cell Wall Fractions Soluble in N-Potassium Hydroxide.

Degradation of cell walls with Helix pomatia enzymes (p- 32) and liberation of a part of the heteroglycan polymers by pronase enzymes (p- 44) suggested that most of the cell wall galactose, mannose, rhamnose, xylose and arabinose was present as distinct polymer(s). Many polymers containing mannose have been separated and purified from other glycans by forming complexes with copper salts (Erskine and Jones,1956; Peat,Whelan and Edwards, 1961). Purification of xylans has also been achieved using copper salts (Adams and Castague, 1951). Barium hydroxide has also been used for the precipitation and purification of polysaccharides containing β-(1→4)-linked D-mannan residues and galactan containing a backbone of β-(1→4)-linked D-galactose residues (Meier, 1965 and references cited therein).

The basis of the methods, described in detail in Expt. 15, was (a) addition of barium hydroxide or Fehling solution to a solution of the cell wall fraction in alkali or water, (b) centrifuging off the precipitate (if any), (c) neutralisation of the supernatant (d) centrifuging off the precipitate (if any). The compositions of the resultant fractions are shown in Table III.7,8,9.

Table III.7 Composition of fractions obtained by fractionation of cell wall Fraction 14BB (N-potassium hydroxide soluble, water insoluble) with barium hydroxide.

Fraction	Wt.% of original Fraction	Per cent (expressed as anhydro sugar)	
		Total Hexose G.M.1	Glucose G.M.3
Starting material Fraction 14BB	100	88.7	85.7
Precipitate on addition of $Ba(OH)_2$	No significant precipitate formed		
Precipitate formed on neutralisation	71.8	92.0	90.0
Neutralised supernatant after centrifugation	15.0	83.3	81.0

Table III.8 Composition of fractions obtained by fractionation of cell wall Fraction 14BA (N-potassium hydroxide soluble, water soluble) with barium hydroxide.

Fraction	Wt.% of original Fraction	Per cent (expressed as anhydro sugar)	
		Total Hexose G.M.1	Glucose G.M.3
Starting material Fraction 14BA	100	64.7	45.5
Precipitate on addition of $Ba(OH)_2$	6.3	Not analysed	
Supernatant after neutralisation	70	64.4	50.0

No ppt. formed on neutralisation

Table III.9 Composition of fractions obtained by fractionation of cell wall Fraction 14BB (N-potassium hydroxide soluble, water insoluble) with Fehling solution

Fraction	Wt.% of original Fraction	Per cent (expressed as anhydro sugar)	
		Total Hexose G.M.1	Glucose G.M.3
Starting material Fraction 14BB	100	88.7	85.7
No precipitate formed on addition of Fehling solution			
Precipitate formed on neutralisation	65.5	90.0	88.0
Precipitate formed on dialysing neutralised supernatant	26.0	85.4	80.2
Dialysed neutralised supernatant	1.5	70.0	60.0

Although the precipitates formed on neutralisation contained less heteroglycan than the original fraction, it was found that the same effect

could be obtained merely by solution in alkali and precipitation on neutralisation. It appears that most of the cell wall heteroglycan polymer(s) could not be precipitated by barium hydroxide or Fehling solution.

D. Further Fractionation of Cell Wall Fraction 14BB with 8M-Urea [Expt. 15(c)] .

Urea solution has been stated by Burk and Greenberg (1930) to be a powerful solvent for starch. 8M-urea has been used to break up the aggregates of partially methylated cellulose by disrupting the hydrogen bonds thus bringing it into aqueous solution (Neely, 1961). It is known to protein chemists as a powerful agent for dissociation of proteins into subunits, and is presumed to act in reducing protein-protein interaction, thus increasing solubility (Woychik, Boundy and Dimler, 1961). Probabely the prime action of urea is that of competitive breaker of hydrogen bonds (Reithel, 1963 and references cited therein).

Cell wall Fraction 14BB was further fractionated on the basis of solubility in 8M-urea as described in Expt. 15(c). The amounts and composition of the fractions are given in Table III.10. All the fractions stained blue-black with iodine solution. Infrared spectra of both the fractions were similar as the original (i.e. they have both 850 cm^{-1} and 890 cm^{-1} absorption).

Table III.10 Composition of fractions obtained by fractionation of Fraction 14BB(N-potassium hydroxide soluble, water insoluble) with 8M-urea.

Fraction	Wt.% of original fraction	Per cent (expressed as anhydro sugar)	
		Glucose G.M.3	Galactose G.M.4b
Starting material Fraction 14BB	100	89.2	1.5
Fraction insoluble in 8M-urea	74	90.4	0.8
Fraction soluble in 8M-urea	17	88.2	2.2

E. Sonication of Cell Wall Fraction 14BB (N-potassium hydroxide soluble, water insoluble) [Expt.15(d)].

Bacon, Farmer, Jones and Taylor (1969) succeeded in dissolving a part of the originally alkali insoluble intact yeast cell glucan in alkali after ultrasonic treatment and also induced an originally insoluble part of yeast glucan to dissolve in dimethyl sulphoxide by ultrasonication. The reason suggested was disruption of a protecting membrane.

Cell wall Fraction 14BB was sonicated in water as described in Expt.15(d). The amounts and composition of fractions are given in Table III.11. The insoluble residue still stained with iodine, and had a very similar infrared spectrum to that of the original fraction.

Table III.11 Composition of fractions obtained by sonication of cell wall Fraction 14BB (N-potassium hydroxide soluble, water insoluble).

Fraction	Wt.% of original Fraction	Per cent (expressed as anhydro sugar)	
		Glucose G.M.3	Galactose G.M.4b
Starting material Fraction 14BB	100	89.2	1.5
Fraction 14BB, still insoluble in water	98	89.0	0.95
Fraction 14BB, material solubilised	Not analysed		

F. Dimethyl Sulphoxide Fractionation

Dimethyl sulphoxide is an excellent solvent for many polysaccharides. It was selected for the extraction of xylan (Bouveng, Garegg and Lindberg, 1960), and pine wood glucomannan (Meier, 1961) because of the

mild nature of the solvent. Dimethyl sulphoxide has also been used for the isolation of tissue glycogen (Whistler and BeMiller, 1962). It has a high dielectric constant, while being relatively free of the degradative effects of the more commonly used alkali solvents.

Cell walls or cell wall fractions were extracted with dimethyl sulphoxide (hot or cold) as described in Expt.16. Tables III.12 and III.13 show the amounts and compositions of the fractions obtained.

All the fractions obtained stained blue black with iodine, and their infrared spectra showed absorption maxima at 850 cm^{-1} and 890 cm^{-1}, characteristic of α and β glucans respectively and very similar to that of unfractionated cell walls.

Table III.12 Solubilities of cell wall preparations in dimethyl sulphoxide

Starting material	Time of extraction	Temperature	Per cent		$[\alpha]_D$ of soluble fraction in N-KOH
			(B) Insoluble	(A) Soluble	
A. Total Cell wall	24hr.	20-25°	80	20.0	+ 86°
B. " " "	5days	20-25°	78	20.4	-
C. 90% Phenol extracted cell wall residue (Expt. 13C)	5days	20-25°	88	11.6	-
D " " "	3hr.	60°	92	8.0	-
E. Pronase deproteinised cell wall (Expt.13A)	2days	20-25°	77	23.0	+126°
F. α-Chymotrypsin deproteinised cell wall (Expt.13B)	2days	20-25°	76	22.0	+102°
G. Fraction 14BB. (N-Potassium hydroxide soluble, water insoluble)	5days	20-25°	60	20.0	+ 73° +153° *

- = Not determined * = Residual fraction.

Table III.13 Carbohydrate composition of fractions obtained by fractionation of cell wall preparations with dimethyl sulphoxide (Table III.12).

Fraction	Per cent (expressed as anhydro sugar)			
	Total Hexose G.M.1	Glucose G.M.3	Galactose G.M.4b	Mannose G.M.7
16AA. Soluble	74.2	62	3.4	2.7
B. Insoluble	81.0	73.0	3.8	3.0
16CA. Soluble	83.7	71.8	3.2	2.7
B. Insoluble	79.2	70.0	3.2	3.0
16EA. Soluble	75.4	66.0	1.8	2.0
EB. Insoluble	85.0	79.9	3.4	1.8
16FA. Soluble	75.4	67.1	1.9	2.2
FB. Insoluble	86.6	79.9	4.2	2.0
16GA. Soluble	78.4	71.0	2.6	1.3
B. Insoluble	89.0	85.4	2.3	1.3

G. Ethylenediamine Fractionation.

Ethylenediamine has been used in the isolation of glycopeptides from yeast cell walls (Korn and Northcote, 1960; Sentandreu and Northcote, 1968). It has the advantage as a polysaccharide solvent of causing less degradation than aqueous alkali.

Cell walls were extracted with ethylenediamine and further separated on the basis of water solubility as described in Expt.17. Tables III.14 and III.15 show the amounts and composition of the different fractions obtained.

Table III.14 Ethylenediamine fractionation of Fusicoccum cell walls.

Fraction	Per cent of original cell wall	$[\alpha]_D$ of soluble fraction in N-KOH
17 A. Ethylenediamine soluble, water soluble	20	-25°
17 B. Ethylenediamine soluble, water insoluble	15	$+120^\circ$
17 C. Ethylenediamine insoluble, water insoluble	60	

Table III.15 Carbohydrate composition of different fractions obtained by fractionation of Fusicoccum cell walls with ethylenediamine.

Fraction	Per cent (expressed as anhydro sugar)		
	Total Hexose G.M.1	Glucose G.M.3	Galactose G.M.4b
17 A.	63.3	50.4	4.7
17 B.	78.5	70.2	3.7
17 C.	83.2	75.0	3.1

All the fractions obtained stained blue-black with iodine and their infrared spectra had absorption maxima at 850 cm^{-1} and 890 cm^{-1}, characteristic of α and β glucan respectively, and very similar to original cell wall, except Fraction 17A, in which the β absorption peak at 890 cm^{-1} was more intense than the others. Fractions 17A and 17B contained small amount of glucosamine as detected by hydrolysis with

5.7N-hydrochloric acid (G.M.15c) and paper chromatography (Solvent b).

H. Chloral Hydrate Fractionation

Aqueous chloral hydrate is an excellent solvent for starch, and treatment with the solution has a negligible effect upon the blue value of potato starch. It has been used for the extraction of starch from the protozoan, Polytomella coeca (Bourne, Stacey and Wilkinson,1950), and for extraction of polysaccharides of the amylopectin type from Clostridium butyricum, Cycloposthium, and Holorich ciliates (Bourne and Weigel, 1965 and references cited therein). It also readily dissolves gum arabic (Mauch, 1901).

Since the cell wall preparations of Fusicoccum stained blue-black with iodine and contained α-D-(1$\rightarrow$4)-glucopyranose linkages, which were attacked by α-amylase producing maltose and maltotriose (result II D), it was of importance to determine whether the α1$\rightarrow$4 linked glucan is an integral part of the cell wall. If it were a starch contaminent from the cytoplasm or medium it should be readily soluble in chloral hydrate.

Cell walls were extracted with 33% (w/v) chloral hydrate at 100° as described in Expt. 18. The results are given in Table III.16.

Table III.16 Chloral hydrate fractionation of cell wall preparations.

Starting material	Per cent (by weight of original fraction)	
	Insoluble	Soluble
Total cell wall	87	10
Fraction 14 FJ	93	4.0

The insoluble residue from the total cell wall and Fraction 14FJ cell wall preparation stained blue-black with 0.1N-iodine solution. All the soluble fractions stained light brown with iodine. N-Sulphuric acid hydrolysis (G.M.15A) of the soluble fraction from the total cell wall

Fig. III 1 Fractionation of Cell Walls for Structural Studies

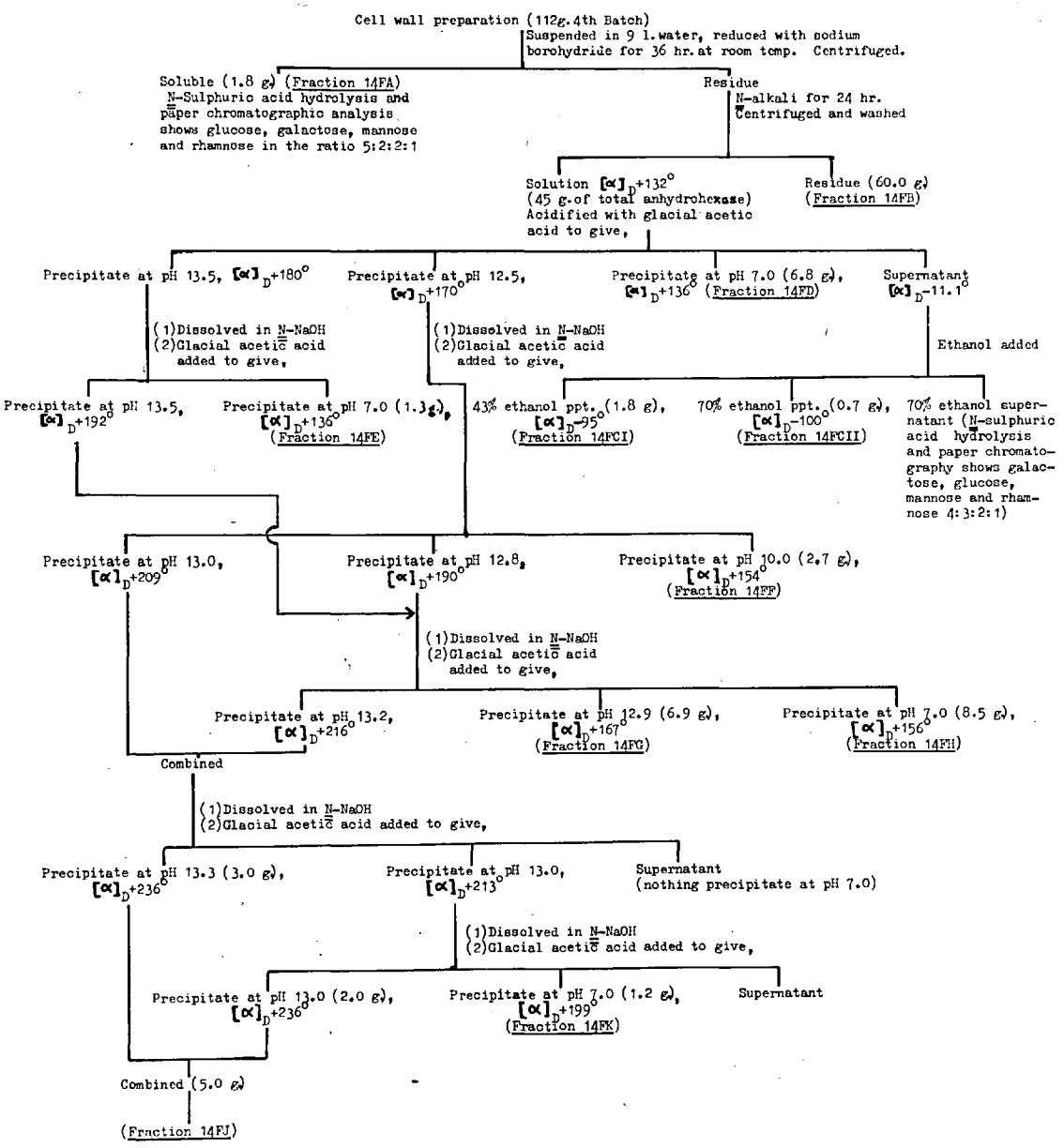


Fig. III.1. Fractionation of Cell Walls for Structural Studies. (For detail see Expt. 14F.).

preparation, followed by paper chromatography (Solvent c) showed mainly galactose, mannose, glucose, rhamnose, xylose and arabinose; in the case of Fraction 14 FJ glucose was the main product of hydrolysis of the chloral hydrate soluble fraction. The infrared spectra of all the soluble fractions gave both the absorption peaks at 850 cm^{-1} and 890cm^{-1} characteristic of α and β linkages; the peak at 890 cm^{-1} was more prominent than in the original cell wall, indicating that β-glucans were preferentially extracted.

J. Fractionation of Cell Walls for Structural Studies (Expt.14F)

Cell wall fractions obtained by potassium hydroxide fractionation (Expt.14F) were fractionated further prior to structural studies. Cell wall fractions soluble in water, could be further fractionated on the basis of solubility in ethanol-water mixtures of varying composition. Fractions soluble in alkali, but insoluble in water could be further fractionated by solution in alkali followed by stepwise precipitation at decreasing pH's. The procedures are described in Expt. 14F and are summerised in Fig. III.1.

The sedimentation profile of Fraction 14 FJ (Fig. VII.1) when examined with the Model E Analytical Ultracentrifuge using Schlieren optics (G.M.29) showed two components with S^{o}_{20} values 6.6S and 2.8S, very similar to that of Fraction 14BB except that the faster moving peak in the former one was smaller in size than the latter one. The infrared spectrum of Fraction 14FJ shows absorption maxima at 850 cm^{-1} and 890 cm^{-1} (i.e. α and β-glucopyranose linkages) very similar to that of Fraction 14BB, except that the βpeak in Fraction 14BB was larger than the β peak in Fraction 14FJ (Fig. 1. appendix).

Further fractionation of Fraction 14FJ (whioh had $[\alpha]_D$ + 236°) with alkali (as described in the Fig.III.1) was not pursued as the recovery goes down in each step of precipitation from alkali solution with acetic acid; in view of the repeated alkali fractionations already carried out it was unlikely that complete separation of the two components would have been obtained by this method. Further fractionation using

enzymic methods and structural studies of the products obtained were described in Sections VII, VIII and IX.

K. Further Fractionation of Cell Wall Fraction 14FCI and 14FCII with Iodine Solution (Expt. 26)

In aqueous solution at neutral pH 7, the amylose molecule forms a a random coil, which consists of linear segments of helical structure, stiffened by hydrogen-bonds and it forms inclusion complexes (e.g. with iodine) only while the hydrogen-bonds exist between the primary and secondary hydroxyl groups in neighbouring turns. On the basis of this type of complex formation, separation and fractionation of amylose from other polymers as well as fractionations of amylose according to the degree of polymerisation have been achieved (Hollo and Szeitli, 1968 and literature cited therein).

Cell wall Fractions 14FCI (1.8g.) and 14FCII (0.7g.) were combined (2.5 g.) and fractionated with iodine solution to give mainly two fractions (Expt.26). The one which precipitated with iodine, Fraction 26U (800 mg.), had $[\alpha]_D$ + 40°, and the other, Fraction 26W (1.0g.), isolated by ethanol precipitation of the supernatant, had $[\alpha]_D$ -110°. With iodine solution Fraction 26U gave a deep blue colour, similar to that given by amylose, while the Fraction 26W gave a very light blue colour.

The optical rotations of these fractions, $[\alpha]_D$ + 40° and $[\alpha]_D$ -110°, compared with those of cell wall Fractions 14FCI and 14FCII, $[\alpha]_D$ -100°, suggest that a further amount of laevorotatory material remained in the supernatant after ethanol precipitation.

Both Fraction 14FCI and Fraction 26W gave a single peak of the same area when examined at a concentration of 10mg./ml. with Schlieren optics in a Model E Analytical Ultracentrifuge at 40,000 r. p.m. (G.M.29), indicating that the two constituent components of Fraction 14FCI and 14FCII had the same sedimentation constant.

L. Search for Nigeran in Cell Wall Fraction 14 FJ.

Cell wall Fraction 14 FJ was extracted with hot water as described in Expt.25. Only 0.2% $^{w}/_{w}$ of soluble material could be recovered from

the supernatant, indicating that the cell walls did not contain a significant amount of nigeran.

IV. CHARACTERISATION OF HEXOSAMINE CONTAINING POLYMERS

A. Search for Chitosan in the Total Cell walls (Expt.19)

Chitosan is a partially or non-N-acetylated form of chitin. The occurrence of natural chitosan in fungi was first reported by Kreger (1954) in the cell walls of the mycelium and sporangiophores of Phycomyces. Natural chitosan was isolated and identified (van Wisselingh test for chitosan, chitosan sulphate test and X-ray diffraction) by Bartnicki-Garcia and Nickerson (1962) in the mycelial and yeast walls of Mucor rouxii.

Fusicoccum cell walls were extracted with N-hydrochloric or N - acetic acid and soluble material was isolated as described in Expt. 19. The following evidence indicated that the extract did not contain chitosan .

(a) It gave a negative van Wisselingh test for chitosan (Tracey, 1955). The original cell walls and the extract gave a blue black and a brownish grey colour on addition of 0.2% iodine in aqueous potassium iodide solution respectively, which did not change on addition of 1% sulphuric acid. Chitosan, prepared from crustacean chitin (Expt.21B) by de-N-acetylation with alkali, gave a brown colour with iodine/potassium iodide solution, which turned, red-violet to blackish on acidification with sulphuric acid.

(b) The extract was soluble in both N - sodium hydroxide, and 0.1N - acetic acid. Chitosan is insoluble in N - sodium hydroxide.

(c) Examination of acid hydrolysates of the extract (G.M.15A and c) by paper chromatography (Solvents a and b) showed the presence of glucose, galactose and mannose with only traces of glucosamine.

B. Chemical Isolation of Cell Wall Chitin (Expt.20)

(a) Cell wall chitin was isolated using a procedure based on that of Hotwink and Kreger (1952) and Kreger (1954), a method which has

Fig.IV.1 Preparation of Chitin from Fusicoccum cell walls (Expt.20A)

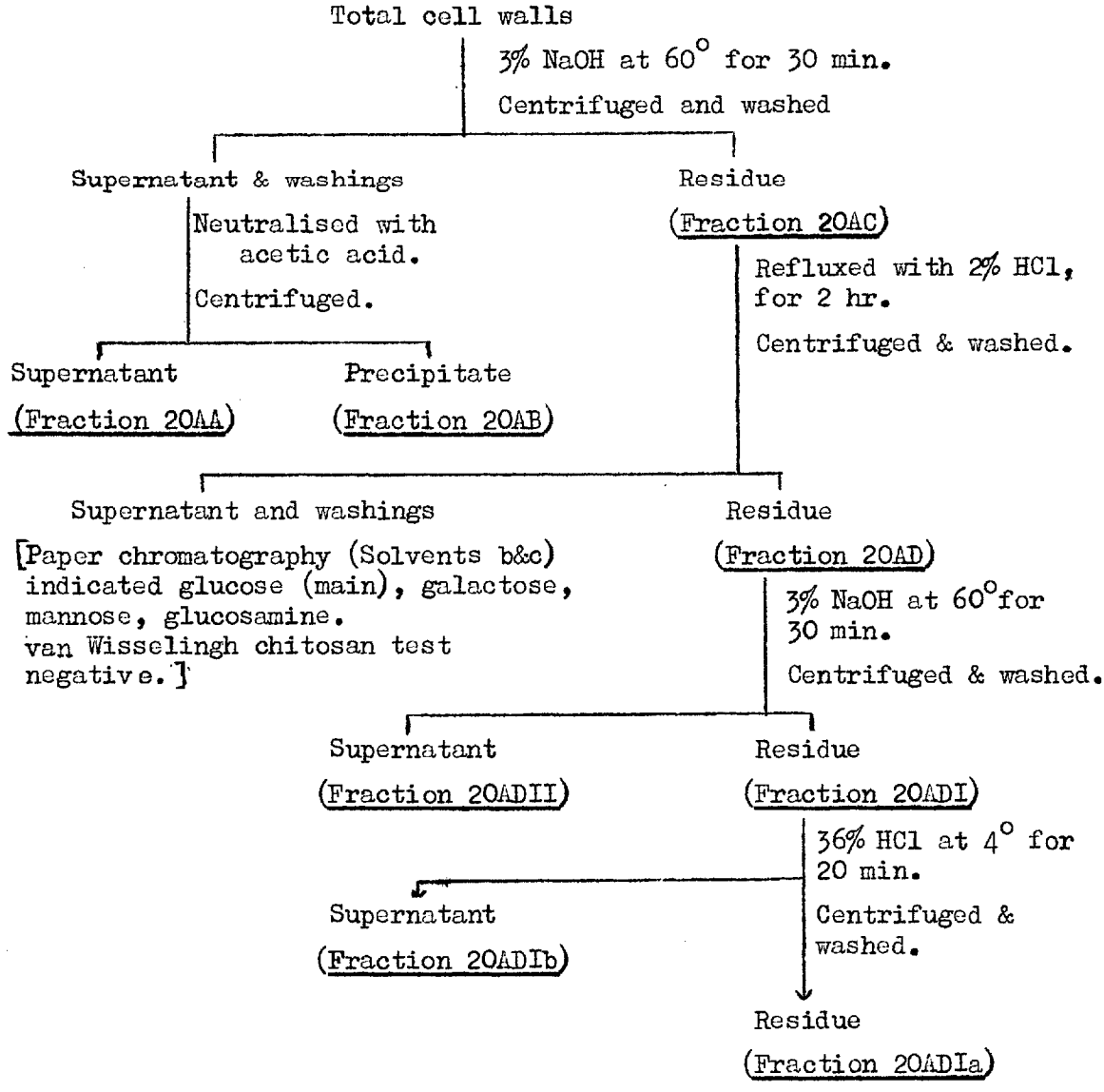


Table IV.1 Composition of fractions at various stages of chitin preparation from Fusicoccum cell walls.

Fraction	Wt. % of original cell wall	Per cent (expressed as anhydro sugar)				Per cent Phosphorus
		Total Hexose G.M.1	Glucose G.M.3	Glucosamine as N-acetyl G.M.6	N-acetyl D-glucosamine liberated by chitinase G.M.5	G.M.10
Total cell walls	100.0	79.2	69.2	5.7	4.8	0.047
20AA	6.8	71.4	36.0	<1	-	0.052
20AB	27.0	87.5	81.2	<0.5	-	0.013
20AC	60.0	79.8	69.6	6.5	-	0.010
20AD	25.0	70.0	67.5	9.0	2.5	-
20ADI	7.0	41.3	32.8	34.0	9.0	-
20ADII	12.5	91.9	88.7	-	-	-
20ADIa	1.9	-	-	-	-	-
20ADIb	2.4	-	-	-	18.0	-

(-) Not determined.

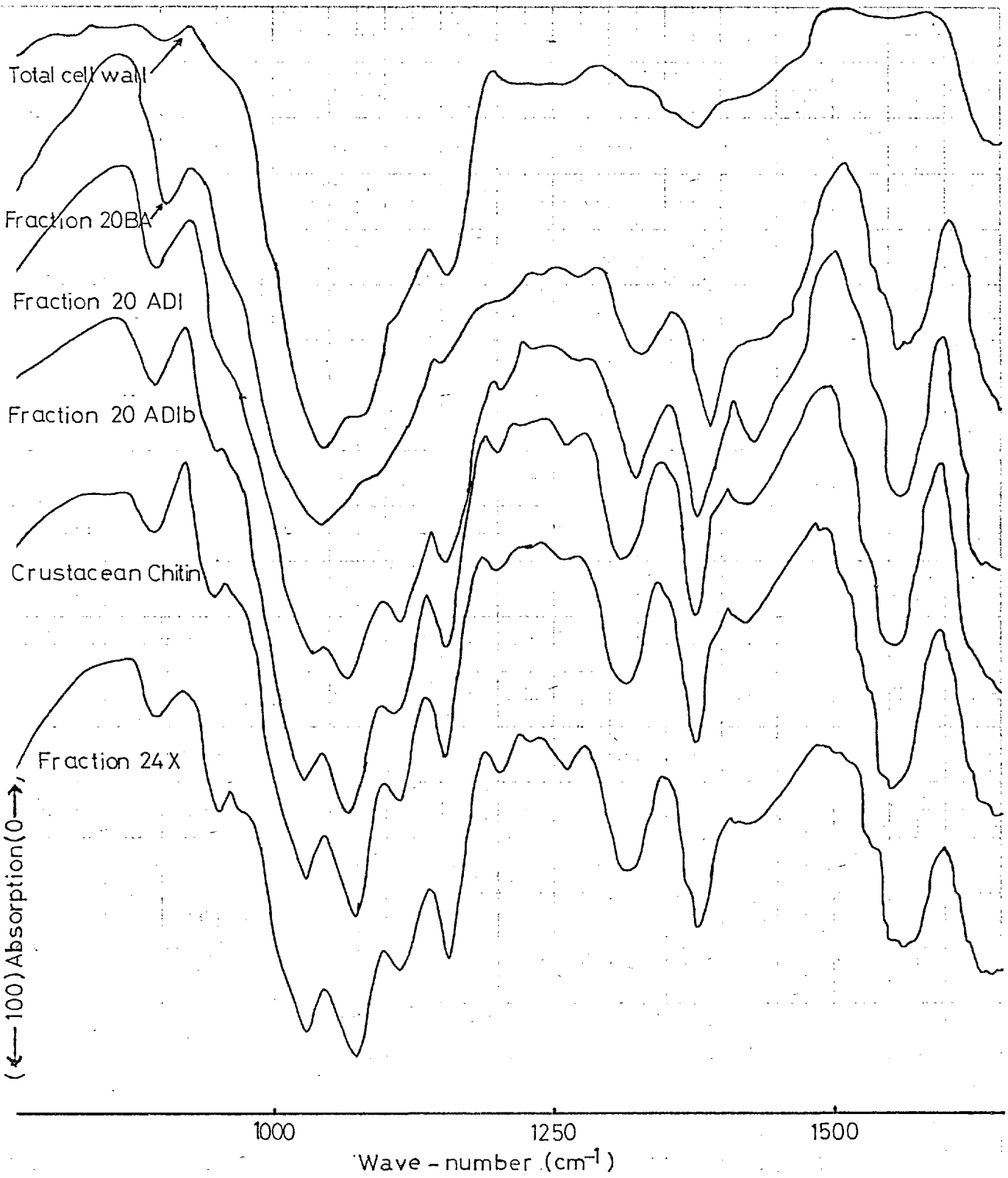
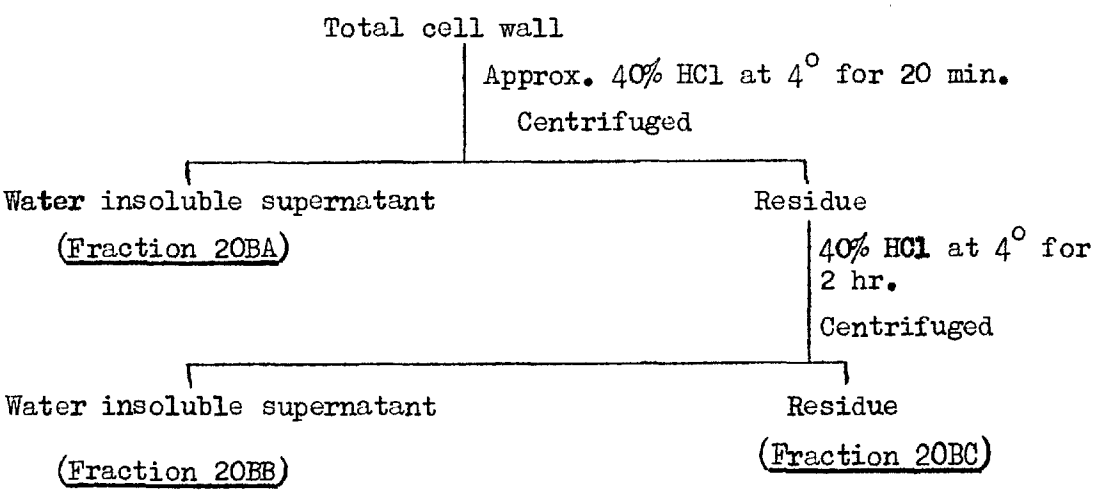


Fig. IV.2. Infrared Spectra of Chitin Preparations from Fusicoccum Cell Walls, Total Cell Walls and Crustacean Chitin.

been used widely for the isolation of chitin from fungal cell wall preparations. However the procedures involved are drastic and may not lead to a pure chitin preparation (Hackman, 1962). It was therefore of interest to determine the composition of fractions at different stages of the procedure. The method is described in detail in Expt. 20A and summarised in Fig.IV.1. The carbohydrate composition of the different fractions is given in Table IV.1. According to Houwink and Kreger (1953) Fraction 20ADI would correspond to chitin; its X-ray diffraction photograph was very similar to that of crustacean chitin, but much less sharp. Moreover chemical analysis (Table IV.1) showed that it contained only 27% glucosamine and a considerable amount of neutral carbohydrate. Fraction 20ADIb obtained after further fractionation by 36% hydrochloric acid gave a sharper X-ray diffraction pattern (Fig. 2 and Table 2 ;appendix), very similar to that of crustacean chitin. The infrared spectra of fractions 20ADI and 20ADIb were almost identical with that of crustacean chitin (Fig.IV.2).

(b) Cell wall chitin was also prepared by a modification of the method given by BeMiller (1965, and references cited therein). The method is described in Expt. 20B and summarised in Fig.IV.3.

Fig.IV.3 Preparation of Chitin from Fusicoccum cell walls.



The carbohydrate composition of the different fractions (Fig.IV.3) is given in Table IV.2. The X-ray diffraction photograph of Fraction 20BA, which should correspond to chitin was very similar to that of crustacean chitin, although not as sharp as that of Fraction 20ADIb. It clearly contained a considerable amount of neutral carbohydrate as well as chitin (Table IV.2). The infrared spectrum of Fraction 20BA was very similar to that of crustacean chitin (Fig.IV.2).

Table IV.2. Composition of fractions at various stages of chitin preparation from Fusicoccum cell walls.

Fraction	Wt.% of original cell wall	Per cent (expressed as anhydro sugar)			
		Total Hexose G.M.1	Glucose G.M.3	Glucosamine as N-acetyl G.M.6	N-acetyl D-glucosamine liberated by chitinase G.M.5
Total Cell Wall	100.0	79.2	69.2	5.7	4.8
20BA	7.4	51.2	49.3	40.0	6.5
20BB	6.6	77.0	75.0	-	2.7
20BC	24.0	76.0	75.0	Traces	-

C. Preparation of Cell Wall Chitosan and Authentic Chitosan

In order to confirm the presence of chitin in the cell walls, cell wall chitin was deacetylated with alkali as described in Expt.21A. The resultant product gave a positive van Wisselingh test for chitosan, and an X-ray diffraction pattern (Table 2. Fig. 2 , appendix) similar to that of chitosan prepared from crustacean chitin (Expt.21B), although not as sharp.

D. Enzymic Method for the Isolation of Cell Wall Chitin.

Cell walls were treated with (a) α-amylase and then (b) exo -β 1$\rightarrow$3 glucanase and the residue was extrated with N-sodium hydroxide as described in Expt. 39 for the estimation of cell wall polysaccharides linkage composition (see results p- 119). The insoluble residue (Fraction 39X) consisted almost entirely of N-acetyl-D-glucosamine (95%, Table IV.4) and had an X-ray diffraction pattern (Table 2 Fig. 2 , appendix) and an infrared spectrum very similar to authentic chitin (Fig.IV.2). The amino acid composition (G.M.8c) of the preparation is given in Table IV.3 (24 hour hydrolysis only).

Table IV.3 Amino-acid Composition of Cell Wall Chitin, prepared enzymically (Fraction 39X).

Amino - acid	% anhydro amino-acid	Molar ratio
Aspartic acid Threonine Serine	Not separated 0.134 (Total)	14.8 (Total) (based on average molecular weight
Alanine	0.076	11.8
Arginine	0.000	0.0
Glutamic acid	0.082	7.0
Glycine	0.045	8.8
Histidine	Not resolved from glucosamine	
Isoleucine	0.032	3.1
Leucine	0.061	6.0
Lysine	0.000	0.0
Methionine	0.024	2.0
Phenylalanine	0.074	5.6
Tyrosine	0.014	1.0
Valine	0.039	4.4

E. Action of a Mixture of Streptomyces Chitinase and Helix pomatia Enzymes on Cell Wall Chitin, Prepared Enzymically (Fractions 24X and 39X).

Cell wall chitin preparation (Fraction 24X and 39X) was hydrolysed almost completely to N-acetyl-D-glucosamine by a mixture of Streptomyces chitinase and Helix pomatia enzymes (Table IV.4). Neither of the enzymes alone hydrolysed the preparation completely in the same time. Table IV.4 shows the composition of the supernatant after incubation with enzymes.

Table IV.4 Liberation of N-acetyl-D-glucosamine by different enzymes from Fraction 24X and 39X (Expt.38).

Chitin preparation (Fraction 24X or 39X) was incubated with Streptomyces chitinase or Helix pomatia enzymes or a mixture of both as described in Expt. 38. After 24 hr. the mixture was assayed for N-acetyl-D-glucosamine, glucose and total hexose.

Sugar	Wt.% of original fraction released into supernatant (expressed as anhydro sugar)		
	Helix pomatia enzymes alone (0.1%)	S.griseus chitinase alone (0.1%)	Helix pomatia enzymes (0.05%) + S.griseus chitinase (0.05%)
N-Acetyl-D-Glucosamine (G.M.5)	50.0	70.0	95.0
Glucose (G.M.3)	-	-	NIL
Total Hexose (G.M.1)	-	-	Traces

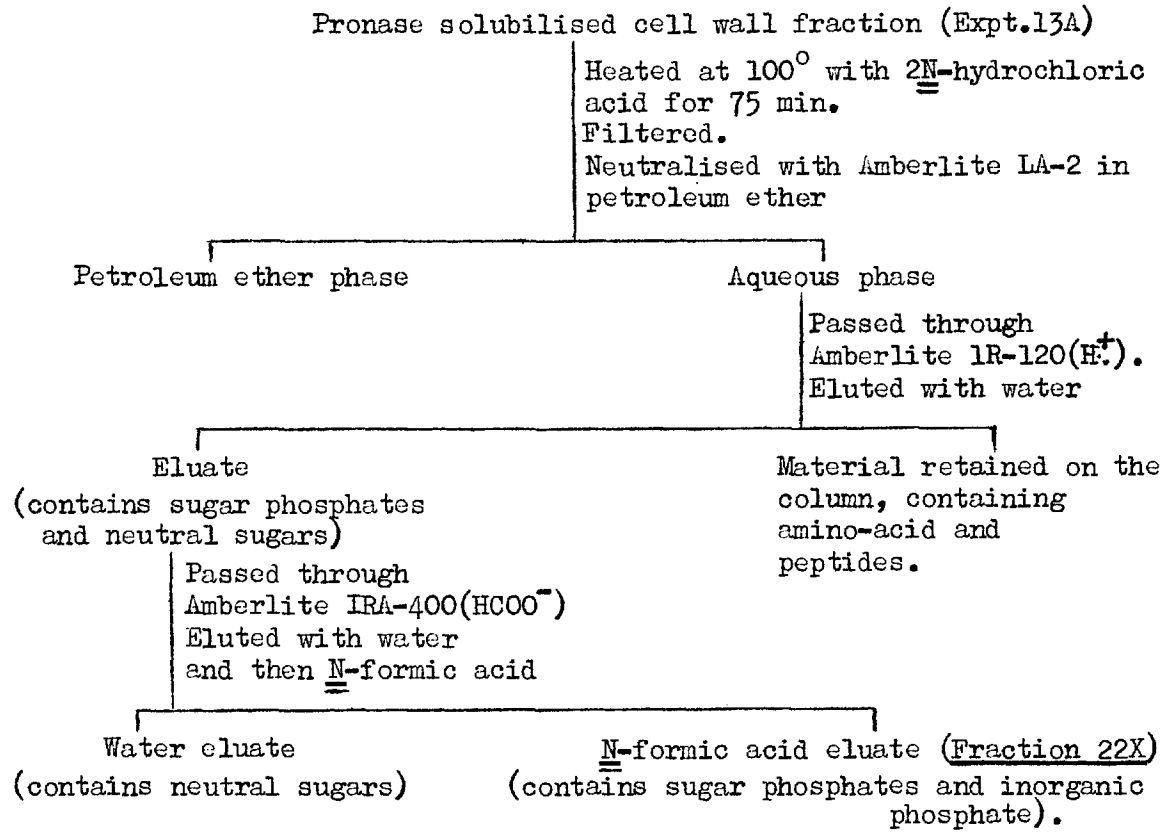
- = Not determined

It is clear that cell wall chitin prepared enzymically (Fraction 24X and 39X) was much less degraded than that prepared by the Kreger or BeMiller methods.

V. ISOLATION AND CHARACTERISATION OF SUGAR PHOSPHATES FROM THE CELL WALL FRACTION SOLUBILISED BY PRONASE

Incubation of Fusicoccum cell walls with pronase releases 38.3% of the cell wall phosphorus into the supernatant in a non-dialysable form. Sugar phosphates were isolated from this supernatant after acid hydrolysis according to the scheme shown in Fig.V.1. (Expt.22).

Fig.V.1 Isolation of sugar phosphates from the cell wall fraction solubilised by Pronase.



The product (Fraction 22X) was free from neutral and amino sugars and oligosaccharides as indicated by paper chromatography in Solvents b and c; and contained three components with R_{Pi} values of 1.00, 1.15 and 1.30, which were separated by chromatography on Whatman 3MM paper with Solvent k. The component with R_{Pi} value 1.00 showed the same kind

of transition in colour from yellow to light blue as inorganic orthophosphate with the molybdate spray (G.M.22d). The component with R_{Pi} value 1.15 (Fraction $22X_1$) had the same R_{Pi} value as hexose-6-phosphate (glucose, galactose and mannose-6-phosphate), developed the characteristic blue colour of hexose-6-phosphate with the molybdate spray (G.M.22d), and reacted positively with the silvernitrate and aniline hydrogen phthalate sprays (G.M.22a and b).

The component with R_{Pi} value 1.3 (Fraction $22X_2$) did not correspond to any sugar phosphate examined (G.M.22, Solvent k). It developed a very bright blue colour with the molybdate spray (G.M.22d) unlike the dark blue colour given by hexose-6-phosphate. This component also reacted positively with spray reagents a and b.

In solvent 1. the R_{Pi} values were 1.00, 0.6 (Fraction $22X_2$) and 0.45 (Fraction $22X_1$) respectively.

(a) Treatment with Acid Phosphatase. The eluted Fractions $22X_1$ and $22X_2$ from a chromatogram irrigated with Solvent k were incubated with acid phosphatase (G.M.21d) and then chromatographed in Solvent c. Fraction $22X_1$ gave rise to three spots, which reacted with both the silvernitrate and aniline hydrogen phthalate spray reagent and had the same R_G values as galactose (main component), mannose and glucose (small amount). Fraction $22X_2$ gave rise to four spots with both the above spray reagents, having the same R_G values and colour as galactose (main component), mannose (small amount), xylose (trace) and glucose (trace).

(b) Periodate Oxidation (Expt.22). Fraction $22X_1$ and $22X_2$ and also authentic hexose-6-phosphates (Glucose, galactose and mannose-6-phosphates) were oxidised with sodium metaperiodate and then chromatographed in Solvent k to give a component with the same mobility as glyceraldehyde-3-phosphate (R_{Pi} value 1.74), detectable with the silvernitrate, aniline hydrogen phthalate and molybdate spray reagent (G.M.22a and b), with traces of unreacted hexose-6-phosphate in all the cases. When the bands corresponding to glyceraldehyde-3-phosphate were eluted with water, treated with acid phosphatase, reduced with sodium

borohydride and chromatographed, all showed a single spot with the same mobility as glycerol in Solvent e.

VI PREPARATION AND PURIFICATION OF ENZYMES FOR USE IN CELL WALL FRACTIONATION AND STRUCTURAL STUDIES.

Enzymes are amoung the most powerful tools for the determination of the structure of biological polymers. However in order to obtain meaningful information it is essential that the enzymes used are of known purity and well defined action. Many enzyme preparations contain unwanted activity and hence it is necessary to devise methods of purification. This section describes the purification of a number of enzymes which were required for the structural studies to be described later.

A. Attempted Removal of Amylase Activity from the Enzymes of the Snail, Helix pomatia.

Enzymes from the gut of the snail, Helix pomatia, contain a wide range of carbohydrases, phosphomono- and-di-esterase, lipase, and little or no protease (Margaret Holden and Tracey, 1950). Fractionations of this complex mixture have been carried out mainly for the isolation of specific enzymes e.g. chitinase and muramidase (Takeda, Strasdine, Whitaker and Roy, 1966) and β-D-(1→3)-glucanases (Noble and Sturgeon, 1968). Anderson and Millbank (1966), fractionated a snail enzyme preparation in order to study the enzymes involved in the formation of protoplasts from yeast.

Incubation of Fusicoccum cell walls with a snail enzyme preparation (Results, p- 32) left a residue, $[\alpha]_D$ + 212°, which stained only faintly with iodine indicating the removal of most of the α-D-(1→4) linkages, possibly all the β-linked glucans and most of the heteroglycan polymer(s). In order to determine the nature of the α-linked polymers in the complete cell walls it was necessary to remove the β-glucans, chitin and heteroglycan polymers from the cell wall. The snail enzyme preparation, without its amylase activity, should

achieved this aim and attempts were therefore made to remove the amylase activities from the crude snail enzyme preparation.

(a) Using enzyme substrate complexing (Expt.4A). Adsorption on columns of starch or precipitation with glycogen has been used for the purification of α-amylase from a wide range of sources (Dube and Nordin, 1961; Greenwood, MacGregor and Milne,1965). However amylase activity could not be completely removed from the snail enzyme preparation by adsorption on defatted starch column or precipitation with glycogen, even on repeated treatment (Expt. 4A). Although α-amylase appeared to be largely removed, as indicated by failure of the partially purified enzyme to remove the iodine staining properties of starch and total cell wall preparation, even on prolonged incubation with a high concentration of the enzyme (1% substrate, 1% enzyme at pH 4.8 or pH 6.9), there was still a trace of amyloglucosidase (or β-amylase +α-glucosidase) activity as shown by the liberation of a small amount of glucose on prolonged incubation and this could not be removed even on repeated adsorption on starch or glycogen. It thus appeared that there were two types of amylase activity in the snail enzyme preparation, only one of which (α-amylase) could be removed by adsorption on starch or glycogen. Attempt was therefore made to selectively denature the residual amylase activity by heating.

(b) Using differential denaturation by heating (Expt.4). The effect of heating the snail enzyme preparation with reduced amylolytic activity (from a) at different temperatures is shown in Table VI.1. Although amylase activity appeared to be reduced more than β-D-(1$\rightarrow$3) glucanase activity, it was not possible to remove it completely.

Table VI.1 Effect of heating on the residual amylase activity of the partially purified (starch column treated) snail enzymes.

Starch column treated (α-amylase removed) snail enzyme preparation in acetate buffer, pH 4.8, was heated at various temperature for 30 min., cooled to 37° and assayed for β1$\rightarrow$3 glucanase and amylase activity as described in Expt. 4B. The amount of glucose liberated from laminarin

without prior heat treatment of the enzyme is taken arbitrarily as 10.

Temperature of heating	Comparative amounts of glucose liberated on incubation with	
	Laminarin	Starch
Without heating	10	Less than 0.1
45°	10	Less than above
50°	10	do.
55°	10	do.
60	8	do.
65	6	do.

B. Isolation and Purification of Endo - β- D - (1→3) - Glucanase from the Digestive Enzymes of Snail, Helix pomatia (Expt.5).

In order to study the structure of the β-glucan components of the cell walls a pure endo- β-1→3 -glucanase was required. Noble and Sturgeon (1968) have described the isolation of an endo-β-D-(1→3) glucanase from a snail enzyme preparation, free from cellulase and chitinase activities but did not state whether the product contained amylase activity. Their method, which involved adsorption on pachyman in 0.1M-sodium citrate buffer, pH 4.8, and desorption with 0.1M-sodium borate buffer, pH 8.2, (Expt. 5B), was therefore used for the isolation of the endo-β-D-(1→3) glucanase from snail enzyme preparation; it was found that the resultant enzyme had no action on starch, pustulan or cellulose, while on incubation with pachyman or laminarin gave rise to a homologous series of laminaridextrins, gentiobiose and free glucose.

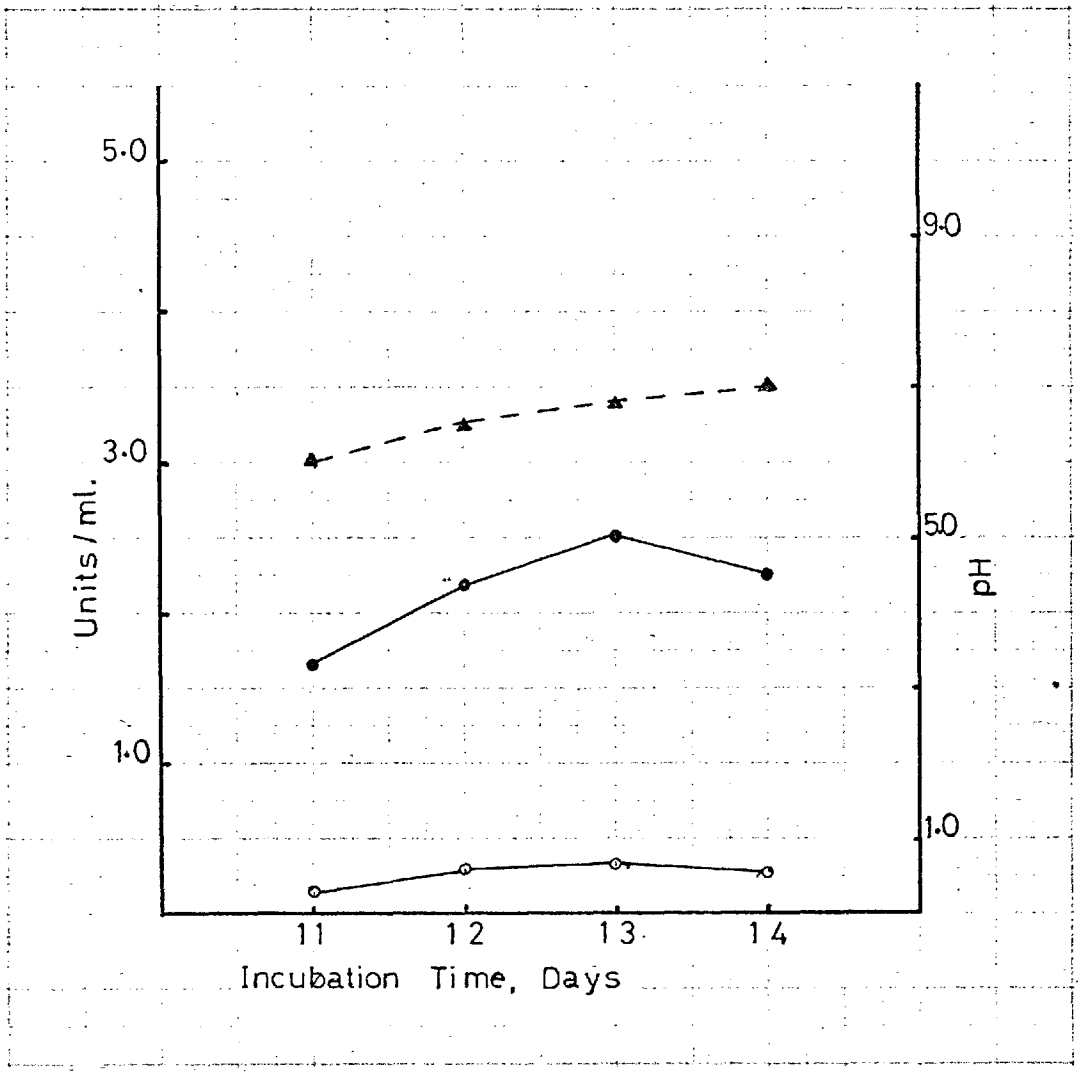


Fig. VI.1. Enzyme Activities and pH Changes in Basidiomycete QM 806 Culture from 11 to 14 Days.

▲ , pH; ● , β-D-(1⟶3)-glucanase activity;

o , Amylase activity.

25 units of β-D-(1→3) glucanase activity were recovered from 150 units by the above purification.procedure, as determined by G.M.19.

C. Preparation and Partial Purification of Exo -β- D -(1→3) glucanase from Basidiomycete QM806. (Expt.6)

For structural studies of the β-glucan components of Fusicoccum cell walls a pure exo-β1→3-glucanase was needed. The culturefiltrate of Basidiomycete QM806 is a convenient source of such an enzyme (Reese and Mandels, 1959; Chesters and Bull, 1963 and Nelson, Scaletti, Smith and Kirkwood, 1963).

The crude exo-β-D-(1→3) glucanase was isolated from the 14 day old culture filtrates of Basidiomycete QM806 by either acetone precipitation (Reese and Mandels, 1959) or by concentration under reduced pressure at 37° (Huotari, Nelson, Smith and Kirkwood, 1968). Activities during the last few days of growth are shown in Fig.VI.1. The preparation had 9 - 13 units/mg. β-D-(1→3) glucanase activity (G.M.19) and 1.1 - 1.6 units/mg. of amylase activity (G.M.19). It had no action on pustulan or cellulose, and very weak activity towards gentiobiose. Attempts were first made to remove the amylase activity.

(a) By substrate enzyme complexing : Amylase activity could not be removed completely from the crude enzyme preparation by adsorption on defatted starch or glycogen (Expt.6). Although α-amylase activity appeared to be largely removed, as indicated by failure to remove the iodine staining properties of starch and total cell wall preparation even on prolonged incubation with a high concentration of the enzyme (1% substrate, 1% enzyme at pH 4.8 and pH 6.9), there was still about half the amylase activity as shown by the liberation of glucose on incubation of starch or amylopectin. It appeared that, as in the case of the snail enzyme preparation, complexing with starch or glycogen removes only the α-amylase, leaving amyloglucosidase (or β-amylase + α-glucosidase). Attempts were therefore made to selectively denature the amylase activity by heating.

(b) Attempted differential denaturation of amylolytic activity by heating : The effect of heating the crude exo-β-D-(1→3) glucanase preparation at different temperatures is shown in Table VI.2. It appeared that both β-D-(1→3) glucanase and amylase activities were lost in parallel, and it was not possible to completely remove amylase activity while retaining β 1→3 glucanase activity.

Table VI.2 Effect of heating on amylase activity of crude Basidiomycete QM806 exo - β- D - (1→3) glucanase preparation.

Crude exo- β 1→3 glucanase from Basidiomycete QM806 in acetate buffer, pH 4.8, was heated at various temperatures for 30 min., cooled to 37° and assayed for β 1→3 glucanase and amylase activity as described in Expt.4B. The amount of glucose liberated from the laminarin without prior heat treatment of the enzyme is taken arbitrarily as 10.

Temperature of heating	Comparative amounts of glucose liberated on incubation with	
	Laminarin	Starch
Without heating	10	3
45°	10	3
50°	10	3
55°	10	2
60°	9	1
65°	1.5	Trace

(c) DEAE Cellulose chromatography : Huotari, Nelson, Smith and Kirkwood (1968) have described a partial purification of Basidiomycete QM806 exo-β1→3 glucanase by chromatography on DEAE-cellulose. However no assays for amylase activity were carried out. This procedure was

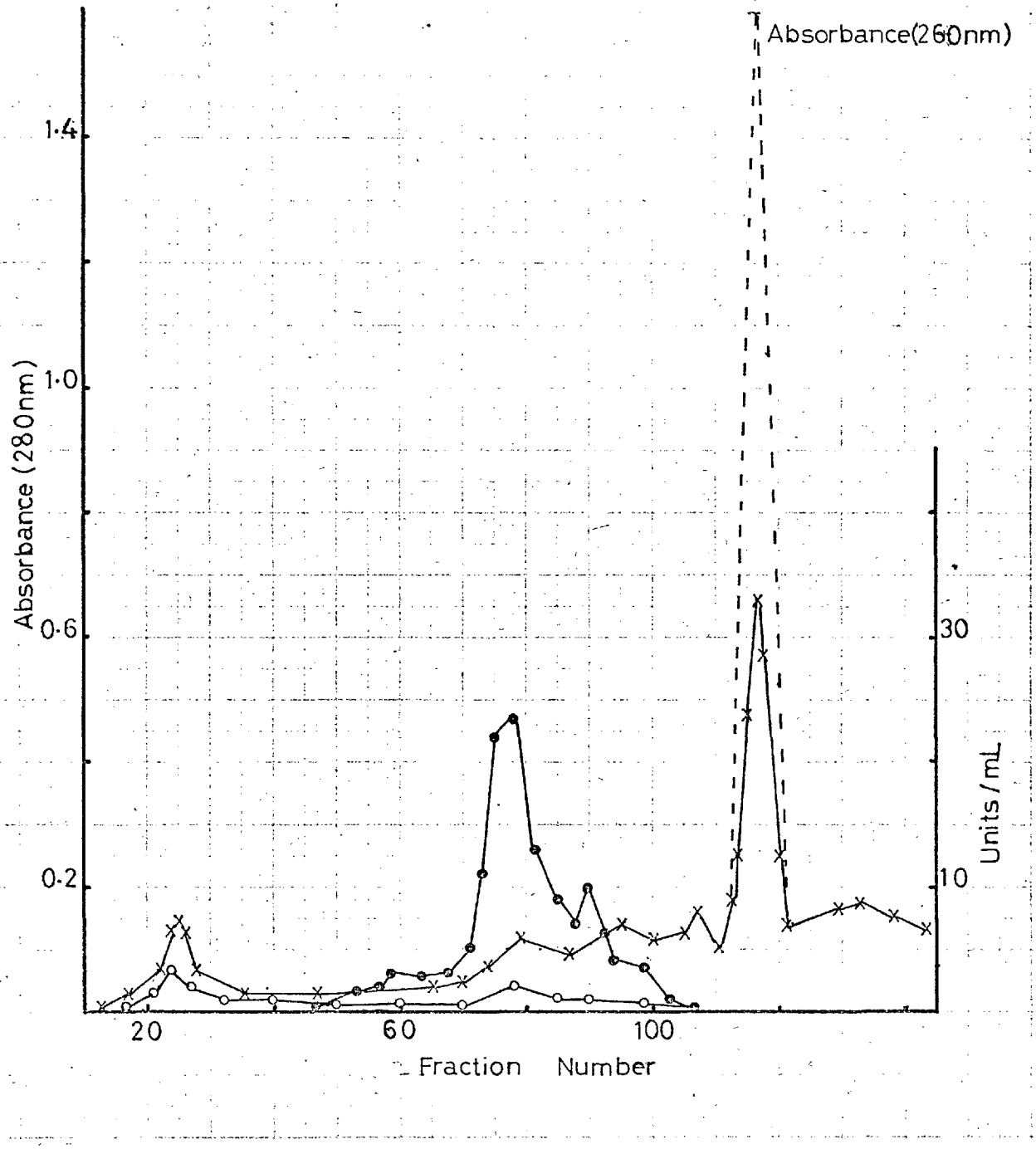


Fig. VI.2. DEAE-Cellulose Chromatography of Basidiomycete QM 806 β-D-(1⟶3)-glucanase.

o , Amylase activity; ● , β-D-(1⟶3)-glucanase activity; x , Absorbance at 280nm.; - - , Absorbance at 260nm.

therefore carried out (Expt.6) and the elution profile on DEAE-cellulose was similar (Fig.VI.2) to that obtained by Huotari, Nelson, Smith and Kirkwood (1968). The large peak (Fractions 110 - 120) was due to nucleic acid which had not been removed by prefractionation with ammonium sulphate. Assay for amylase (G.M.19) showed that there were at least two types of amylase activity, one of which overlaps the β1→3 glucanase peak area. This method was therefore not suitable for obtaining an amylase free preparation of exo-β1→3 glucanase.

(d) Adsorption of β-D-(1→3) glucanase activity on pachyman : It was found that Basidiomycete QM806 exo β1→3 glucanase could be obtained almost free from amylase activity by complexing on pachyman (Expt.6c). The enzyme was adsorbed on pachyman in McIlvine citrate-phosphate buffer, pH 3.5, and washed free of other enzymes which did not adsorb. It was then recovered by incubation at pH 4.8 which hydrolysed most of the pachyman to glucose with a small amount of gentiobiose. The resultant exo-β1→3 glucanase, which could be recovered almost quantitatively, had no action on pullulan, pustulan, cellobiose or gentiobiose and only a trace of activity towards amylose and amylopectin.

D. Preparation and Purification of Pullulanase from Aerobactor aerogenes.

Pullulanase was required for testing for α1→6 glucopyranose linkages in the cell wall after amylase action.

Crude pullulanase enzyme was isolated from culture filtrates of A. aerogenes by the method of Bender and Wallenfels (1961) (Expt.7) and had pullulanase activity 0.3 units/mg. with some endo β1→3 glucanase and α-amylase activity, producing a homologous series of laminaridextrins and free glucose from laminarin and a homologous series of maltodextrins from amylose, but it had no action on cellulose or maltose, under similar conditions (G.M.17).

As the enzyme was required to test for the presence of α-D-(1→6) linkages as branch points in the cell wall α-D-(1→4) linked glucan, it was necessary to remove the β1→3 glucanase and amylase activity from the enzyme preparation (as both of those linkages are present

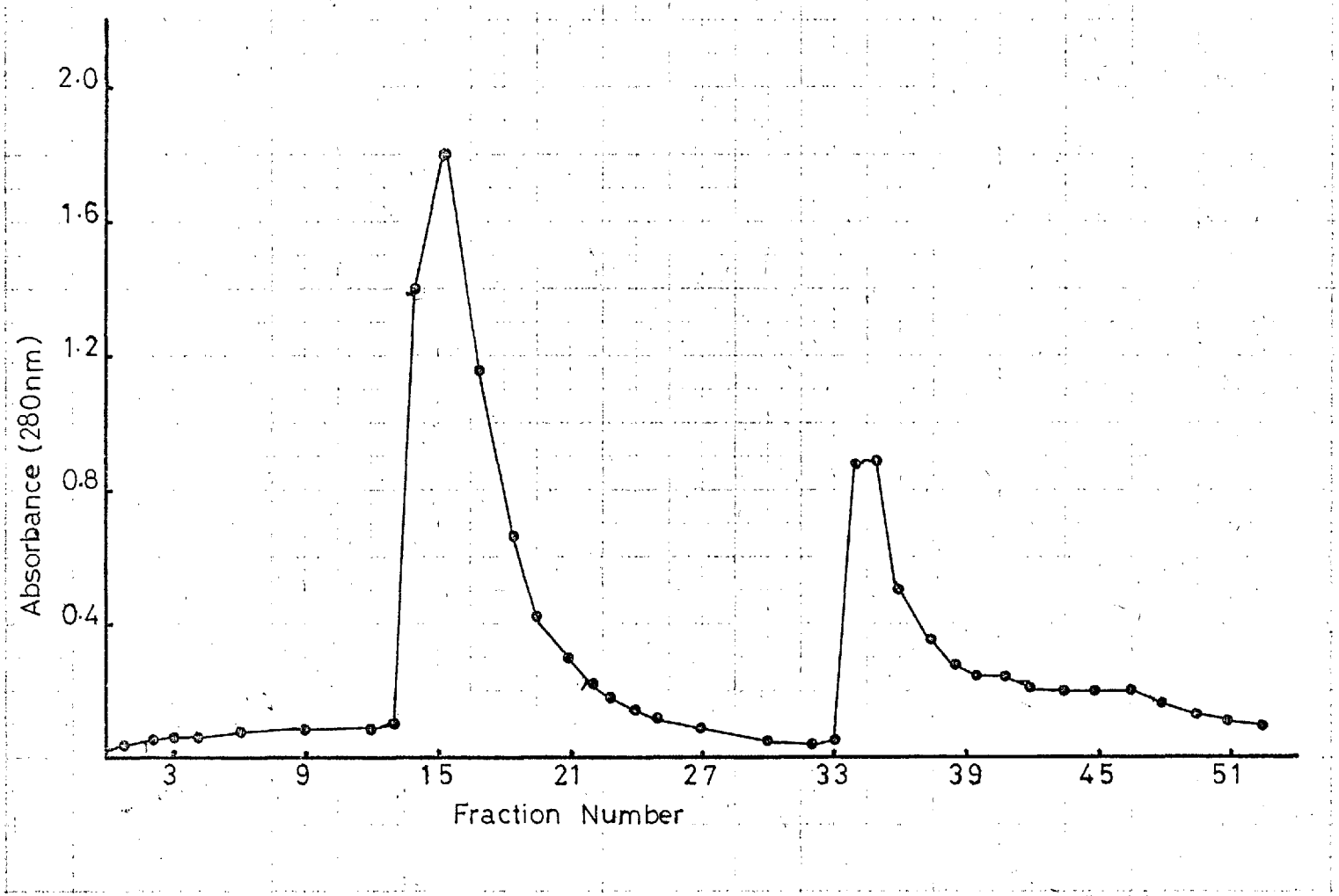


Fig. VI.3. Stepwise Elution Profile of Crude Pullulanase Preparation from Aerobactor aerogenes on a DEAE-Cellulose Column. (Expt.7.).

in the cell walls). The crude enzyme preparation was therefore fractionated on DEAE-cellulose and the result are shown in Fig.VI.3. Fractions 14 to 17 were combined as Fraction A, 18 to 24 Fraction B and 34 to 38 as Fraction C on the basis of the amount of maltotriose liberated by individual fractions from pullulan. Fractions A and B were found to act on both laminarin and amylose in addition to pullulan. Fraction C had 5 units/mg. pullulanase activity (G.M.17), but no detectable action on laminarin or amylose under the same incubation conditions.

E. Preparation and Partial Purification of Human Salivary α- Amylase.

The salivary α-amylase was prepared according to the method of Bornfeld(1955) omitting the crystallisation (Expt.8). The enzyme was used as a solution in the 0.02M-sodium phosphate buffer, pH 6.9, containing 0.0067M-sodium chloride, and had activity towards amylose, amylopectin and maltose, but no action on pullulan, pustulan, pachyman, laminarin, cellulose, cellobiose and nigeran.

VII. ACTION OF ENZYMES ON CELL WALL FRACTIONS AND FRACTIONATION OF THE PRODUCTS.

A. Wall Fraction 14FJ $[\alpha]_D$ + 236°.

Examination of cell wall Fraction 14FJ by analytical centrifugation showed the presence of two components (Fig.VII.1), with S°_{20} values of 6.6 and 2.8. Since the repeated alkali fractionation (Fig.III.1) had failed to separate these two components, the action of enzymes on the mixture was investigated to determine if selective action on one component could be obtained, with the aim of thus fractionating the products of enzyme action.

(a) Salivary α-amylase : Cell wall Fraction 14FJ was incubated with salivary α-amylase for 24 hr. as described in Expt. 23a. After 3 hr. the fraction no longer stained with iodine and examination of the supernatant by paper chromatography (Solvent b) revealed sugars

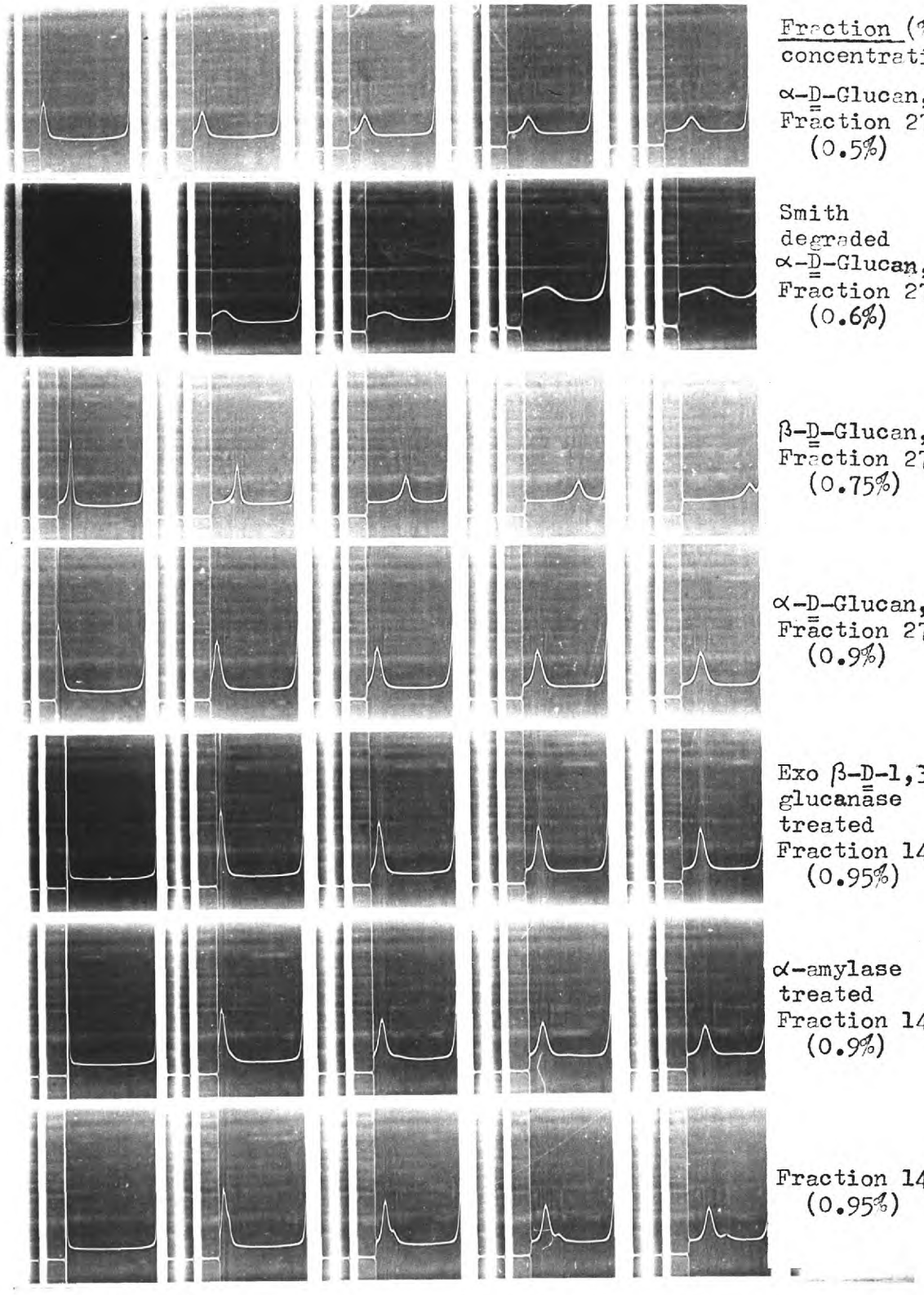


Fig.VII.1. Sedimentation Photographs of Cell Wall Fractions. Cell wall fractions (in N- sodium hydroxide) were centrifuged at 48,000 r.p.m. in a Beckman Model E Analytical Ultracentrifuge (G.M.29). Schlieren photographs were taken at an interval of 64 min.

with the mobilities of glucose, maltose and maltotriose. The identities of the oligosaccharides were confirmed after eluting the sugars concerned from the chromatogram followed by incubating aliquots with emulsin and β-amylase separately and rechromatographing the incubation mixtures in Solvents b and f. The oligomers were unafftected by emulsin and had the same mobilities as maltose and maltotriose in Solvent b and f. β-amylase on the other hand acted on the component with the same mobility as maltotriose, producing glucose and maltose, but had little action on the compound with the same mobility as maltose. Traces of unidentified higher oligosaccharides were also detected.

The residual cell wall after 24 hr. incubation, which was resistant to further attack by α-amylase, had $[\alpha]_D$ + 240°. Table VII.1 shows the result of the incubation for 24 hr.

Table VII.1. Action of salivary α-amylase on wall Fraction 14FJ. (Expt.23).

Component	Per cent of total
Residual cell walls	87.2
Supernatant (expressed as anhydro sugars)	
Total hexose (G.M.1)	12.0
Glucose (G.M.3)	1.2
Reducing sugar as glucose (G.M.2)	4.8

Analytical centrifugation of the residual cell wall using a Beckman Model E Analytical Ultracentrifuge (G.M.29) showed mainly a slow component with only traces of a faster moving component (Fig.VII.1). The residue was not excluded from Sephadex G-200, its elution profile is shown in Fig. 3 (appendix).

(b) Hog-pancreatic α-amylase : The results obtained from hog-pancreatic α-amylase treatment of the Fraction 14FJ were essentially the same as those obtained with salivary α-amylase. Table VII.2 shows the results obtained from a 24 hr. incubation.

Table VII.2 Action of hog-pancreatic α-amylase on Fraction 14FJ (Expt. 23).

Component	Per cent of total
Residual cell wall, $[\alpha]_D$ + 239°	87.2
Supernatant (expressed as anhydro sugars)	
Total hexose (G.M.1)	11.7
Glucose (G.M.3)	2.0
Reducing sugar as glucose (G.M.2)	5.2

Glucose, maltose and maltotriose were the only products detected after paper chromatography as in the case of the salivary α-amylase incubation.

(c) β-amylase : The glucan was relatively resistant to β-amylase incubation and appreciable action could only be obtained using large amounts of enzymes. When Fraction 14FJ (20 mg.) was incubated with β-amylase (1,140 units) as described in Expt. 23b, maltose was the only sugar detected in the supernatant by paper chromatography (Solvent b). Table VII.3 shows the results.

(d) Pullulanase : In order to assess whether the α1→4 D glucan contained any α1→6 D-glucopyranose branch points, as in amylopectin or pullulan, cell wall Fractions 14FJ (before or after treatment with α-amylase or β-amylase) was incubated with pullulanase as described in Expt. 23c and the soluble products examined by paper chromatography (Solvent b). Pullulanase did not liberate any soluble sugar from Fraction 14FJ either before or after treatment with α-amylase or β-amylase.

Table VII.3 Action of β-amylase on Fraction 14FJ (Expt.23b)

Component	Per cent of total
* Residual cell wall	95.0
Supernatant (expressed as anhydro)	
Total hexose (G.M.1)	2.5
Glucose (G.M.3)	0.0
Reducing sugar as glucose (G.M.2)	0.9

* The residue was completely resistant to further action of β amylase and still stained with iodine.

(e) Exo - β- D - (1→3)-glucanase : Glucose and gentiobiose were the only sugars detected by paper chromatography (Solvent b) which were released by the action of Basidiomycete QM806 exo -β-D-(1→3)-glucanase, on Fraction 14FJ, either before or after treatment with α-amylase as described in Expt.23d. The compositions and optical rotations of the supernatants and the residues are shown in Table VII.4.

The β1→3 glucanase resistant fraction, $[\alpha]_D$+ 279°, still stained with iodine. The infra-red spectrum (Fig. 1 ,appendix) had an absorption peak at 850 cm^{-1}, characteristic of α-glucan and no absorption peak at 890 cm^{-1}, characteristic of β-glucan. Examination of the residue (obtained by the pachyman purified exo-β1→3 glucanase action, which has not been previously treated with amylase and still stained with iodine) on the Beckman model E Analytical Ultracentrifuge, as described in G.M.29, indicated the complete absence of the 6.6 S component (Fig.VII.1). The residue was not excluded from Sephadex G-200, its elution profile is shown in Fig 3 (appendix).

(f) Starch column treated snail enzymes: Starch column purified snail enzymes (Expt.23e) liberated mainly glucose with traces of gentiobiose and laminaribiose from Fraction 14FJ, either before or

Table VII.4 Action of exo-β-D-(1$\rightarrow$3) glucanase on Fraction 14FJ. (Expt.23d)

Fraction 14FJ, was incubated with pachyman purified exo-β1$\rightarrow$3 glucanase as described in Expt.23d. After 24 hr. the mixture was assayed for total hexose, reducing sugar and glucose.

Glucan Fraction	Wt.% of Fraction 14FJ	Wt.per cent of Fraction 14FJ					$[\alpha]_D$ of residue
		Sugars released in the supernatant (expressed as anhydro sugar)				Residual cell wall	
		Total Hexose G.M.1	Reducing sugar as glucose G.M.2	Glucose G.M.3	Gentiobiose *		
Fraction 14FJ	100	22.5	18.5	16.4	3.0	74	+279°
Fraction 14FJ after treatment with α-amylase as described in Expt.23a	88	22.0	18.3	16.2	2.9	67.5	+292°

* Gentiobiose = $\frac{\text{Total hexose} - \text{Glucose}}{2}$

after treatment with α-amylase. The results are given in Table VII.5.

The enzyme resistant residue still stained with iodine, and its infrared spectrum was identical with that of the residue obtained after treating cell wall Fraction 14 FJ with exo-β1$\rightarrow$3 glucanase (Expt.23d).

(g) Glucamylase : Incubation of Fraction 14FJ with A.Niger glucamylase for 4 hr., as described in Expt. 23d and G.M. 21b, liberated 11.6% glucose (expressed as anhydro) (kindly done by Dr.B.E.Ryman); the residue did not stain with iodine. The glucamylase preparation contained a small amount of α-amylase and hence the products obtained are the result of the combined activities of the two enzymes.

Table VII.5 Action of starch column treated snail enzymes on Fraction 14FJ (Expt. 23e).

Cell wall Fraction 14FJ was incubated with starch column treated snail enzymes, before and after α-amylase treatment as described in Expt. 23e. The sugars released after 24 hr. of incubation were estimated by glucose oxidase (G.M.3), and phenol/sulphuric acid (G.M.1).

Glucan Fraction	Wt.% of Fraction 14FJ	Wt. per cent of Fraction 14FJ			$[\alpha]_D$ of residue
		Sugars released in the supernatant (expressed as anhydro sugar)		Residual cell wall	
		Total Hexose G.M.1	Glucose G.M.3		
Fraction 14FJ	100	19.8	19.0	76	$+280^{\circ}$
Fraction 14FJ (after treatment with -amylase Expt.23a)	88	19.0	18.0	68.4	$+285^{\circ}$

B. Fractionation of the Products of α-Amylase Action on Cell Wall Fraction 14FJ (Expt. 27)

Cell wall Fraction 14FJ was incubated with hog pancreatic α-amylase for 18 hr. as described in Expt. 27 and the residue was dissolved in N-sodium hydroxide and then neutralised:

(a) The precipitate, $[\alpha]_D + 270^{\circ}$, still released glucose and gentiobiose when incubated with pachyman purified exo-$\beta 1 \rightarrow 3$ glucanase, as described in Expt.27; after incubation for 18 hr with this enzyme the residue was dissolved in N-sodium hydroxide and reprecipitated by adjusting to pH 10.0. The final product, α-D-Glucan, Fraction 270 had $[\alpha]_D + 290^{\circ}$.

(b) 3 Volumes of ethanol were added to the supernatant and the resultant precipitate, β-D-Glucan, Fraction 27K, had $[\alpha]_D$ + 16°.

The fractionation is summarised in Fig.VII.2 and the compositions of supernatants S_I and S_{II} are given in Table VII.6.

Fig.VII.2 Fractionation of the Products of α-Amylase Action on Cell Wall Fraction 14FJ (Expt.27)

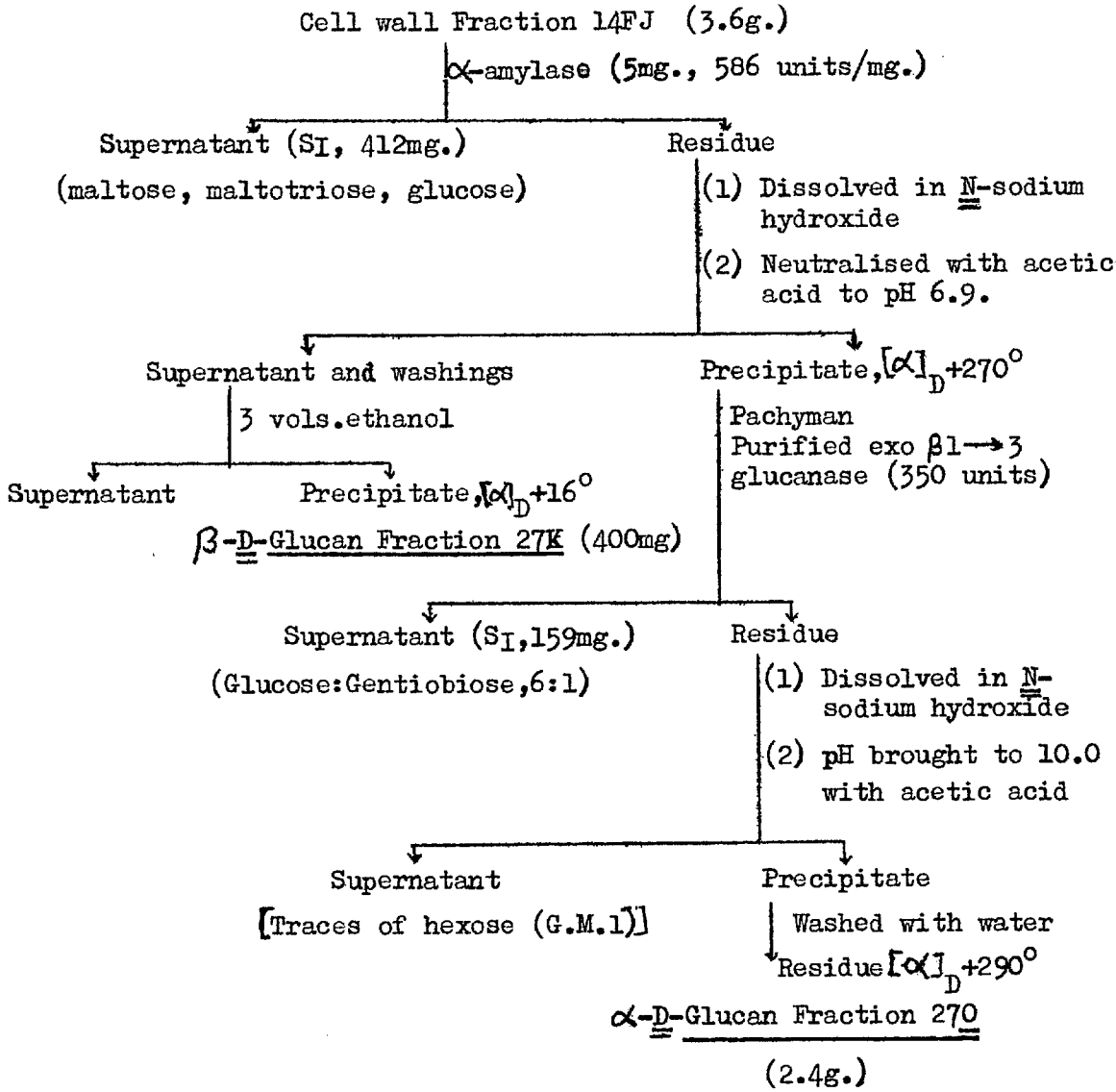


Table VII.6 Composition of fractions described in Fig.VII.2. (Compositions are given as anhydro sugars as per cent of Fraction 14FJ)

Fraction	Hexose G.M.1	Reducing sugars as glucose G.M.2	Glucose G.M.3	Wt.% of Fraction 14FJ	$[\alpha]_D$
Supernatant (S_I)	11.4	6.2	1.0		
β-D-Glucan, Fraction 27K				11.1	+16°
Supernatant (S_2)	4.4	4.0	3.3		
α-D-Glucan, Fraction 27O				66.6	+290-292°

C. Action of Basidiomycete Exo-β-D-(1→3) glucanase on the Cell Wall Fraction Insoluble in Alkali (Fraction 14FB). (Expt. 24).

Cell wall Fraction 14FB contains N-acetyl-D-glucosamine (probably as chitin), glucose (probably as α and β glucans, the infrared spectrum shows absorption peaks at 850 cm^{-1} and at 890 cm^{-1}) small amounts of galactose, mannose and rhamnose and traces of protein and phosphorus.

Since it has been found that glucan Fraction 14FJ could be fractionated into two completely different glucan fractions, after incubation with α-amylase (Result VII B), it was of interest to know if it were possible to fractionate all the remaining glucan in the Fraction 14FB by the simple removal of a particular glucan type with purified enzymes.

Incubation of cell wall Fraction 14FB with pachyman purified Basidiomycete QM806 exo-β-D-(1→3)-glucanase as described in Expt.24a

liberated glucose and gentiobiose as revealed by paper chromatography (Solvent b). The results of a 48 hr. incubation are shown in Table VII.7.

Table VII.7 Action of exo-β-D-(1→3) glucanase on Fraction 14FB.

Component		Per cent of Fraction 14FB
Residual cell wall		50.0
Supernatant (expressed as anhydro)		
Total hexose	(G.M.1)	50.0
Glucose	(G.M.3)	39.3
Reducing sugar as glucose	(G.M.2)	45.0

Using the relationship Gentiobiose = $\frac{\text{Total hexose - Glucose}}{2}$ the molar ratio of glucose to gentiobiose may be estimated as 7.3:1. The residual glucan still stained blue-black with iodine solution, and could be divided into alkali soluble and alkali insoluble fractions as described in Expt.24b. The results are given in Table VII.8.

Table VII.8 N-Potassium hydroxide fractionation of Fraction 14FB after exo-β-D-(1→3)glucanase action.

Fraction	% of Fraction 14FB	$[\alpha]_D$
24Y (Soluble in N-potassium hydroxide)	72.0	+238°
24X (N-Potassium hydroxide insoluble)	22.0	

The infrared spectrum of Fraction 24Y had an absorption peak at 850 cm^{-1}, characteristic of α-glucopyranose linkage and no absorption peak at 890 cm^{-1}, characteristic of β-glucopyranose linkage and the spectrum was similar to Fraction 14FJ (Fig. 1 , appendix) obtained after treatment with purified exo $\beta 1 \rightarrow 3$ glucanase.

The infrared spectrum, X-ray diffraction pattern and the action of a mixture of chitinase and snail enzymes on the Fraction 24X (Results IV E) shows that it is very similar to crustacean chitin and contains 95% anhydro-N-acetyl-D-glucosamine.

VIII. STRUCTURAL STUDIES OF α-D-GLUCAN, FRACTION 27O.

The following experiments were carried out to study the structure of α-D-Glucan, Fraction 27O, using physical, chemical and enzymic methods.

A. Physical Properties of α-D-Glucan, Fraction 27O.

α-D-Glucan, Fraction 27O $[\alpha]_D$ + 290 - 292°, was insoluble in water and soluble in N-alkali. Its infrared spectrum (p. 232) showed absorption maxima at 855 (strong & broad) and 930 (strong) cm^{-1} characteristic of α-linked glucopyranose residues. There was no absorption at 890 cm^{-1}, characteristic of β-linkages. When examined with the Beckman Model E Analytical Ultracentrifuge at 160,000 g. with Schlieren optics as described in G.M.29 only one peak with S^{o}_{20} 1.7S (N-sodium hydroxide) was observed. α-D-Glucan, Fraction 27O was not excluded from Sephadex G-200; its elution profile is shown in Fig. 4 (appendix).

B. Chemical Composition of α-D-Glucan, Fraction 27O

The carbohydrate composition of Fraction 27O, determined after N-sulphuric acid hydrolysis (G.M.15A), is shown in Table VIII.1 The amino acid analysis, determined after 6N - hydrochloric acid hydrolysis for 24 hr. (G.M.15B), is shown in Table VIII.2.

Table VIII.1 Carbohydrate composition of α-D-Glucan, Fraction 270.

	Method	Per cent anhydro sugar
Total Hexose	G.M.1	95.7
Glucose	G.M.3	93.2
Galactose	G.M.4a	0.2

Table VIII.2 Amino acid composition of α-D-Glucan, Fraction 270.

Amino acid	% anhydro amino acid	Molar ratio
Aspartic acid, Threonine, Serine (Not separated)	0.0133 (total)	16.00 (total) (based on average molecular weight)
Alanine	0.0044	7.8
Arginine	0.0164	13.1
Glutamic acid	0.0068	6.5
Glycine	0.0038	8.3
Histidine	0.0031	2.8
Isoleucine	0.0025	2.8
Leucine	0.0057	6.3
Lysine	0.0020	1.9
Phenylalanine	0.0042	3.5
Tyrosine	0.0013	1.0
Valine	0.0032	4.1
Total	0.0667	

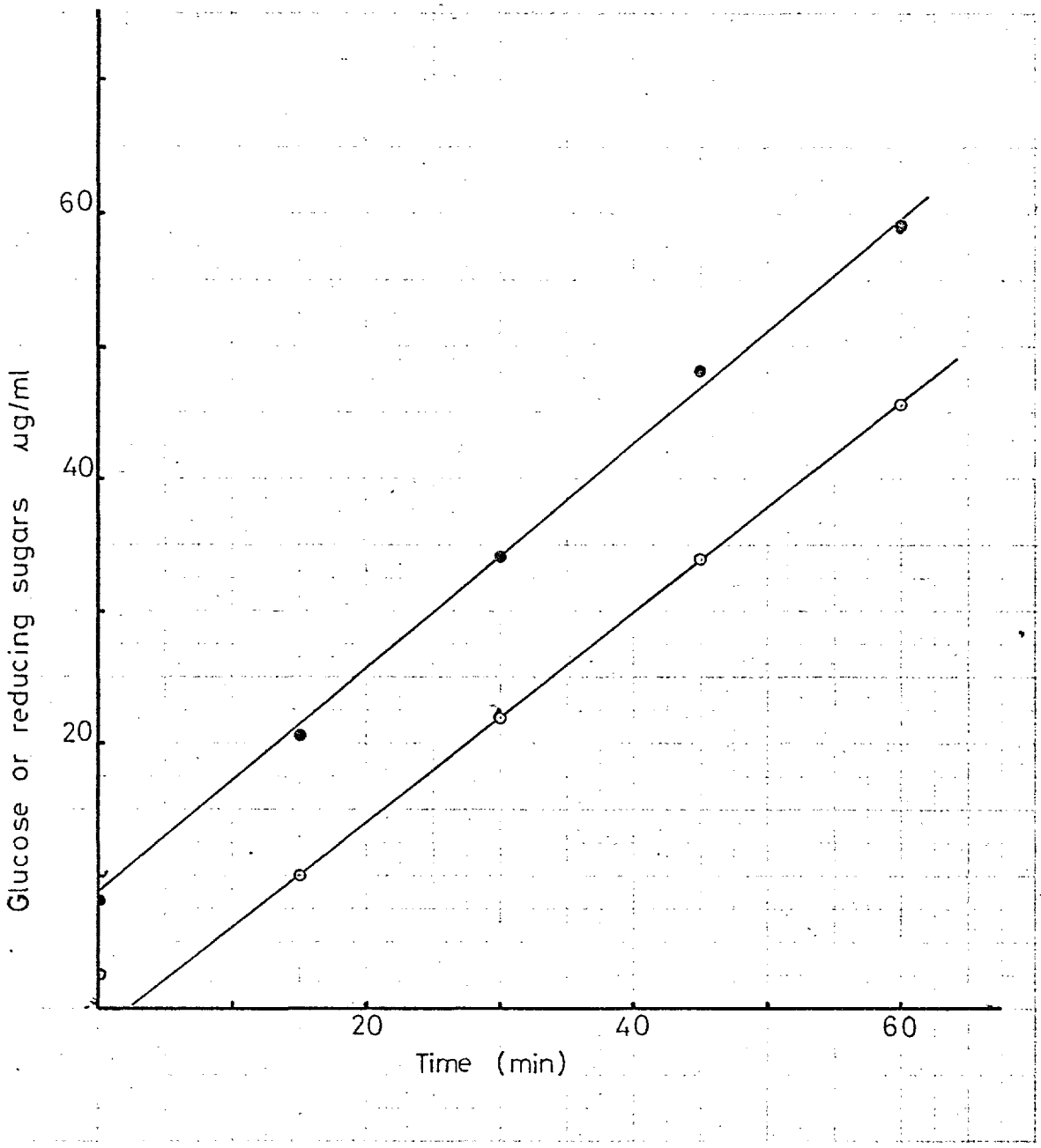


Fig. VIII.1. Partial Hydrolysis of α-D-Glucan, Fraction 27O.
α-D-Glucan, Fraction 27O was hydrolysed and glucose and total reducing sugars were measured as described in Expt. 33.

● , Reducing sugars; o , Glucose.

Paper chromatography of the N-sulphuric acid hydrolysates showed the presence of glucose with only traces of galactose and mannose. Amino acid analysis indicated that only traces of protein (peptide) remained.

C. Action of Enzymes

(a) Action of purified Aspergillus niger glucamylase on the α-D-Glucan, Fraction 270. Incubation of the α-D-Glucan, Fraction 270 with A.niger glucamylase for 24 hr. (Expt.32a) did not result in the liberation of any sugar, which could be detected by paper chromatography.

(b) Action of Trichoderma viride cellulase preparation on the α-D-Glucan, Fraction 270. Incubation of the α-D-Glucan, Fraction 270, before and after Smith degradation, with T.viride cellulase preparation for 24 hr. (Expt.32b) liberated small amounts of glucose only, which could be detected by paper chromatography with Solvent b.

(c) Action of Helix pomatia enzymes on the α-D-Glucan, Fraction 270. α-D-Glucan, Fraction 270, was incubated with H.pomatia enzymes preparation for 24 hr. as described in Expt.10. Examination of the supernatant by paper chromatography (Solvent b) showed that no detectable sugars had been liberated into the supernatant. The supernatant also gave no detectable colour with the Nelson-Somogyi reagent (G.M.2). It appears that Helix pomatia enzymes have no action on this fraction.

D. Partial Acid Hydrolysis of α-D-Glucan, Fraction 270

The α-D-Glucan, Fraction 270, was partially hydrolysed by first heating with 98% formic acid and then with 0.44N-sulphuric acid as described in Expt.33. Fig.VIII.1 shows the liberation of total reducing sugars and glucose as a function of time. The proportion of oligosaccharides appeared to remain constant throughout the hydrolysis and this was confirmed at different intervals of time. Paper chromatography (Solvents g and j) indicated the products to be nigerodextrins detectable upto nigerohexaose, identified by R_G values

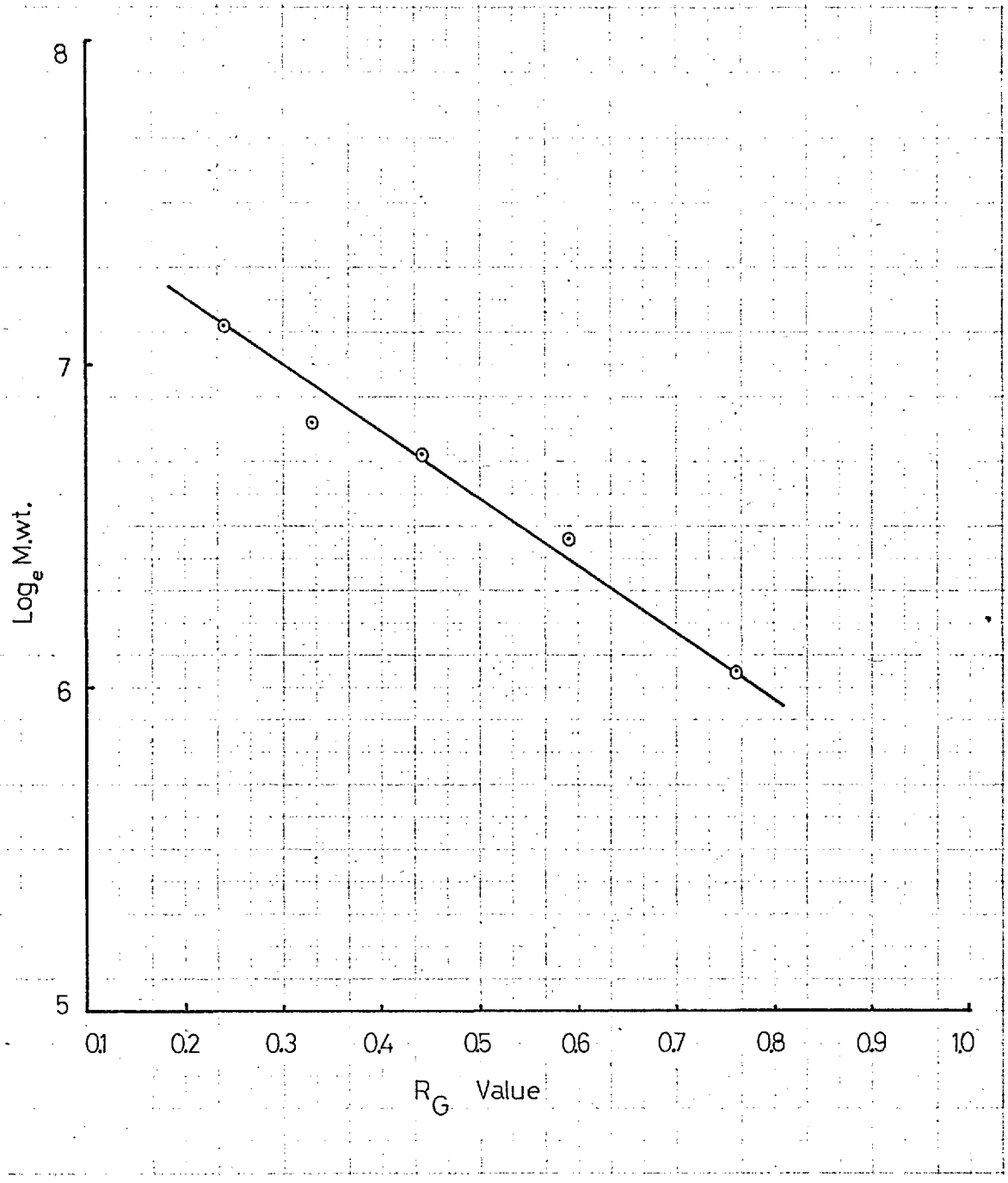


Fig. VIII.2. Relationship Between Log$_e$ Molecular Weight and R_G Values for the Nigerodextrins Released During Hydrolysis of α-D-Glucan, Fraction 270 in Butanol: Pyridine:Water:: 6:4:3, by vol., (Solvent system g).

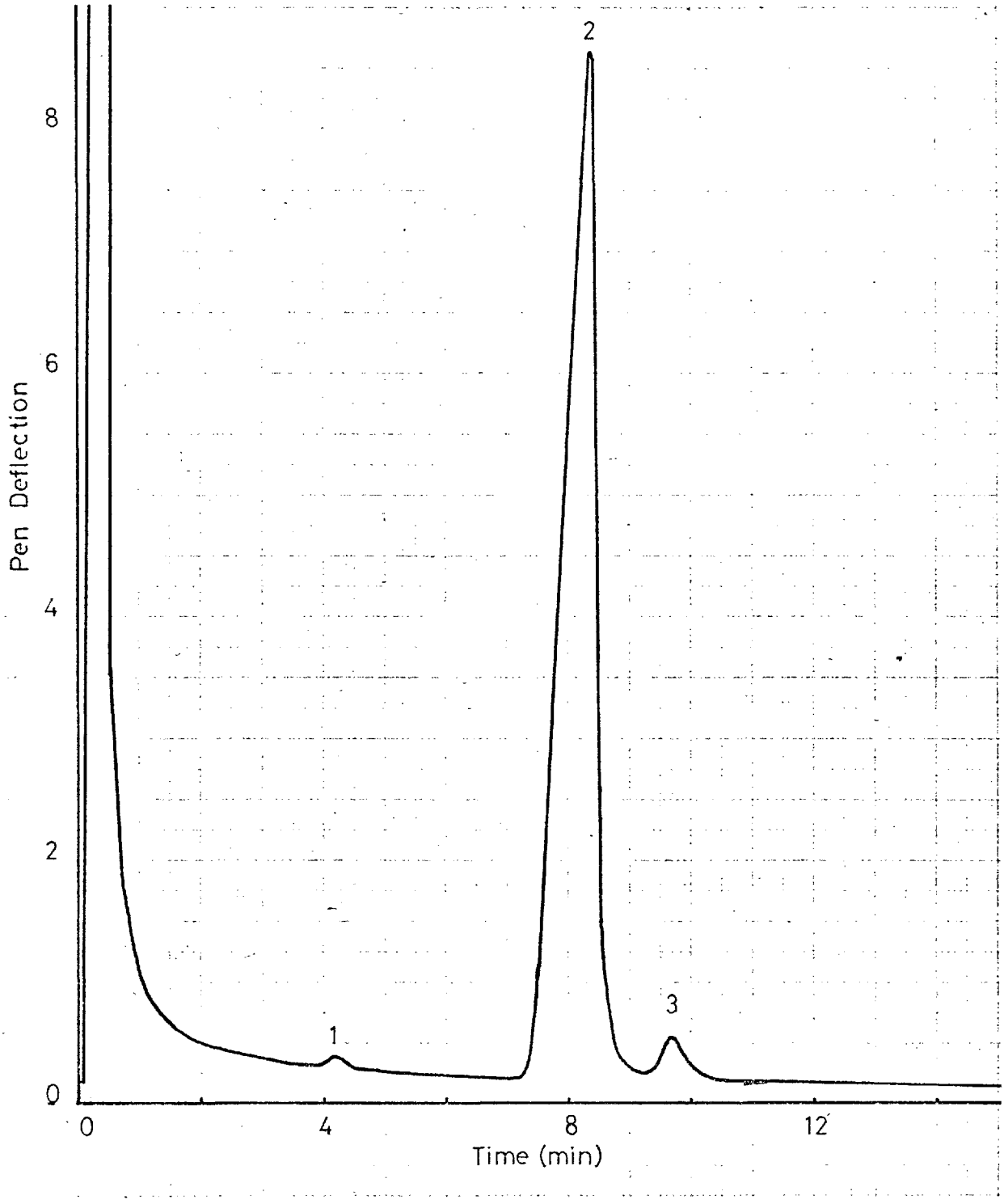


Fig. VIII.3. Gas Chromatographic Analysis of methylated ∝-D-Glucan, Fraction 270 after N-sulphuric acid hydrolysis, followed by reduction and acetylation on ECNSS-M column.

and a graph of log M.wt. against R_G value which was linear (Fig.VIII.2). Maltose was also detected.

E. Methylation Analysis of α-D-Glucan, Fraction 270

α-D-Glucan, Fraction 270 was methylated in good yield using the Haworth methylation technique; followed by the methyl iodide, sodium hydride, dimethyl sulphoxide method of Anderson and Cree (1966) as described in Expt.34. The absence of absorption at 3500 cm^{-1} in the infrared spectrum of the methylated product indicated that the methylation was complete.

The methylated glucan was hydrolysed with N-sulphuric acid (G.M.15A) and the partially methylated sugars formed were reduced with sodium borohydride and acetylated with acetic anhydride/sodium acetate (Expt.35). These derivatives were separated and quantitated by gas-liquid chromatography on ECNSS-M (G.M.24) and identified by their relative retention times and by their mass spectra (Bjorndal, Lindberg and Svensson, 1967a and 1967b).

The results are shown in Fig.VIII.3 and Table VIII.3.

Table VIII.3. Methylation analysis of α-D-Glucan, Fraction 270

Peak No.	Relative retention time (T)	Molar ratio	Identified by mass spectroscopy (M) and or relative retention time (T)
1	1.0	1.0	1,5-di-O-acetyl- 2,3,4,6 -tetra-O-methyl-D-Glucitol (M and T)
2	1.9	223.6	1,3,5-tri -O-acetyl- 2,4,6 -tri-O-methyl-D-Glucitol (M and T)
3	2.3	6.1	1,4,5-tri-O-acetyl- 2,3,6 -tri-O-methyl-D-Glucitol and/or 1,5,6-tri-O-acetyl- 2,3,4 -tri-O-methyl-D-Glucitol (T)
4	3.6	0.25	Unidentified di-O-methyl D-hexitol tetraacetates.
5	4.7	0.12	

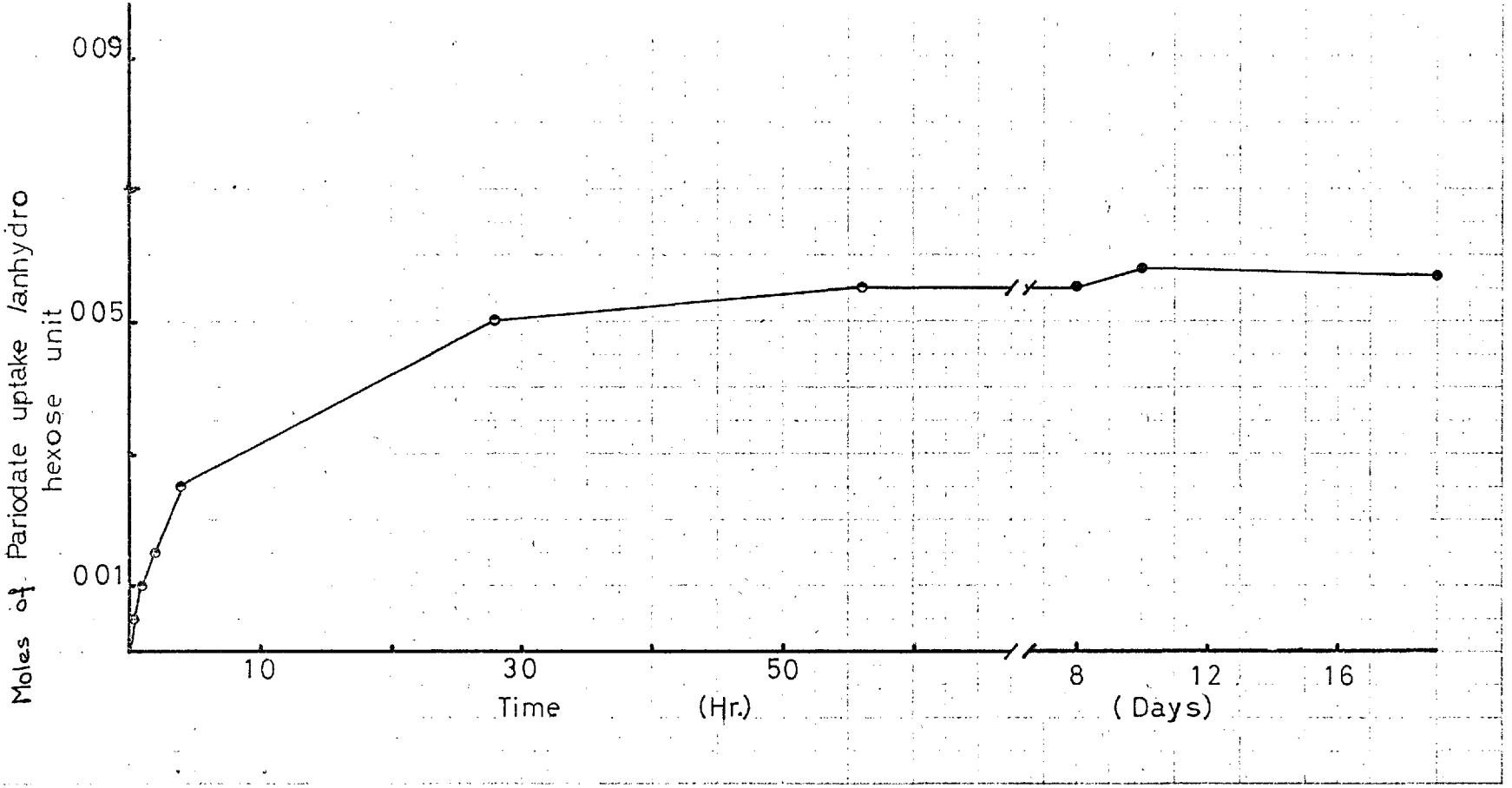


Fig. VIII.4. Periodate Oxidation of α-D-Glucan, Fraction 270.
(For details see Expt.36.).

F. Periodate Oxidation of α-D-Glucan, Fraction 270

α-D-Glucan, Fraction 270, was oxidised with an excess of sodium metaperiodate (0.500 - 1.136 mole/anhydro hexose unit) as described in Expt.36. The results are given in Table VIII.4. and Fig.VIII.4.

Table VIII.4. Periodate oxidation of α-D-Glucan, Fraction 270

Time	Moles per anhydro hexose unit		
	Periodate consumed G.M.31	Formic acid produced G.M.33	Formaldehyde produced G.M.32
0 hr.	-	-	-
0 hr.20min.	0.005	-	-
1 hr.	0.010	-	-
2 hr.	0.015	-	-
4 hr.	0.025	0.016	-
28hr.	0.050	-	-
56hr.	0.055	0.023	-
8days	0.055	-	-
10days	0.058	0.025	0.006
19days	0.057	0.022	0.006

- = Not determined.

G. Smith Degradation of α-D-Glucan, Fraction 270

Periodate oxidised, sodium borohydride reduced α-D-Glucan, Fraction 270 was hydrolysed at room teperature in 0.1N-sulphuric acid for 24 hr. as described in Expt.37. The insoluble residue was analysed after total acid hydrolysis by gas-liquid chromatography on ECNSS-M (G.M.24), after reduction of the product formed with borohydride and acetylation with acetic anhydride/sodium acetate as

described in Expt.37(b). The results are shown in Table VIII.5 together with the results obtained from the total hydrolysis of periodate oxidised borohydride reduced α-D-Glucan, Fraction 270, in N-sulphuric acid (G.M.15A).

Table VIII.5 Composition of fractions obtained by Smith degradation of α-D-Glucan, Fraction 270

	Molar % of sugars and glycitols	
	α-D-Glucan, Fraction 270 (1) Periodate oxidised (2) Borohydride reduced	α-D-Glucan Fraction 270 Residue after Smith degradation
Glucose	94.92	98.37
Arabinose	1.00	Traces
Xylose	0.37	Traces
Threitol	0.36	Traces
Erythritol	2.62	1.13
Glycerol	0.73	0.50

Paper chromatography (Solvent e) of the soluble product formed on Smith degradation showed the presence of glycerol, threitol and erythritol (main component) with traces of products of R_G value 0.09 and 0.29.

Smith degraded residual α-D-Glucan, Fraction 270 had $[\alpha]_D$ + 279°. Analytical Ultracentrifugation on Beckman Model E of the Smith degraded residue at 160,000g gave a broad peak which diffused rapidly at lower concentration, and hence no accurate S value could be obtained; however it was definitely much less than original α-D-Glucan, Fraction 270 (Fig. VII.1 and Fig. 4 appendix).

IX STRUCTURAL STUDIES OF β-D- GLUCAN, FRACTION 27K

The following experiments were carried out to study the structure of β-D-Glucan, Fraction 27K, using, physical, chemical and enzymic methods.

A. Physical Properties of β-D-Glucan, Fraction 27K:

β-D-Glucan, Fraction 27K had $[\alpha]_D$+ 16°. After freeze drying it was not soluble in water but it stayed in solution after neutralisation of a N-sodium hydroxide solution of the glucan. Its infrared spectrum (p. 233) showed an absorption maximum at 890 cm^{-1}, characteristic of β-glucopyranose linkages and the whole range of spectrum was very similar to laminarin. There was no absorption at 850 cm^{-1}, characteristic of α-glucopyranose linkages. When examined with the Beckman Model E Analytical Ultracentrifuge at 160,000 g. with Schlieren optics as described in G.M.29 only one peak with S^{o}_{20} 4.6 (N-sodium hydroxide) was observed. β-D-Glucan, Fraction 27K, was almost completely excluded from Sephadex G-200; its elution profile is shown in Fig. 4 (p. 236).

B. Chemical Composition of β-D-Glucan, Fraction 27K :

The carbohydrate composition of β-D-Glucan, Fraction 27K, determined after N-sulphuric acid hydrolysis (G.M.15A) is shown in Table IX.1. Examination of the hydrolysates by paper chromatography (Solvents b & c) showed, glucose (main componont), galactose, mannose, rhamnose, arabinose and xylose. The amino acid analysis, determined after 6N-hydrochloric acid hydrolysis for 24 hr. (G.M.15 B) is shown in Table IX.2.

Table IX.1 Carbohydrate composition of β-D-Glucan, Fraction 27K

	Methods	% anhydro sugar
Total Hexose	G.M.1	96.0
Glucose	G.M.3	88.4
Galactose	G.M.4a	4.4

Table IX.2 Amino acid composition of β-D-Glucan, Fraction 27K

Amino acid	% anhydro amino acid	Molar ratio
Aspartic acid, Threonine, Serine (Not separated)	0.0273 (total)	13.4 (total) (based on average molecular weight)
Alanine	0.013	9.2
Arginine	0.030	9.6
Glutamic acid	0.011	4.5
Glycine	0.008	7.0
Histidine	0.010	3.5
Isoleucine	0.004	1.8
Leucine	0.007	3.1
Lysine	0.004	1.5
Phenylalanine	0.006	2.1
Tyrosine	0.003	1.0
Valine	0.010	5.0
Total	0.133	

C. Action of Enzymes :

(a) Action of Basidiomycete QM806 exo-β-D-(1⟶3)-glucanase on β-D-Glucan, Fraction 27K : β-D-Glucan, Fraction 27K, was hydrolysed and almost completely solubilised by pachyman purified exo-β-D (1⟶3)-glucanase as described in Expt.28a. Examination of the incubation mixture by paper chromatography (Solvent b) revealed the presence of

only glucose and gentiobiose; after N-sulphuric acid hydrolysis (G.M.15A) galactose and traces of mannose, xylose, arabinose and rhamnose were also detected. The composition of the incubation mixture is given in Table IX.3.

Table IX.3 Action of exo-β1→3-glucanase on β-D-Glucan, Fraction 27K

	Method	Wt.% anhydro sugar
Total Hexose (H)	G.M.1	95.4
Reducing sugars (as glucose)	G.M.2	83.7
Glucose (G)	G.M.3	78.1
Gentiobiose	(Total Glucose) -(Glucose released by β1→3 glucanase)	10.3

Molar Ratio of Glucose : Gentiobiose released = 15.1:1

% β1→3 linkages in β-D-Glucan, Fraction 27K = 83.25
(= Molar % Glucose + Molar % Gentiobiose released)

% β1→6 linkages in β-D-Glucan, Fraction 27K = 5.15
(= Molar % Gentiobiose released)

(b) Action of Helix pomatia endo-β-D-(1→3)-glucanase on β-D-Glucan, Fraction 27K : Purified H.pomatia endo-β-D-(1→3)-glucanase solubilised β-D-Glucan, Fraction 27K completely, forming a number of oligomers [Expt.28(b)] . The oligomers were identified by a technique which involved paper chromatography on one direction, spraying the oligosaccharide zones with Basidiomycete QM806 exo-β-D-(1→3)-glucanase and then chromatography at right angles to the first direction. The results with two different Solvent systems are

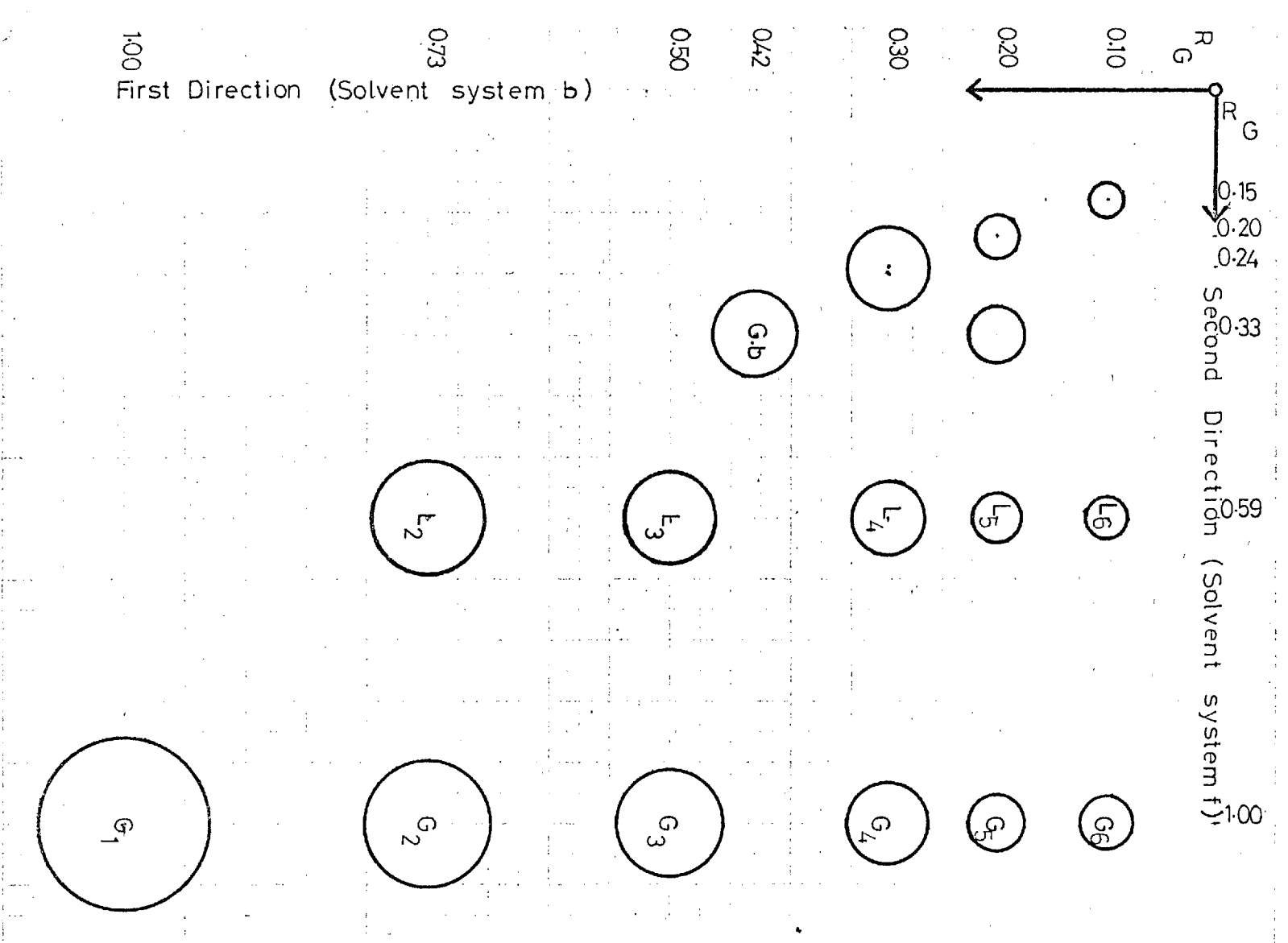


Fig. IX.1. Two dimensional paper chromatography of oligomers formed by the action of endo β-D-(1→3)-glucanase on β-D-Glucan, Fraction 27K.
(For detail see Expt. 28b.).

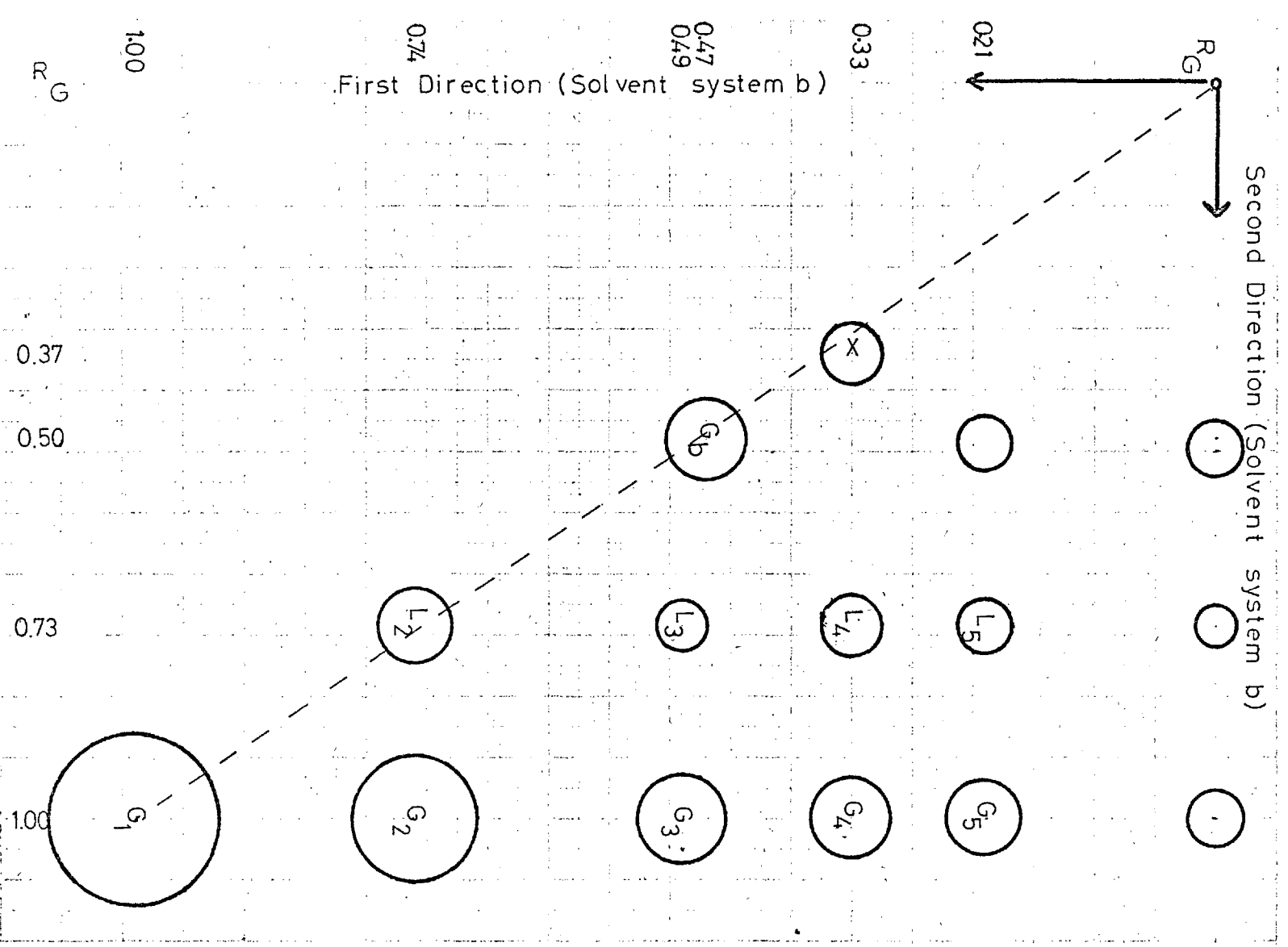


Fig. IX.2. Two dimensional paper chromatography of oligomers formed by the action of endo β-D-(1→3)-glucanase on β-D-Glucan, Fraction 27K.
(For detail see Expt. 28b.)

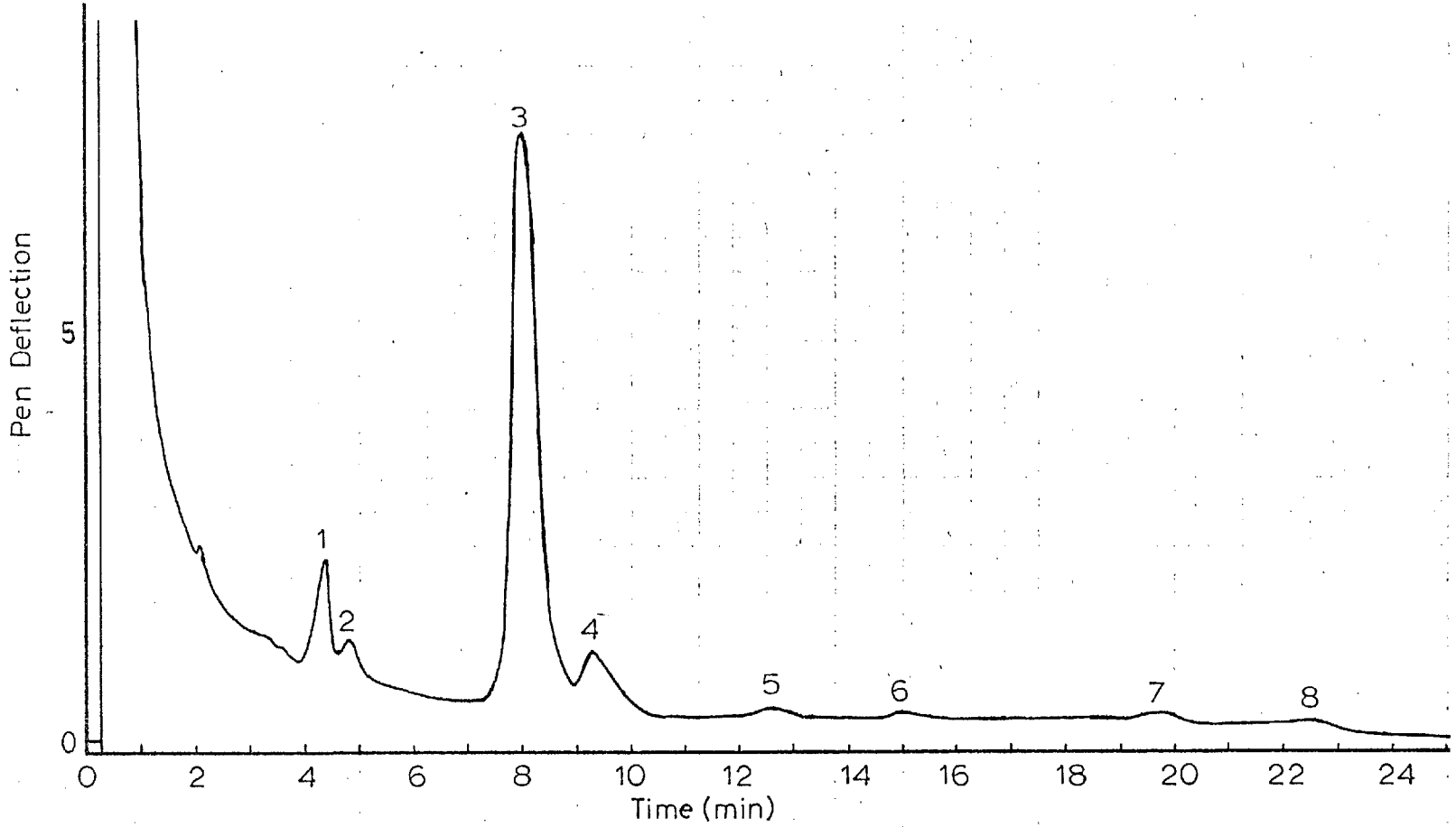


Fig. IX.3. Gas chromatographic analysis of methylated β-D-Glucan, Fraction 27K after N-sulphuric acid hydrolysis followed by reduction and acetylation. (For detail see Expt. 29.).

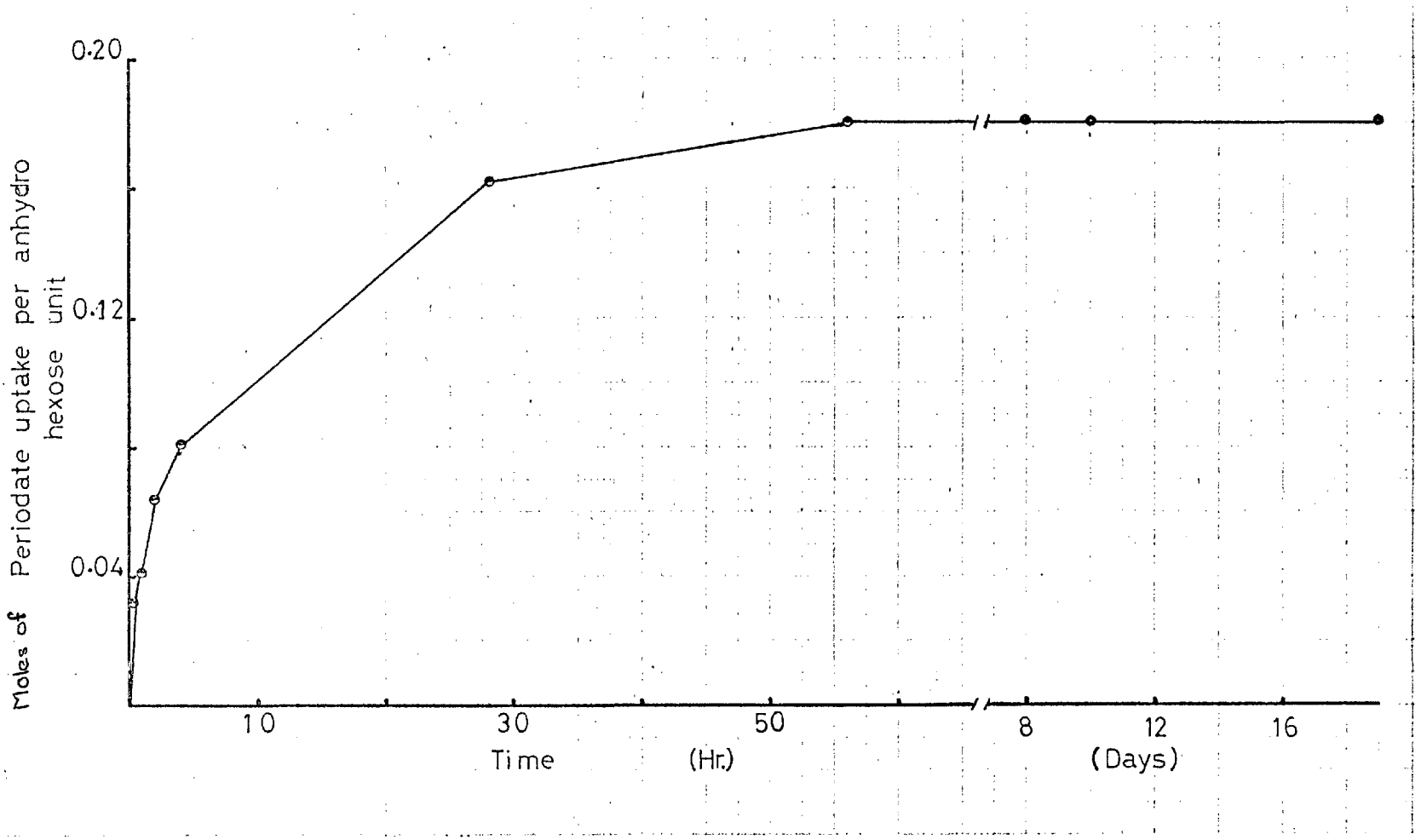


Fig. IX.4. Periodate oxidation of β-D-Glucan, Fraction 27K.
(For detail see Expt. 30a.).

shown in Fig.IX.1 and in Fig.IX.2, where the same solvent system is used in both directions. Spots on the diagonal (Fig.IX.2) are those resistant to the exo-$\beta 1 \rightarrow 3$ glucanase, while other spots are the result of the enzyme action. The results indicated that the products of action of the H.pomatia endo $\beta 1 \rightarrow 3$ glucanose include gentiobiose, laminaribiose, and all the laminaridextrins upto laminariheptaose. The unidentified spot X could be branched $\beta 1 \rightarrow 3$, $\beta 1 \rightarrow 6$ oligomer.

D. Methylation Analysis of β-D-Glucan, Fraction 27K.

β-D-Glucan, Fraction 27K was methylated in good yield by the methyl iodide, sodium hydride, dimethyl sulphoxide method of Anderson and Cree (1966) as described in Expt.29. The absence of absorption at 3500 cm^{-1} in the infrared spectrum of the methylated product indicated that the methylation was complete.

The methylated glucan was hydrolysed with N-sulphuric acid (G.M.15A) and the partially methylated sugars formed were reduced with sodium borohydride and acetylated with acetic anhydride/sodium acetate (Expt.29). These derivatives were separated and quantitated by gas-liquid chromatography on ECNSS-M (G.M.24) and identified by relative retention times and by their mass spectra (Bjorndal, Lindberg and Svensson, 1967a and . 1967b).The results are shown in Fig.IX.3 and Table IX.4.

E. Periodate Oxidation of β-D-Glucan, Fraction 27K

β-D-Glucan, Fraction 27K, was oxidised with an excess of sodium metaperiodate (1.62 mole/anhydro hexose unit) as described in Expt. 30(a). The results are given in Table IX.5 and Fig.IX.4.

F. Smith Degradation of β-D-Glucan, Fraction 27K

Periodate oxidised, borohydride reduced β-D-Glucan, Fraction 27K, was hydrolysed by two different methods and the products formed were analysed by gas-liquid chromatography after reduction of the product with borohydride followed by acetylation as described in Expt. 30(a). The results are given in Table IX.6. Paper chromatography (Solvent e) of the N-sulphuric acid hydrolysis products showed the

Table IX.4 Methylation analysis of β-D-Glucan, Fraction 27K

Peak No.	Relative retention time (T)	Molar ratio	Identified by mass spectroscopy(M) and or relative retention time (T)
1	1.00	1.00	1,5-di-O-acetyl -2,3,4,6-tetra-O-methyl -D-Glucitol (M and T).
2	1.12	0.24	Tentatively identified as 1,4 (1,5)-di-O-acetyl -2,3,5,6(2,3,4, 6)-tetra-O-methyl -D-Galactitol. (T)
3	1.90	9.20	1,3,5-tri-O-acetyl- 2,4,6-tri-O-methyl -D-Glucitol. (M and T)
4	2.30	1.05	1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-Glucitol (and/or mannitol and/or Galactitol). 1,5,6 -tri-O-acetyl-2,3,4-tri-O-methyl -D-Glucitol (T).
5	3.02	0.27	
6	3.62	0.22	Unidentified di-O-methyl
7	4.70	0.32	D-hexitol tetraacetates.
8	5.30	0.27	

presence of glucose (main component), traces of xylose, an unresolved mixture of arabinose, threitol and erythritol (identified after elution from the paper followed by acetylation and gas-liquid chromatography) and glycerol. The soluble product formed on Smith degradation contained glycerol, erythritol, threitol, and traces of products of R_G value 0.06, 0.33, 0.52 and 0.68 detectable by silver-nitrate spray reagent a, but not with aniline hydrogen phthalate spray reagent b.

Table IX.5. Periodate oxidation of β-D-Glucan, Fraction 27K.

Time	Moles per anhydro hexose unit		
	Periodate consumed G.M.31	Formic acid produced G.M.33	Formaldehyde produced G.M.32
0 hr.	-	-	-
0 hr. 20 min.	0.032	-	-
1 hr.	0.041	-	-
2 hr.	0.064	-	-
4 hr.	0.081	0.023	-
28 hr.	0.162	-	-
56 hr.	0.180	0.065	-
8 days	0.180	-	-
10 days	0.180	0.065	0.0178
19 days	0.180	0.065	0.017

- = Not determined.

Table IX.6 Composition of fractions obtained by Smith degradation of β-D-Glucan, Fraction 27K.

- = Not determined

	Molar % of sugars and glycitols	
	β-D-Glucan, Fraction 27K (1) Periodate oxidised (2) Borohydride reduced Residue	β-D-Glucan, Fraction 27K Residue after one Smith degradation
Glucose	88.9	96.6
Arabinose	1.3	1.5
Xylose	-	0.3
Threitol	3.4	0.3
Erythritol	1.9	0.3
Glycerol	4.5	1.0

G. Comparison of the Action of Basidiomycete QM806 Exo-β-D-(1→3)-Glucanase Action on β-D-Glucan, Fraction 27K, and the Residues after One and Two Smith Degradations.

β-D-Glucan, Fraction 27K, before and after one and two Smith degradations was incubated with exo- β-D-(1→3) glucanase as described in Expt. 31. Glucose and gentiobiose were the only products detected by paper chromatography (Solvent b). The composition of the products is shown in Table IX.7.

Table IX.7 Composition of the products of action of exo-β1→3 - glucanase on β-D-Glucan, Fraction 27K, before and after one and two Smith degradations.

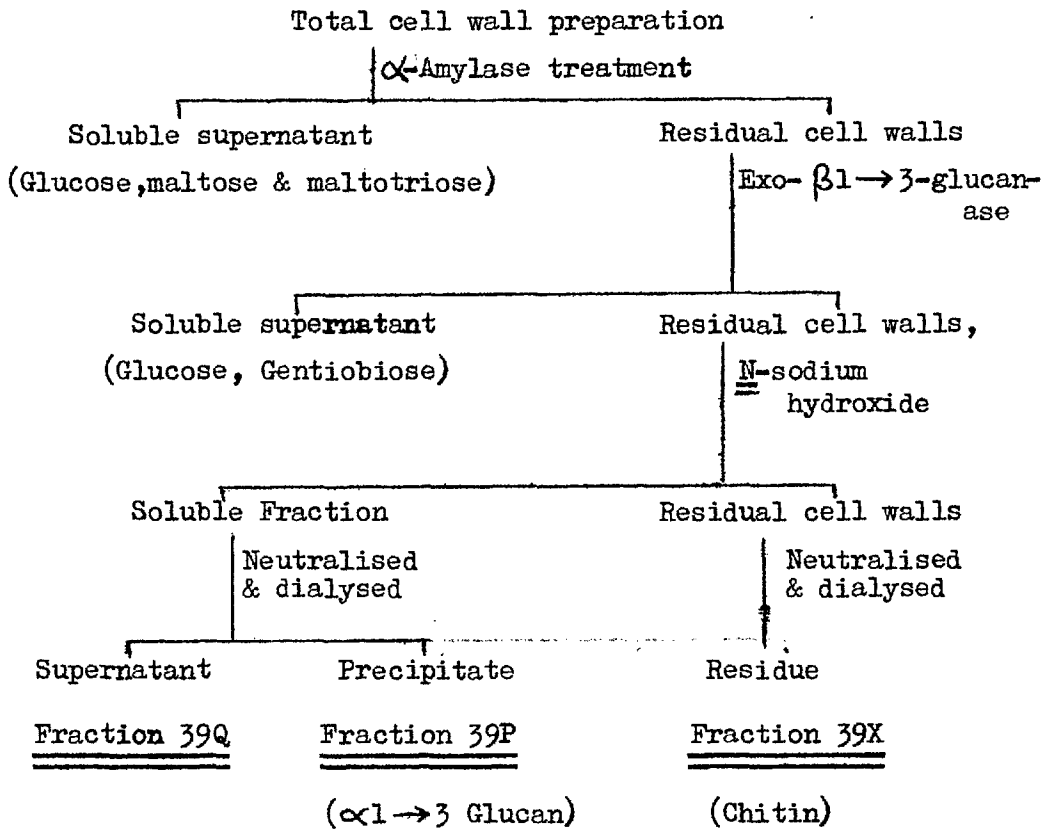
	Method	β-D-Glucan, Fraction 27K Wt. % of total anhydro hexose		
		Before Smith degradation	After one Smith degradation	After two Smith degradations
Total Hexose	G.M.1	100	100	100
Reducing sugars as glucose	G.M.2	87.7	80.2	91.4
Glucose	G.M.3	81.9	67.0	79.3
Molar ratio of Glucose : Gentiobiose	$\frac{\text{(Total Glucose-Glucose liberated)}}{2}$ = Gentiobiose	15.1:1	4.1:1	7.7:1
	(Reducing sugars - Glucose) = Gentiobiose	14.1:1	5.1:1	6.6:1

X. SEQUENTIAL ENZYMIC HYDROLYSIS OF CELL WALLS

A. Semiquantitative Determination of the Polysaccharide Linkage Composition of Fusicoccum Cell Walls

The method is described in detail in Expt.39 and is summarised in Fig.X.1.

Fig.X.1. Scheme for determination of polysaccharide linkage composition of Fusicoccum cell walls.



α 1⟶4 Glucopyranose linkages

α-Amylase acts on Fusicoccum cell walls liberating glucose, maltose and maltotriose. The amount of total hexose liberated was taken as the α1 ⟶ 4 linkages (Table II.5 p. 44).

The average $\bar{DP}$ of oligosaccharides in the supernatant

$$= \frac{\text{Total Hexose - Glucose}}{\text{Reducing sugar- Glucose}} \quad \text{(as anhydro)}$$

(assuming equivalent reducing power for oligomers)

$$\text{For 1st Batch } \bar{DP} = \frac{42.0 - 13.0}{24.3 - 13.0} = 2.57$$

$$\text{For 4th Batch } \bar{DP} = \frac{48.6 - 15.0}{28.0 - 15.0} = 2.58$$

i.e. little if any polymeric carbohydrate had been released into solution.

The value obtained 8.4% for the 1st Batch and 9.7% for the 4th Batch) may be low because α-amylase may leave stubs of $\alpha 1 \rightarrow 4$ linked glucose residues attached to the residue.

$\beta 1 \rightarrow 3$ and $\beta 1 \rightarrow 6$ Glucopyranose linkages

Basidiomycete QM806 exo-$\beta 1 \rightarrow 3$ glucanase acted on the residue after α-amylase treatment liberating only glucose and gentiobiose. Maltose and maltotriose could not be detected, indicating that stubs of $\alpha 1 \rightarrow 4$ linked glucose had not been liberated. The composition of the supernatant is given in Table X.1.

Table X.1 The action of exo-β-D-$(1 \rightarrow 3)$glucanase on the residue obtained after α-amylase action on the Fusicoccum cell walls.

Original cell wall preparation	Wt.taken (mg.)	Sugar in supernatant measured as anhydro glucose (mg.)			Residue in mg.	% recovery
		Total Hexose G.M.1	Reducing Sugar G.M.2	Glucose G.M.3		
1st Batch	204.5	118.8	105.3	82.8	80.0	97.2
4th Batch	203.0	111.6	100.8	83.0	92.0	100.3

The amount (mg.) of gentiobiose present in the supernatant

$$= \frac{\text{Total Hexose - Glucose}}{2} \text{ (as anhydro)}$$

$$\text{For 1st Batch} = \frac{118.8 - 82.8}{2} = 18.0$$

$$\text{For 4th Batch} = \frac{111.6 - 83.0}{2} = 14.3$$

The number of glucose + gentiobiose molecules liberated was taken as the $\beta 1 \longrightarrow 3$ linkages. The number of gentiobiose molecules liberated was taken as the $\beta 1 \longrightarrow 6$ linkages

For the 1st Batch $\bar{D}P$ of oligosaccharides in the supernatant

$$\sim \frac{36}{22.5} = 1.6$$

For the 4th Batch $\bar{D}P$ of oligosaccharides in the supernatant

$$\sim \frac{28.6}{17.8} = 1.6$$

The values are very approximate because of the non-equivalent reducing power of gentiobiose but indicate that little, if any, polymeric carbohydrate had been released into solution at this stage.

$\alpha 1 \longrightarrow 3$ Glucopyranose linkages

N-Sodium hydroxide solubilises all the glucan from the cell wall left after consecutive treatments of the Fusicoccum cell walls with α-amylase and exo -$\beta 1 \longrightarrow 3$ glucanase. The alkali soluble, water insoluble glucan (Fraction 39P), $[\alpha]_D$ + 290°, obtained after neutralisation of the supernatant, had an infrared spectrum identical to that of α-D-Glucan, Fraction 270. The X-ray diffraction pattern was similar, but not identical to that of α-D-Glucan, Fraction 270, but different from Johnston's α-D-Glucan, Fraction IVR (Johnston, 1965) obtained from A.niger (Table 2 , Fig. 2 , appendix). The weight of

Fraction 39P (Table X.2) was taken as $\alpha 1 \rightarrow 3$ glucan; it may be slightly high due to the presence of small amounts of galactose, mannose and protein and possibly $\alpha 1 \rightarrow 4$ and $\alpha 1 \rightarrow 6$ glucopyranose linkages.

Table X.2 N-Sodium hydroxide fractionation of Fusicoccum cell wall residue after consecutive treatments with α-amylase and $\beta 1 \rightarrow 3$ glucanase

Original cell wall preparation	Wt. taken (mg.)	Fractions from N-sodium hydroxide fractionation (mg.)			% recovery
		Water soluble	Neutralised precipitate	Insoluble residue	
1st Batch	55	3.0	43.5	6.0	95.4
4th Batch	55	2.7	42.5	6.0	93.3

Chitin

The final alkali insoluble residue (Fraction 39X) was composed of 95% N-acetyl-D-glucosamine (Table IV.4 p. 76) and some protein, but no hexose, and had an infrared spectrum (Fig.IV.2 p. 72) and X-ray diffraction pattern very similar to those of crustacean chitin (Fig. 2 and Table 2,appendix).

The overall composition is shown in Table X.3.

Table X.2 Semiquantitative estimation of polysaccharide linkage composition of Fusicoccum cell walls.

Linkage	% 1st Batch	% 4th Batch
$\alpha 1 \rightarrow 4$ Glucopyranose	8.4	9.7
$\beta 1 \rightarrow 3$ Glucopyranose	48.4	43.3
$\beta 1 \rightarrow 6$ Glucopyranose	16.2	12.7
$\alpha 1 \rightarrow 3$ Glucopyranose	27.8	31.3
Chitin	3.8	4.4

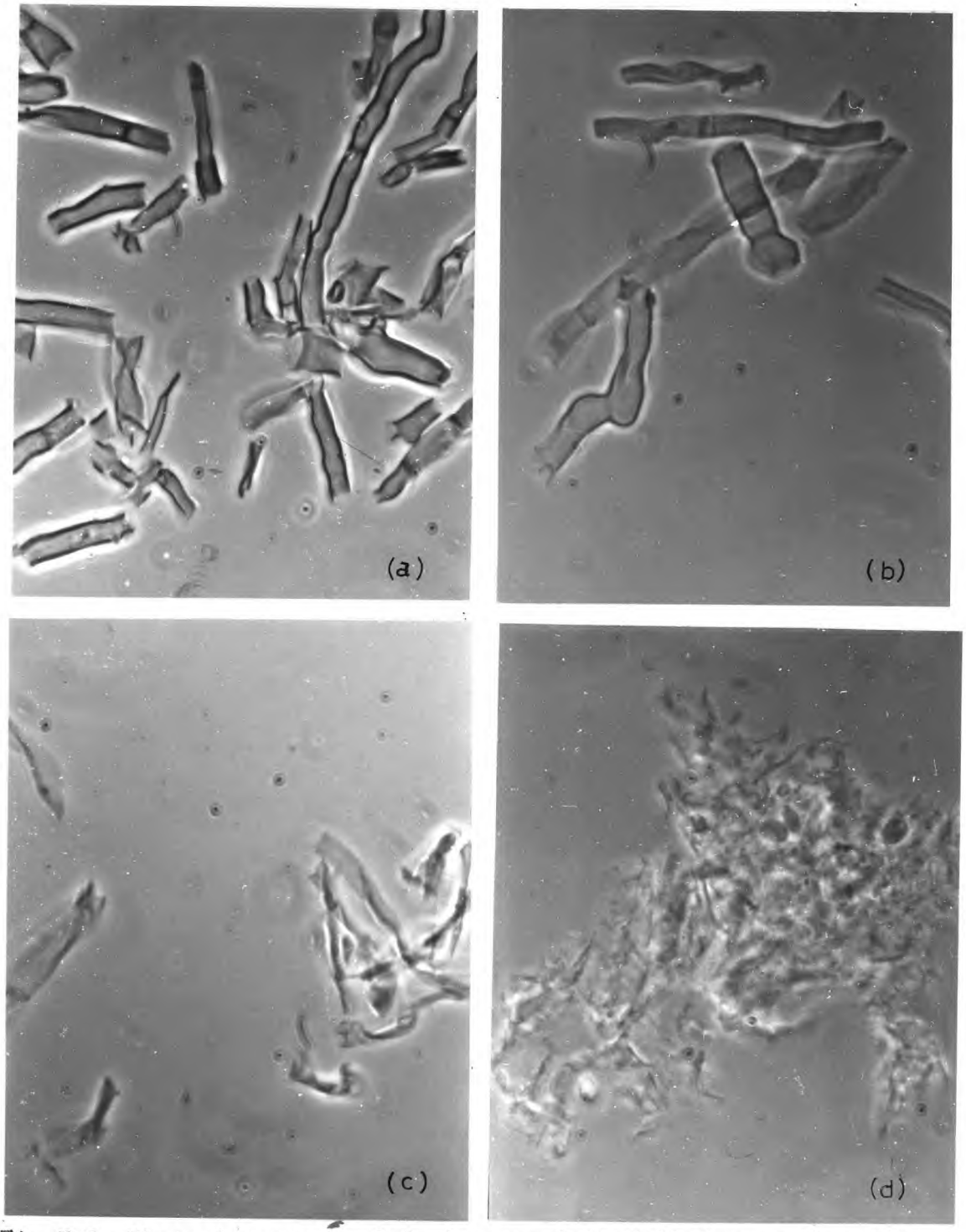


Fig.X.2. Photomicrograph of Fusicoccum Cell Wall Preparation After Different Enzymic and Chemical Treatments.
(a) Total cell walls. (b) Cell walls after α-amylase action.
(c) α-amylase and β1,3-glucanase treated cell walls.
(d) Residue after treatment of cell walls with α-amylase and β1,3-glucanase, followed by N-sodium hydroxide extraction. (Chitin preparation).

The percentages were determined on the basis of the total cell wall and did not take into account losses during the different processes.

B. Microscopic Examination of Fusicoccum Cell Walls During Different Stages of Enzyme Treatment.

Fusicoccum cell walls appeared thick and rigid when examined under the phase contrast microscope (magnification 100 x 10); they stained blue-black with 0.1N-iodine solution (Fig.X.2a). After treatment with α-amylase they appeared much thinner, and had lost their rigidity as indicated by their tendency to fold up very easily (Fig.X.2b). The cell wall residue after consecutive treatments with α-amylase and exo-β1→3 glucanase had lost all the rigidity and shape of the original walls (Fig.X.2c). The final residue after further N-sodium hydroxide extraction (Fraction 39X = chitin) had no characteristic shape or size (Fig.X.2d).

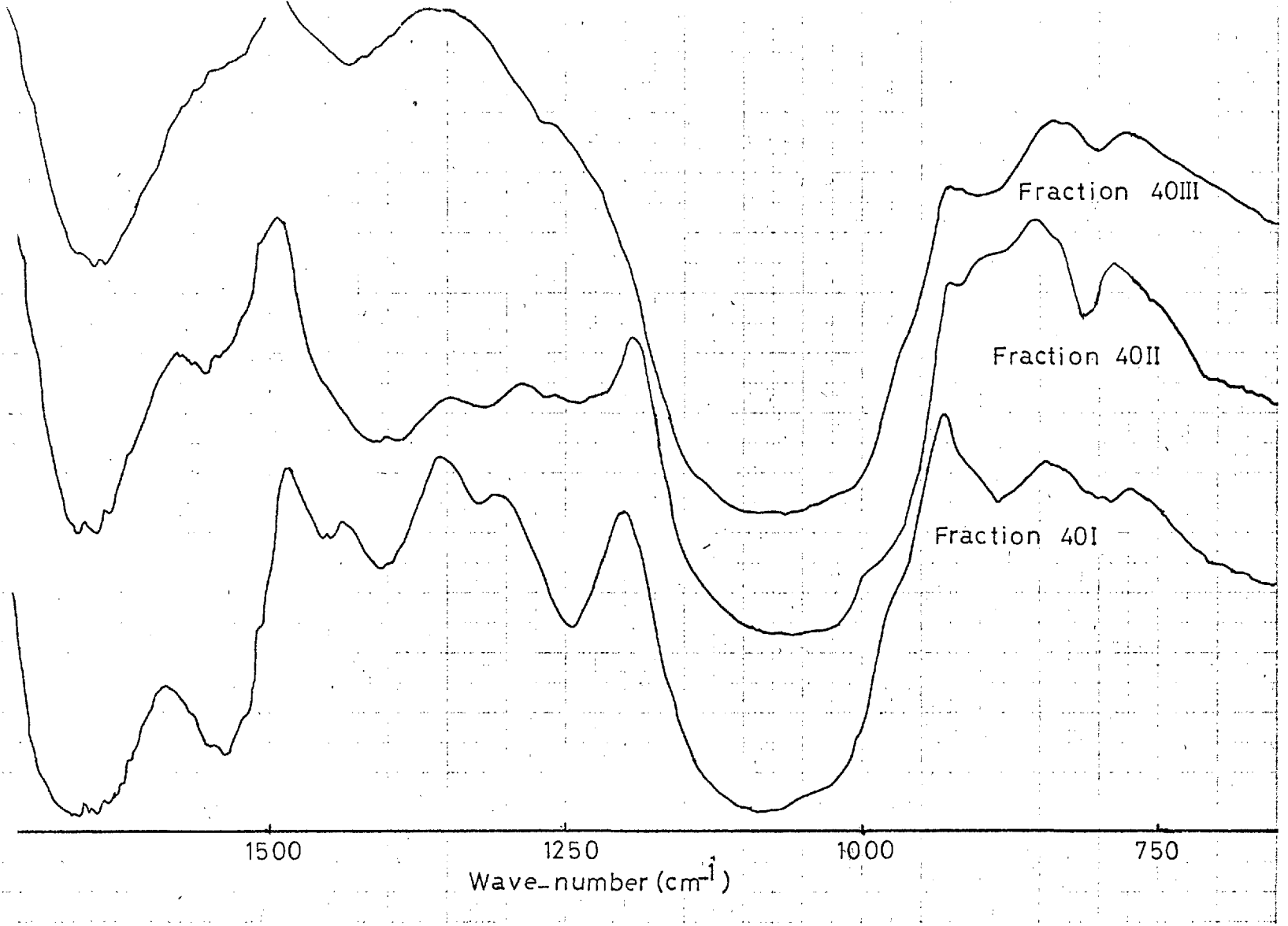


Fig. XI.1. Infrared Spectra of Different Extracellular Polysaccharides from Fusicoccum Culture Medium.

XI ISOLATION AND PROPERTIES OF EXTRACELLULAR POLYSACCHARIDES.

Three types of extracellular polysaccharides were isolated from $3\frac{1}{2}$ day old cultures grown in shaken flasks on Medium IIa as described in Expt. 40.

Fraction I. Dialysable; precipitated by ethanol.

Fraction II. Non-dialysable; water soluble.

Fraction III. Non-dialysable; precipitates out on dialysis.

The carbohydrate composition of the three fractions is given in Table XI.1.

Table XI.1 Carbohydrate composition of extracellular polysaccharides.

	Ratio of different sugars (G.M.7)		
	Fraction I	Fraction II	Fraction III
Galactose	Traces	37	37
Glucose	30	11	11
Mannose	10	26	26
Arabinose	0	0	0
Xylose	50	Traces	Traces
Rhamnose	10	26	26

The infrared spectra of the three fractions were different from one another. Fraction II stained blue black with iodine but Fraction I and III did not.

DISCUSSION

A. Chemical Composition

The cell walls of Fusicoccum amygdali consisted of 85% polysaccharides with small amounts of lipid (5%) and protein (4-6%); these components accounted for 94-96% of the cell walls.

Seven of the twelve naturally occurring sugars so far identified as fungal cell wall constituents (see introduction and Bartnicki-Garcia, 1968) were found. Of these only three are consistently found in most fungi, namely D-glucose, D-glucosamine and D-mannose (Crook and Johnston, 1962; Bartnicki - Garcia, 1968). Small amounts of D-galactose, L-rhamnose, xylose and arabinose were also found. D-galactose is fairly characteristic of the Ascomycetes, while L-rhamnose has been reported in the cell walls of Penicillium chrysogenum (Hamilton and Knight, 1962), and a number of Phycomycetes (Novaes-Ledieu, Jimenez-Martinez and Villanueva, 1967). There have been very few reports of the occurrence of pentoses in fungal cell walls. Hamilton and Knight (1962) found small amounts of xylose in P.chrysogenum and it was also reported in Polystictus sanguinens (Crook and Johnston, 1962). Arabinose has been found in the cell walls of Aspergillus niger (Johnston, 1965) and P.chrysogenum (Greenawalt, 1960). The role of these minor components in cell wall construction is unknown.

The cell walls of F.amygdali contained a small amount of protein (4 - 6%). The quantitative determination of protein in association with polysaccharides is difficult (Gottschalk, 1965). Of the methods used, Method I [Total protein = 6.25 (Total nitrogen - Hexosamine nitrogen)] is approximate because of the varying nitrogen contents of different proteins, possible error in the hexosamine assay and the possible presence of other nitrogenous materials. Method II (Ninhydrin colouration after acid hydrolysis, calibrated against bovine serum albumin) does not allow for destruction of amino acids during hydrolysis; moreover the extinctions of different protein vary with amino acid content. Method III (Sum of individual amino

acids) is potentially the most accurate, but in this case did not include values for cysteine, cystine, methionine and proline and the value obtained (3.7%) was therefore probably low. Most of the common amino acids were detected, namely alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalamine, proline, serine, threonine, tyrosine and valine. Hydroxyproline, which has been detected only in cell walls containing cellulose (Bartnicki - Garcia, 1968), was not detected.

There was abundant evidence that most of the protein in the cell wall of F.amygdali was a genuine cell wall component and not contamination from the cytoplasm or cytoplasmic membrane.

(a) Very little (about 10% of the protein) could be extracted with phenol.

(b) Incubation of cell walls with pronase released 25% of the cell wall galactose and 20% of the cell wall mannose as water soluble polymeric material, suggesting that this material was linked to the cell walls by peptide bonds. 33% of the cell wall protein was not solubilised by pronase indicating that either the remaining protein was inaccessible to the enzyme or bound by linkages resistant to pronase action (Result II E a). The action of α-chymotrypsin was similar, but less extensive than that of pronase (Result II E b).

(c) Cell walls could be fractionated with N-alkali into three portions (i) alkali insoluble (36.0%) (ii) alkali soluble, water insoluble (36.5%) (iii) water soluble (20.0%). When the fractionation was carried out after prior pronase treatment, the total amount of the water soluble fraction increased to 36.0%. Solubilisation by alkali may of course itself be due in part to hydrolysis of peptide linkages.

(d) Similarly when cell walls were extracted with DMSO, 20% was dissolved. However when pronase action preceded DMSO extraction the total amount solubilised increased to 32%. In contrast when phenol extraction preceded DMSO extraction the total amount solubilised was 20% i.e. the total solubility was not increased. This increase in solubility after enzymic deproteinisation and not by phenol extraction

is probably due to removal of peptide linkages between the different polymers.

(e) Small amounts of protein remained in cell wall fractions insoluble in alkali. This suggests that some of the protein is "hidden" or very firmly bound, although the possibility of wall contamination by alkali insoluble protein cannot be completely excluded.

The above evidence indicated that protein is an integral part of the cell wall structure and probably serves to link polysaccharide chains. Evidence for a structural role of protein in other walls e.g. Saccharomyces cerevisiae was described in the Introduction.

Fusicoccum amygdali cell walls contained 2.2% readily extractable lipids (free lipids) and 2.7% bound lipids (lipoproteins, glycolipids, etc.). The extent to which these values could be contributed by contamination with cytoplasmic membrane fragments could not be assessed, as electron microscope facilities were not available, and membranes would not be clearly visible in the light microscope. Removal of the free lipids with ethanol and ether did not alter the alkali solubility of the cell walls (K.W. Buck, unpublished results), whereas removal of bound lipids with acidified ethanol and ether followed by chloroform, probably resulted in hydrolysis of some of the carbohydrate linkages in the cell walls, as evidenced by loss of their iodine staining capacity. Although small amounts of lipid have been reported in most studies on fungal cell walls, little is known of its structural role (see Introduction).

No definite evidence could be obtained for the presence of nucleic acids in the cell walls of F.amygdali from the U.V. spectrum or paper chromatography of acid hydrolysates. Moreover failure to detect either ribose or deoxyribose in the carbohydrate analysis excludes the presence of significent amounts of RNA or DNA. This result is in agreement with studies on fungal cell walls in general and also provides more evidence for wall preparations free from cytoplasmic contamination.

Fusicoccum amygdali cell walls contained 0.044 - 0.103% phosphorus, equivalent to 0.145 - 0.340% phosphate (as $NaPO_3$).

Two types of phosphorus in the cell wall were distinguished on the basis of acid lability. In the case of the 4th cell wall Batch 48% of the phosphorus was labile to dilute acid and probably represents polyphosphate. All of the acid labile phosphorus was released after incubation of cell walls with pronase, suggesting that the phosphate was bound either to the protein portion of the cell wall or to the galactomannan polymer which is also released. The remaining phosphorus may be largely accounted for as sugar phosphate (see Result V), but the possibility of a contribution from phospholipid cannot be excluded. Small amounts of phosphorus have been reported in many fungal cell walls. Mannose-6-phosphate has been isolated from yeast cell walls (Sentandreu and Northcote, 1968) and Harold (1962) has shown that a considerable amount of polyphosphate is bound to a polygalactosamine component in the cell walls of Neurospora crassa.

B. Nature of the Galactomannan Heteropolymers

The cell walls of Fusicoccum amygdali contain, in addition to 70% glucose, other neutral sugars, namely galactose (3.9%), mannose (2.8%), rhamnose (1.2%), xylose (0.9%) and ~~mannose~~ arabinose (0.5%). Most of these were released as water soluble polymer(s), with traces of glucose, on incubation of cell walls with Helix pomatia enzymes (Result II A). This heteropolymeric material was mainly excluded from Sephadex G-200 gel, indicating a molecular weight above about 200,000. In contrast, treatment of the cell walls with pronase released about 30% of the above heteropolymer, which gave a broad elution profile on sephadex G-200 in the molecular weight range 160,000 - 50,000 (Fig. II 6); the ratio of sugars in different fractions of the elution profile varied considerably (Table II.8) indicating that the material was heterogeneous. The above evidence indicates that (a) most of the neutral sugars,other than glucose, form a distinct heteropolymer or heteropolymers, (b) the polymers released by Helix pomatia enzymes probably consist of smaller units which are joined together by peptide linkages, (c) the ability of pronase to release only a fraction of the total heteropolymer suggests that this may be linked to the cell wall glucan

by glycosidic bonds. An alternative explanation is that some of the cell wall peptide bonds were unavailable to pronase.

Sugar phosphates were isolated from acid hydrolysates of the heteroglycans released by pronase. These were tentatively identified on the basis of (a) phosphatase action and (b) sequential periodate oxidation, borohydride reduction and phosphatase action as galactose-6-phosphate and mannose-6-phosphate, but further studies are required to distinguish them unequivocally from the corresponding 2-phosphates. Unidentified sugar phosphates, containing galactose, mannose, xylose and glucose were also detected and their chromatographic mobilities suggested that they may be sugar diphosphate (or cyclic phosphate). It is thus likely that some of the linkages in the heteroglycans are through phosphate bonds. Such linkages have been postulated to hold together mannan units in the yeast cell wall (Sentandreu and Northcote, 1968) and are found in a number of extracellular polysaccharides of fungi e.g. the phosphomannan of Hansenula holstii (Jeanes and Watson, 1962) and the phosphogalactan of Sporobolomyces spp. Y-6493 and Y6502 (Slodki, 1966; Gorin and Spencer, 1968).

Further evidence that the heteropolymers were joined to the cell wall via peptide bonds was provided by the fractionation experiments. Aqueous alkali released much more of the cell wall galactose and mannose than the milder reagents dimethyl sulphoxide or ethylenediamine. It is possible that the aqueous alkali caused β-elimination reactions to occur in bonds involving threonine and serine (peptide or glycosidic linkages), whereas these linkages would be stable to ethylenediamine and dimethylsulphoxide (Sentandreu and Northcote, 1968).

Some of the galactose in the cell walls was probably linked in the 4 position, as the purified α and β-D-Glucans isolated from the cell walls which contain small amounts of galactose, gave rise to

threitol after periodate oxidation, borohydride reduction and acid hydrolysis (see later in the discussion). Further evidence to be discussed later suggested that some of the galactomannan heteropolymer was responsible for linking an $\alpha 1 \rightarrow 4$ and $\beta 1 \rightarrow 3$, $1 \rightarrow 6$ glucan.

Extracellular polysaccharides were isolated from the culture medium. The non-dialysable fraction had a very similar ratio of galactose, mannose and rhamnose (see Table II.2 and XI.1) as the non-dialysable heteroglycans obtained after treatment of the cell walls with the Helix pomatia enzymes. The almost total absence of xylose from both the nondialysable extracellular polysaccharide fractions and also the heteroglycans obtained by snail enzyme as well as pronase treatment on the cell wall is probably due to the lower molecular weight and dialysable properties of the xylose oligosaccharides. In agreement with this was the fact that the dialysable fraction of the extracellular polysaccharides contained 50% xylose.

The above evidence is in agreement with the view that the extracellular polymers were once constituents of the cell walls and were released during the growth process, which will involve a balance between synthetic and lytic enzyme systems.

C. Evidence of a linked $\alpha 1 \rightarrow 4$, $\beta 1 \rightarrow 3$, $1 \rightarrow 6$ Glucan in the Cell Walls

One of the most interesting characteristics of the Fusicoccum amygdali cell wall was its iodine staining reaction, which was first observed when testing for the possible presence of free chitosan. Chitosan gives a brown colour with iodine solution, which changes to red-violet on addition of 1% sulphuric acid. However the Fusicoccum cell walls gave a blue-black colour with iodine, which did not change on addition of 1% sulphuric acid. When living mycelium was stained with iodine the cell walls stained blue-black, whereas the cytoplasm was stained reddish-brown, characteristic of glycogen. The iodine staining characteristics suggested the presence of an amylaceous glucan and this was confirmed by incubation of cell walls or intact mycelium with α- amylase, when the iodine staining properties were lost and glucose,

maltose and maltotriose (equivalent to 12 - 14% of the total cell wall glucose) were liberated.

It was then necessary to determine if this amylaceous glucan was a true structural component of the cell wall, or if it were a contaminant. Contamination would be possible from three sources (a) an insoluble component of the medium; (b) intracellular storage polysaccharides; (c) extracellular polysaccharides. The medium used for growing Fusicoccum (Medium IIb) contained small amounts of insoluble material arising from soya bean meal, which stains brownish blue with iodine and is known to contain polymers of the starch/glycogen type (K.W.Buck and J.M. Tyler, unpublished results). In order to eliminate soya bean meal as a possible cell wall contaminant, the organism was grown on a completely soluble medium (Medium IIa). However the iodine staining properties of mycelium and cell walls were the same as when the organism was grown on Medium IIb, eliminating a medium contaminant as a source of the amylaceous glucan. The second possibility was suggested by the work of Northcote (1963), who showed that in yeast, glycogen granules were bound to the cell wall by fine membranes. They however stained brown with iodine and were not present in mechanically isolated cell walls. In the case of Fusicoccum no granules could be observed with the light microscope (X1000) and the prepared cell walls or cell walls of intact mycelium were rigid with a uniform thickness and stained uniformly with iodine solution. Moreover after incubation with α- amylase the cell walls appeared very much thinner and had lost their rigidity (Fig.X.2b). The ease of accessibility of α-amylase for its substrate suggests, but does not prove, an outer location in the cell wall for the $\alpha 1 \longrightarrow 4$ glucan. It was therefore considered unlikely that membrane bound granules were responsible for the iodine staining capacity of the cell walls. The third possibility was eliminated because, although three extracellular polysaccharide fractions could be isolated from culture filtrates, only one of them gave a colour with iodine solution and this was water soluble making contamination of the cell walls unlikely.

Further evidence that the α1→4 linked glucan is a true cell wall constituent and not a contaminant was provided from fractionation studies. Firstly chloral hydrate, known to be a powerful starch solvent (Bourne and Weigel, 1965 and references cited therein) failed to extract any material which stained blue-black with iodine; in fact β-glucan was preferentially extracted. Secondly extraction with a number of solvents, namely N-potassium hydroxide, ethylenediamine, dimethyl sulphoxide and urea, all known to be good starch solvents, only partially dissolved the amylaceous glucan, Both soluble and insoluble cell wall fractions in each case still stained blue-black with iodine.

Further investigation of this amylaceous glucan has suggested that it is linked to a β-glucan in the cell wall. It was found that a water soluble polymer obtained by fractionation of cell walls with alkali (Fraction 14FCI and 14FCII, Results IIIJ and K) stained deep blue with iodine solution. Although this fraction showed only one peak when examined by ultracentrifugation with Schlieren optics it was shown to contain (at least) two components, one of which could be easily precipitated with iodine solution. The component which precipitated, had $[\alpha]_D$ + 40° and stained deep blue with iodine. It gave only one peak when examined by Schlieren optics on the analytical ultracentrifuge. Since this rotation was much lower than those reported for amylose [$[\alpha]_D$ + 162°, N,NaOH (Dimler, 1964)] , amylopectin[$[\alpha]_D$ + 163°, N, NaOH (Dimler, 1964)] or glycogen[$[\alpha]_D$ + 200, water (Liddle and Manners, 1957)] , the material may well contain both α and β linkages. Although it is likely that the α, β linked polymers were joined together in some way, because a β linked glycan would not be expected to precipitate with iodine, the evidence was not conclusive.

Perhaps the most convincing evidence comes from the study of cell wall Fraction 14FJ. This fraction was obtained by repeated alkali fractionation of the cell walls (Result IIIJ), was soluble in alkali, but insoluble in water; it stained blue-black with iodine and had $[\alpha]_D$ + 236°. Analytical ultracentrifugation (Fig.VII.1) showed it

to contain two components with S values 6.6 S and 2.8 S. When Fraction 14FJ was treated with α-amylase the slower moving component was almost unaffected in quantity, but the faster moving peak was very much reduced in quantity and moved more slowly on ultracentrifugation (Fig. VII.1). Moreover the elution profile on Sephadex G-200 had changed considerably. Before incubation with α-amylase Fraction 14FJ was almost completely excluded from Sephadex G-200, whereas after incubation an elution profile (Fig. 3 , appendix) was obtained, almost completely in the fractionation region of Sephadex G-200, indicating a drop in average molecular weight from above 200,000 to the range 160,000 - 70,000. When the products of α-amylase incubation were fractionated (Result VII B) there was obtained a water insoluble α1→3 glucan (1.7 S) (to be discussed later) and a water soluble β-glucan, which appeared homogeneous (4.6 S) when examined by analytical ultracentrifugation. The structure of this β-glucan (β-D-Glucan, Fraction 27K) will be discussed later.

When Fraction 14 FJ was treated with pachyman purified Basidiomycete QM806 exo β1→3 glucanase and the product was examined by analytical ultracentrifugation the slower moving component was almost unchanged in quantity, whereas the faster moving component could no longer be detected (Fig.VII.1). The product still stained blue-black with iodine. The elution profile on Sephadex G-200 (Fig. 3 , appendix) indicated a drop in average molecular weight from above 200,000 to the range 160,000 - 70,000 as was obtained with α-amylase. The fact that the 6.6 S component was destroyed by both α-amylase and exo-β1→3-glucanase is strong evidence that it contains α and β-glucans linked together. Consistant with this belief is the liberation of the water soluble β-glucan, Fraction 27K after α-amylase action.

The isolated β-D-Glucan, Fraction 27K contained 96% hexose (88.4% glucose, 4.4% galactose, small amounts of mannose and rhamnose and traces of xylose and arabinose). It was almost completely excluded from Sephadex G-200 gel indicating a M. Wt. > 200,000, and appeared

homogeneous on sedimentation analysis (4.6 S). Its optical rotation, $[\alpha]_D$ + 16°, and its infrared spectrum (ν max. 890 cm^{-1}), which was very similar to that of laminarin (Fig. 1 appendix), indicated the presence of mainly β-glucopyranose linkages. It did not give a sharp X-ray diffraction pattern as is given by paramylon (Bull and Chesters, 1966).

The major structural features of this glucan were determined by a combination of enzymic and chemical methods. The glucan was completely solubilised by a purified Helix pomatia endo β1→3 glucanase giving rise to a homologous series of laminaridextrins up to laminariheptaose together with gentiobiose and an oligomer thought to contain β1→3 and β1→6 linkages (Fig.IX.1 and IX.2 result IX C b), indicating that the glucan consisted of chains of, β1→3 linked glucopyranose residues upto at least seven residues long with some branches.

Methylation followed by hydrolysis gave 2, 3, 4, 6 tetra -O- methyl-D-glucose, (1 part), 2, 3, 4, 6 tetra -O- methyl-D-galactose (0.24 part), 2, 4, 6 tri -O- methyl-D-glucose (9.2 parts), 2, 3, 6 and/or 2, 3, 4 tri - O - methyl-D-hexose (1 part) (probably a mixture of 2, 3, 6 tri -O- methyl galactose, glucose and mannose, since periodate oxidation followed by borohydride reduction gave rise to some threitol and erythritol) and four unidentified di -O- methyl hexoses (0.27, 0.22, 0.32 and 0.27 parts respectively). This indicates that the glucan contained chains of 1→3 linked glucopyranose residues, on an average ten units long, which form a highly branched structure with at least four types of branching. The high proportion of galactose present as non-reducing end group indicates that galactose is present in rather short chains in a highly branched structure.

The glucan consumed, per anhydro hexose unit, 0.18 mole of periodate producing 0.017 mole of formaldehyde and 0.065 mole of formic acid. This indicates one 1→6 pyranose linkages and/or non-reducing end group in every 15 residues, a lower value than obtained by methylation analysis. It is possible that methylation results in some degradation with the exposure of more non-reducing end groups. The

amount of formaldehyde liberated is in excess of that expected for a polymer of molecular weight $>$ 200,000 and may have arisen from cleavage of furanose residues; cleavage of 1, 2 or 1, 3 linked galactofuranose residues would give rise to arabinose, which was in fact detected (1.2 mole per cent) in the periodate oxidised product after borohydride reduction. However since Fraction 27K also contained trace amounts of arabinose, a part of this could have arisen from periodate resistant arabinose units. The large amount of glucose (89 mole per cent) present after periodate oxidation/borohydride reduction is consistant with the presence of 1$\rightarrow$3 glucopyranose links or periodate resistant branch units. This product also contained threitol (3.4 mole per cent) and erythritol (1.9 mole per cent), most of which was released on selective acid hydrolysis (Smith degradation). The threitol probably arose from chains of 1$\rightarrow$4 linked galacto - pyranose residues, where as the erythritol could have arisen from 1$\rightarrow$4 linked glucopyranose or mannopyranose residues. The periodate oxidised/borohydride reduced product contained also glycerol (4.5 mole per cent) about $\frac{3}{4}$ of which was released on Smith degradation. The value is somewhat lower than that expected from the formic acid production, suggesting that some formic acid may have arisen from overoxidation. The small amount of residual glycerol (1 mole per cent) after Smith degradation indicates that some unbranched 1$\rightarrow$6 pyranose linkages were present in the original glucan.

Further structural information was obtained from the action of an exo-β1$\rightarrow$3-glucanase from Basidiomycete QM806, which had been purified by complexing on pachyman. The action pattern of this enzyme has been studied recently by Nelson, Johnson, Jantzen and Kirkwood (1969). It was found that it will remove a glycosyl residue which has a free C - 3 hydroxyl and which is attached to another glucosyl residue by a β1$\rightarrow$3 linkage. Although an exo-enzyme, in that it will cleave laminarin but has little action on periodate oxidised laminarin, it can by-pass β1$\rightarrow$6 or β1$\rightarrow$4 linkages giving it an endo action on certain

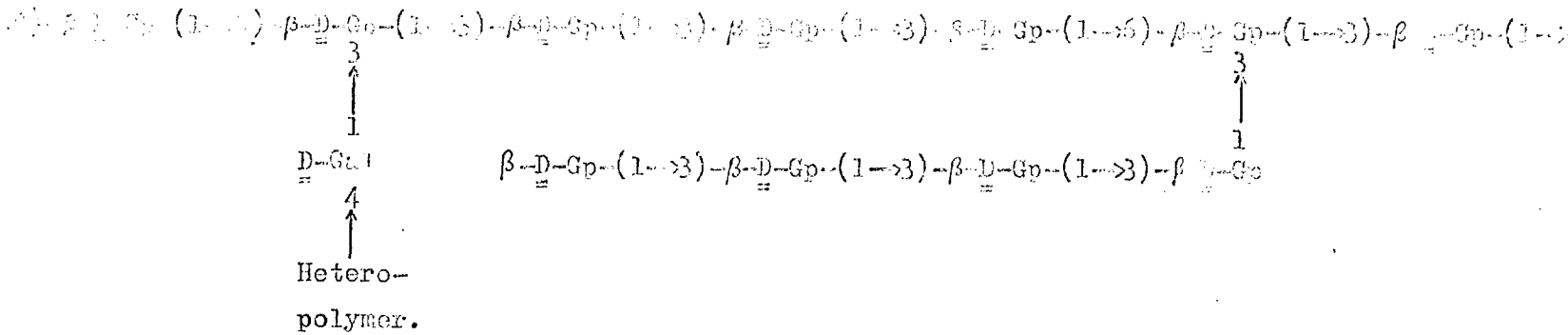
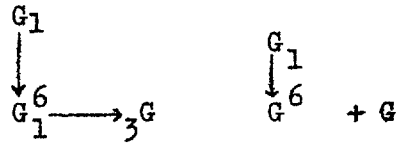


Fig. D.1. A Suggested Structural Feature of β-D-Glucan, Fraction 27K.

structures e.g. 

It follows that the enzyme would cleave all the $\beta 1 \rightarrow 3$ glucopyranose linkages in a glucan of mixed linkages. The enzyme completely solubilised the β Glucan, Fraction 27K, giving glucose and gentiobiose (14 or 15:1) as the only mono- and oligosaccharide detectable. This confirmed the presence of $\beta 1 \rightarrow 3$ and $\beta 1 \rightarrow 6$ linkages and indicates that the $\beta 1 \rightarrow 6$ linkages occur singly and not in blocks of two or more units. Since no other oligosaccharides were detected, it follows that the only branches involving glucose alone are 1, 3, 6. However since four dimethyl sugars were obtained on methylation analysis all of them in much smaller amount than the number of glucose non-reducing end groups, it follows that there was extensive branching involving galactose, mannose or the other neutral sugars.

Further evidence was obtained from the action of the exo - $\beta 1 \rightarrow 3$ glucanase on the Fraction 27K after one and two Smith degradations. In each case glucose and gentiobiose were the only mono- and oligosaccharide detectable. The ratio Glucose : Gentiobiose was 4 or 5:1 (after 1 Smith degradation) and about 7:1 (after 2 Smith degradations). The increase in the gentiobiose formed after one Smith degradation compared with that formed originally suggests (a) that most of the $\beta 1 \rightarrow 6$ glucopyranose residues are resistant to periodate oxidation i.e. are present as 1, 3, 6 branch points and (b) that some of these branch points involved galactose or one of the other monosaccharides attached at the 3 position. Since the enzyme releases no short galactose containing oligosaccharide, the galactose is presumably attached also to a heteropolymer. A possible structure, which would account for these results is shown in Fig.D4. The slightly lower amount of gentiobiose obtained after the 2nd Smith degradation could be due exposure of periodate susceptible $1 \rightarrow 6$ linkages after removal of the hetero polymer side chain.

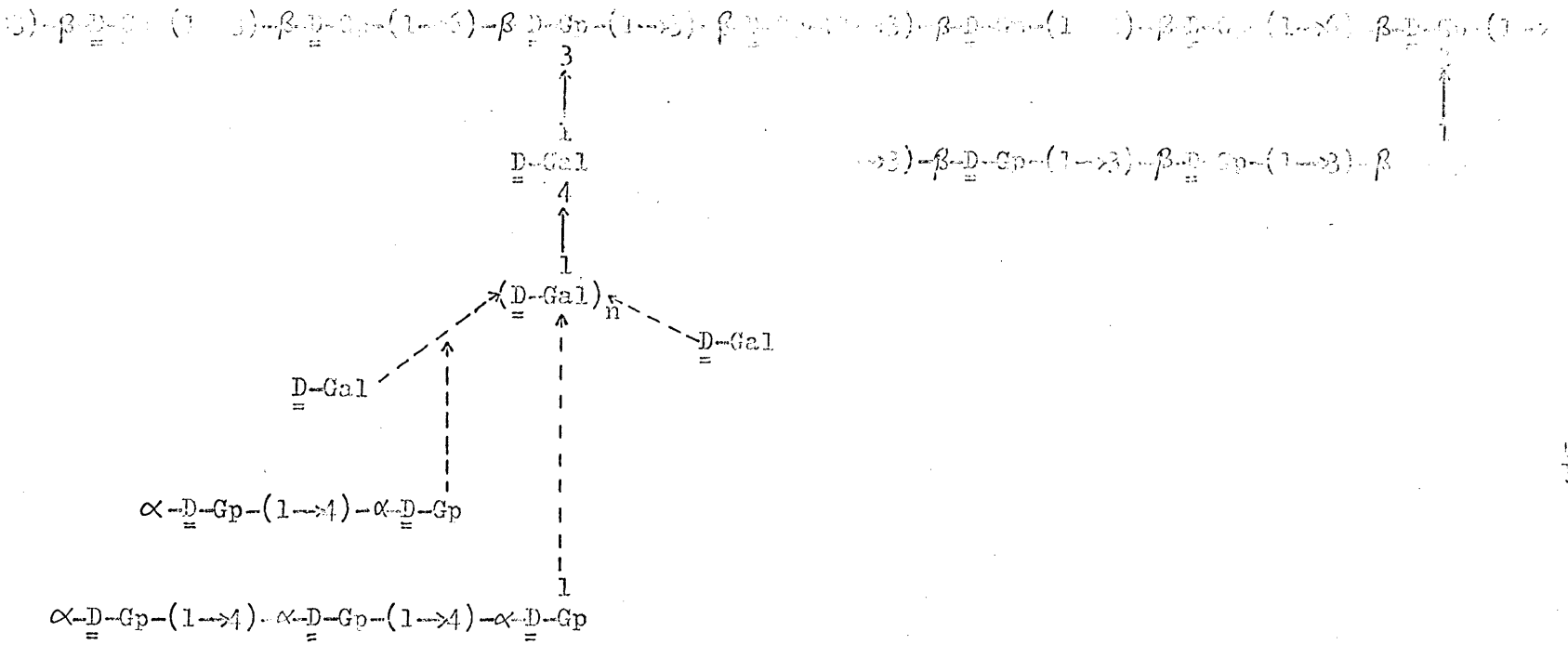


Fig. D.2. A Suggested Partial Structure of β-D-Glucan, Fraction 27K.

This partial structure indicates the probable linkages in β-D-Glucan, Fraction 27K, but is not intended to imply chain lengths.

——→ linkage of α(1—4 D-glucopyranose stubs to galactomannan heteropolymer (the nature of the linkage or the structure of the heteropolymer is not known).

A considerable amount of evidence has been advanced to show that the β-Glucan, Fraction 27K, is attached in some way to an amylaceous glucan. Since α-amylase action on starch or glycogen usually leaves stubs of two or three α 1→4 glucopyranosyl units in the region of the 1, 4, 6 branch points, (Manners, 1962; Greenwood and Milne, 1968) it was anticipated that Fraction 27K may contain α1→4 glucopyranose stubs. However the exo-β1→3 glucanase failed to release any maltose or maltotriose, which would be expected if the α1→4 linkages were attached to the main glucose chain. It was also shown that the only branch point involving glucose was 1, 3, 6. However the amount of 2,4, di-O-methyl glucose obtained in methylation analysis (although not identified) could not have been more than about ¼ of the amount of 2, 3, 4, 6 tetra-O-methyl-D- glucose. This suggested that non-reducing end groups of glucose were attached to the hetero-polymer, and it is postulated that these were the α1→4 glucose linked stubs remaining after α-amylase action. The formation of erythritol after Smith degradation was evidence that 1→4 pyranose linkages were present in the structure. An overall structure for Fraction 27K, which would account for most of the results obtained, is shown in Fig.D.2.

The α1→4 and β1→3, 1→6 glucans present in the original wall would then be joined glycosidically via the galactomannan hetero-polymer. One unexplained feature is the lack of action of β-amylase on the α,β glucan. It is possible that (a) the non-reducing α1→4 glucose end groups were sterically inacessible to the enzyme or (b) non-reducing end groups of other types were present e.g. α1→3 linkages. It is also of interest that incubation with pullulanase after α-amylase action, released no further soluble sugars, indicating the absence of α1, 4, 6 glucopyranosyl branch points of the type found in amylopectin or glycogen.

Although the suggested structures account for most of the results, other structures are possible and further work is needed to determine all the details of the fine structure. The model does, however, provide a useful working hypothesis for further study.

The amylaceous glucan was not confined to Fraction 14FJ, but was found also in the water soluble and alkali insoluble cell wall fractions. $\alpha 1 \rightarrow 4$ glucopyranosyl linkages accounted for 12 - 14% of the cell wall polysaccharides and the α, β glucan in Fraction 14 FJ contained approximately 34 % $\alpha 1 \rightarrow 4$, 61% $\beta 1 \rightarrow 3$ + $\beta 1 \rightarrow 6$ glucopyranose linkage together with 5% galactomannan heteropolymer. If these ratios are constant for the polymer in all the cell wall fractions, it may be estimated that the linked α, β glucan accounts for 35 - 41% of the cell wall polysaccharide. This appears to be the first report of a linked $\alpha 1 \rightarrow 4, \beta 1 \rightarrow 3, \beta 1 \rightarrow 6$ glucan in fungal cell walls.

There have been very few reports of $\alpha 1 \rightarrow 4$ glucans in fungal cell walls. A glucan, stated to be a $\beta 1 \rightarrow 3$ and $\alpha 1 \rightarrow 4$ linked polysaccharide was isolated from the cell walls of Aspergillus oryzae (Horikoshi and Iida, 1964), but no evidence for the structure was produced. A glycogen-like polymer was isolated from an alkali insoluble fraction of the cell wall of Dictyostelium discoideum (Ward and Wright , 1965) after removal of cellulose with "Schweitzer's" reagent. Toama and Raper (1967) isolated an alkali soluble glucan, assumed to be glycogen, from the microcyst walls of Polysphondylium pallidum; however no evidence to support the assumption was presented. Despite the paucity of definitive information regarding the occurrence of $\alpha 1 \rightarrow 4$ glucans in fungal walls, nevertheless the reaction of I_2- KI with cell wall and cytoplasmic contents has been employed as a technique for the classification of higher fungi (Aronson, 1965). The results, however, have rarely been correlated with biochemical analysis. Further investigations in this field may well reveal many more examples of $\alpha 1 \rightarrow 4$ glucans in fungal cell walls.

D. $\alpha 1 \rightarrow 3$ Glucan

The major, 2.8 S, component in Fraction 14 FJ was isolated by degradation of the α, β glucan with α-amylase, reprecipitation from alkali, removal of residual β-glucan with exo-$\beta 1 \rightarrow 3$ glucanase and finally reprecipitation of the residue from alkali. The product,

Fraction 270, (1.7 S) consisted mainly of glucose with only traces of other hexoses and amino acids. Its optical rotation, $[\alpha]_D$ + 290°, indicated it to be an α-glucan and this was confirmed from its infrared spectrum, which had ν max. 855 and 930 $cm.^{-1}$ and was different to that of nigeran, but almost identical to an $\alpha 1 \rightarrow 3$ glucan isolated from the cell walls of Aspergillus niger (Fraction IV R; Johnston, 1965). However the A.niger glucan had $[\alpha]_D$ + 260° and its X-ray diffraction pattern was not identical with that of Fraction 270 (Table 2, Appendix). The detection of a homologous series of nigerodextrins, after partial hydrolysis and paper chromatography, confirmed that $\alpha 1 \rightarrow 3$ glycopyranose linkages predominated in Fraction 270; maltose was also found indicating a small number of $\alpha 1 \rightarrow 4$ glucopyranose linkages. It is of interest that the A.niger glucan Fraction IV R, also contained small numbers of $\alpha 1 \rightarrow 4$ linkages and it is possible that the differance between the two glucans lies in the relative positions of the $\alpha 1 \rightarrow 3$ and $\alpha 1 \rightarrow 4$ linkages.

Further information on the structure of Fraction 270 was obtained from methylation analysis and periodate oxidation. Methylation followed by acid hydrolysis gave 2, 3, 4,6tetra-O-methyl-D-glucose (1 part), 2, 4, 6 tri-O-methyl-D-glucose (224 parts) and 2, 3, 6 and/or 2, 3, 4 tri-O-methyl-D-glucose (6 parts) with only traces of dimethyl sugars. This indicated a linear polymer composed mainly of $1 \rightarrow 3$ glucopyranose linkages with small numbers of $\alpha 1 \rightarrow 4$ and/or $\alpha 1 \rightarrow 6$ glucopyranose linkages.

The glucan consumed, per anhydro hexose unit, 0.057 mole of periodate, producing 0.006 mole of formaldehyde and 0.022mole of formic acid indicating one non-reducing end group or $1 \rightarrow 6$ pyranose linkage per 45 glucose units. The product after borohydride reduction contained (moles per cent) glucose(95), arabinose(1.0), xylose (0.4), threitol (0.4), erythritol (2.6) and glycerol (0.7). Since erythritol would arise from $1 \rightarrow 4$ glucopyranose linkages, this determination suggests 2.6% $\alpha 1 \rightarrow 4$ linkages, the same value obtained for $1 \rightarrow 4 + 1 \rightarrow 6$ linkages by methylation analysis. The value for glycerol indicated one

1→6 pyranose linkage and/or non-reducing end group per 143 glucose units, suggesting that some of the formic acid may have arisen from over oxidation. Arabinose, xylose and threitol probably arose from the minor constituents of the glucan. On Smith degradation glycerol and erythritol were released into the supernatant suggesting that some adjacent α1→4 linkages were present, but presumably in a block of only 2 or 3 units since the glucan was resistant to α-amylase. The Smith degraded glucan contained (mole per cent) glucose (98.3), erythritol (1.1) and glycerol (0.5). The presence of glycerol would tend to confirm the presence of 1→6 linkages, but further evidence is needed for complete confirmation.

Methylation analysis would indicate a $\bar{D}P \sim 224$ ($M_n \sim 36,300$) whereas the amount of glycerol released during Smith degradation suggests a $\bar{D}P \sim 500$ ($M_n \sim 80,000$), if it is assumed that the glycerol was released solely from non-reducing end groups (If there were any adjacent 1→6 or 1→2 linkages or if the reducing end contained the sequence Gp (1→4) Gp (1→2,3 or 4) glycitol or Gp (1→6) Gp (1→ 2,3 or 4) glycitol, the average molecular weight would be higher). The lower value obtained from methylation analysis may have been due to degradation of the polymer during methylation. The elution profile on Sephadex G-200 suggested a molecular weight range of 70,000 - 160,000. On the other hand the elution profile after Smith degradation showed that the product was polydisperse, about 40% being in the molecular weight range 50,000 - 120,000 and the remainder from 50,000 to less than 20,000 (Fig. 4 , appendix). This was confirmed by sedimentation analysis, which gave a broad peak with S_{20} 1.2 . The number average molecular weight of the Smith degraded glucan, calculated from its total erythritol and glycerol, (each indicating an end group) was 12,300. It is clear that Smith degradation causes a considerable drop in molecular weight due to cleavage of internal 1→4 linkages in the main α1→3 chain.

In summary, Fraction 270 is a linear α1→3 glucan containing about one α1→4 glucopyranose linkage per 37 units. The majority of

the $\alpha 1 \rightarrow 4$ linkages appear to occupy internal positions in the chain and some of these are present in blocks of two or three residues. In addition the presence of small numbers (less than 1%) of $1 \rightarrow 6$ and/or $1 \rightarrow 2$ linkages cannot be ruled out.

The $\alpha 1 \rightarrow 3$ glucan appears to be a major component probably about 28 - 31%, of the cell wall and was found in both alkali soluble and insoluble cell wall fractions and also in the cell wall fraction resistant to the action of the Helix pomatia enzymes. Moreno, Kanetsuna and Carbonell (1969) reported an $\alpha 1 \rightarrow 3$ glucan in the cell walls of Paracoccidioides brasiliensis, which was resistant to the digestive juice of Helix pomatia. The Fusicoccum $\alpha 1 \rightarrow 3$ glucan was also resistant to glucamylase, but a Trichoderma viride cellulase preparation acted slowly to release glucose, probably as a result of an $\alpha 1 \rightarrow 3$ glucanase impurity. An $\alpha 1 \rightarrow 3$ glucanase has recently been isolated and purified from T.viride (Hasegawa, Bordin and Kirkwood, 1969). It is clear that cellulases used for detection of cellulose in fungal cell walls must be subjected to rigorous tests of purity if reliable results are to be obtained.

E. Cell Wall β-Glucans

In addition to the $\beta 1 \rightarrow 3$, $1 \rightarrow 6$ glucan component which appears to be linked via a galactomannan heteropolymer to chains of $\alpha 1 \rightarrow 4$ linked glucopyranose units, the Fusicoccum cell walls contain other $\beta 1 \rightarrow 3$, $1 \rightarrow 6$ glucans. Alkali fractionation of cell walls, followed by removal of $\alpha 1 \rightarrow 4$, glucans with iodine gave a β-glucan fraction, with very low optical rotation ($[\alpha]_D$ - 110°, Fraction 26 W, Results III K). Although the structure of this glucan was not determined, no evidence was obtained in the cell wall for β-glucan linkages other than $1 \rightarrow 3$ and $1 \rightarrow 6$. The total amounts 50 - 56.5% of cell wall $\beta 1 \rightarrow 3$ and $\beta 1 \rightarrow 6$ glucopyranose linkages, estimated from the amounts of glucose and gentiobiose liberated on incubation of total cell walls with exo-$\beta 1 \rightarrow 3$ glucanase, were 43 - 48% and 13 - 16% respectively. Tests for cellulose in the cell walls e.g. by extraction with Schweitzer's reagent, were negative.

F. Cell Wall Glucosamine Polymers

Cell walls of *Fusicoccum amygdali* contain 4.5% anhydro-D-glucosamine, equivalent to 5.7% anhydro-N-acetyl-D-glucosamine, out of which about 85% was released by *Streptomyces* chitinase or *Helix pomatia* enzymes as N-acetyl-D-glucosamine, suggesting that most of the cell wall glucosamine was in the form of chitin. This was confirmed by the infrared spectrum and X-ray diffraction patterns of cell wall chitin isolated by a number of methods. The chitin could be isolated in almost quantitative yield with 95% purity by successive removal of $\alpha 1 \rightarrow 4$ glucan with α-amylase, β- glucans with exo $\beta 1 \rightarrow 3$ glucanase and $\alpha 1 \rightarrow 3$ glucans (and any residual galactomannan hetero-polymer) with dilute alkali in the cold. The product had an X-ray diagram and infrared spectrum almost identical with those of crustacean chitin and was converted in 95% yield to N-acetyl-D-glucosamine on incubation with a mixture of *Streptomyces* chitinase and *Helix pomatia* enzymes. In contrast, chitin isolated by the chemical methods described by Kreger (1953, 1954), which involve treatments with hot acids and alkalis, while giving a similar X-ray diffraction pattern and infrared spectrum, was only hydrolysed to a limited extent ($\sim$ 18%) by *Streptomyces* chitinase. It is clear that the chemical methods, commonly used for isolating cell wall chitin, cause extensive degradation of the chitin (probably mainly de-N-acetylation) and it is recommended that in general enzymic methods should be used for chitin isolation from fungal cell walls.

Tests for cell wall chitosan were negative, but small amounts of glucosamine were found in the alkali soluble and ethylenediamine soluble fractions of the cell wall indicating that all the glucosamine was not in the form of chitin. It is possible that linkages of the polysaccharide and peptide components of the cell wall are linked via glucosamine residue. In the case of the yeast cell wall, Sentandreu and Northcote (1968) obtained evidence for a linkage between glucosamine and aspartamide in an ethylenediamine soluble glycopeptide.

G. Protoplast Formation

The results sofar discussed indicate that the cell walls of Fusicoccum amygdali consist largely of polysaccharides with small amounts of peptide and lipid. Two crystalline components, chitin, and an $\alpha 1 \rightarrow 3$ glucan presumably formed the backbone of the walls, with the other components forming the amorphous matrix. Evidence was obtained for separate galactomannan polymers (containing other minor sugars), some of which appeared to be linked by peptide and phosphate bonds, and some of which was postulated to link together glycosidically a $\beta 1 \rightarrow 3$, $\beta 1 \rightarrow 6$ glucan and an $\alpha 1 \rightarrow 4$ glucan. The amorphous matrix also contained separate $\beta 1 \rightarrow 3$, $\beta 1 \rightarrow 6$ glucans. It is known that Helix pomatia enzymes will release protoplasts, albeit in low yield, from the mycelium of Fusicoccum amygdali. From the results obtained here it is evident that the snail enzymes will release most of the heteroglycan polymers and hydrolyse nearly all of the $\beta 1 \rightarrow 3$, $\beta 1 \rightarrow 6$ polymers, the $\alpha 1 \rightarrow 4$ glucan, and the chitin. The only resistant polymer was the $\alpha 1 \rightarrow 3$ glucan. It is possible therefore that a more efficient method of preparing protoplasts may be to use a mixture of the snail enzymes together with an endo $\alpha 1 \rightarrow 3$ glucanase e.g. the Trichoderma viride enzyme (Hasegawa and Nordin, 1969) and possible also α-amylase, since the amylase content of different snail enzyme preparations was variable. An alternative would be to use a mixture of the Trichoderma viride $\alpha 1 \rightarrow 3$ glucanase (as above), Basidiomycete QM806 exo $\beta 1 \rightarrow 3$ glucanase, α-amylase and Streptomyces griseus chitinase.

H. The Structure of the Fusicoccum Cell Wall in Relation to its Classification.

Fusicoccum amygdali is an organism from the Sphaeropsidales group of the Fungi Imperfecti. From the results obtained here regarding the cell wall structure it clearly falls into Bartnicki-Garcia's Group V i.e. Chitin-Glucan (Bartnicki-Garcia, 1969), which includes the Chytridiomycetes, Ascomycetes, Basidiomycetes and Deuteromycetaceae. The Fungi Imperfecti (Deuteromycetes) are those whose perfect stage

is not known. When the perfect stage of a Deuteromycete is discovered the organism is frequently found to be an ascomycete and this may well be the case here. However the knowledge of cell wall structure in this group is very incomplete at the present time and the relationship of the Fusicoccum cell wall to those of other members of this group must await further progress in this field.

Expt. 1 Preparation of Cell Walls

Two methods were employed for the preparation and purification of cell walls.

A. Using the X - press for Cell Breakage

This method was based on that of Edebo (1960) and Yoshida, Heden, Cedergren and Edebo (1961).

Three and a half day old mycelium (25 g. wet weight) was placed in an X - press (Type X - 25, AB Biox, Box 235, Nacka 2, Sweden), cooled to -25° using cardice/industrial spirit, and then pressed at a pressure of 15 to 20 tons/sq. in. Almost complete breakage occurred after 5 to 6 pressings, as revealed by light microscopy using an Ortholux microscope, magnification 100 x 10 with phase contrast (Leitz, Germany).

The cell walls were purified by differential centrifugation by a modification of the method of Mendoza and Villanueva (1963).

The pressed mycelium was suspended in 60% (w/v) sucrose solution (100 ml.) at 4° and centrifuged at 5,000 g. for 20 min. The sedimented material was resuspended in 10% (w/v) aqueous sucrose (100 ml.) at 4° and centrifuged at the same speed and time. The latter treatment was repeated five times. The resulting sediment was resuspended in 1% (w/v) aqueous sodium chloride (100 ml.) at 4° and centrifuged as before. This operation was repeated twice. Finally the residual cell wall preparation was washed in distilled water at 4° by resuspension and centrifugation until the final supernatant was clear.

The cell wall preparation thus obtained was freeze dried and stored in a stoppered vessel in a desiccator over phosphorus pentoxide at room temperature.

Assessment of purity using light microscopy was done after staining the cell wall preparation with cotton-blue stain (0.05 % w/v water soluble aniline blue in lactophenol). Residual cytoplasm and intact cells stain with cotton blue, but cell walls do not. Purity could also be assessed by differential staining of the cell walls and cytoplasm with 0.1N - iodine solution.

B. Using the APV Manton - Gaulin Homogeniser for Cell Breakage

A suspention of distilled water washed mycelium (3 kg. Wet weight)

in distilled water (9 l.) at 0 - 4° was homogenised by two passages through an APV Manton - Gaulin Homogeniser (The APV Co. Ltd., Manor Royal, Crawley, Sussex) at 7,500 lb/sq. in. The homogenate was centrifuged (1,500 g., 30 min.) and the residue was washed with 0.03 M- sodium phosphate buffer, pH 7.6, containing 1% (W/V) sodium lauryl sulphate, at 4° by resuspending and centrifuging (1,500 g., 30 min.) three times. Finally the residual cell wall was washed with distilled water at 4° by resuspension and centrifugation as before, until free from sodium lauryl sulphate.

The preparation was used immediately for the preparation of cell wall fractions (Expt. 14F) for structural studies. A small portion was freeze dried prior to determination of chemical composition and linkage analysis.

Expt. 2 Isolation of Sugars and Preparation of Derivatives from Cell Wall Hydrolysates

A.(i) D - Glucosamine

Cell wall preparation (500 mg.) was hydrolysed and the hydrolysate was neutralised as described in G.M. 15C. The neutralised hydrolysate was chromatographed on Whatman 3MM paper using Solvent a. Amino - sugars were located on strips of the chromatogram, cut from the edges and the centre of the paper, using spray reagent c and the band having the same R_G value as glucosamine hydrochloride was eluted with water by downward irrigation. The eluted glucosamine hydrochloride was further purified by chromatography on Whatman 3MM paper using Solvent b and eluted as before. It was recrystallised from a concentrated solution in water by gradual evaporation in vacuo at 4° in a desiccator over calcium chloride. Yield 27 mg., $[\alpha]_D$ + 99° (15 min.) ⟶ + 73° (2 days, c 1.0, water). Authentic D - glucosamine hydrochloride had $[\alpha]_D$ + 100° (15 min.) ⟶ + 72° (2 days c 1.0, water).

(ii) N - Benzyloxycarbonyl - D - Glucosamine

To an aqueous solution (0.1 ml.), containing cell wall D - glucosamine hydrochloride (15 mg.) and sodium acetate (15 mg.), was added, with shaking, benzyloxycarbonyl chloride (15 µl.) in three equal

portions, during one hr. The mixture, containing needle crystals of N - benzyloxycarbonyl - D - glucosamine, was stored at 4° for 2 hr. Chloroform (0.1 ml.) was added to the paste and the precipitate was washed with chloroform and dried. Yield 20 mg. (70%). Recrystallisation from 30% aqueous methanol gave white needles of N - benzyloxycarbonyl - D - glucosamine, m.p. and mixed m.p. with authentic material, prepared from authentic D - glucosamine hydrochloride, 214 - 215.5° (with decomposition). The infrared spectrum was identical with that of the authentic compound.

B (i) D - Glucose

Cell wall preparation (500 mg.) was hydrolysed and the hydrolysate was neutralised as described in G.M. 15A. The neutralised hydrolysate was chromatographed on Whatman 3MM paper using Solvent b. Reducing sugars were located on strips of the chromatogram, cut from the edges and centre of the paper, using spray reagent b and the band having the same R_G value as glucose was eluted with water by downward irrigation. The eluted glucose was further purified by chromatography on Whatman 3MM paper using Solvent c and eluted as before. The eluate was concentrated by evaporation under reduced pressure and the residue was recrystallised from water by gradual evaporation in vacuo at -20° over calcium chloride to give D - glucopyranose (250 mg.); $[\alpha]_D$ + 52° at equilibrium (c 1.0, water). Authentic D - glucopyranose had $[\alpha]_D$ + 52° at equilibrium (c 1.0, water).

(ii) β - D - Glucopyranose Pentaacetate

A suspention of cell wall D - glucose (70 mg.) and anhydrous sodium acetate (70 mg.) in acetic anhydride (1.4 ml.) was heated for 30 min. at 160° on an oil bath in a stoppered flask. The cooled mixture was then poured with stirring onto cracked ice (10 g.). After stirring for 1 hr. at 0°, the crystalline precipitate was filtered under reduced pressure, washed thoroughly with cold water, and dried. Yield 115 mg. (73%). Recrystallisation from aqueous alcohol gave β - D glucopyranose pentaacetate, m.p. 132°, $[\alpha]_D$ + 3.8° (c 0.5, chloroform), identical (m.p. mixed m.p., optical rotation and infrared spectrum) with authentic material, prepared from D - glucose under

identical conditions.

C. (i) D - Galactose

D - Galactose was isolated from the same hydrolysate and paper chromatogram used to isolated cell wall D - glucose. The eluted band, having the same R_G value as galactose, was further purified twice by chromatography on Whatman 1 paper using Solvent b in order to remove the last traces of glucose. The eluate was evaporated to dryness under reduced pressure and then taken up into distilled water (18 ml.) Yield, as measured by galactose oxidase (G.M.4b) and phenol/sulphuric acid (G.M.1), 15 mg. $[\alpha]_D$ + 80° at equilibrium (c 0.85, water). Authentic D - galactose had $[\alpha]_D$ + 81° at equilibrium (c 1.0, water).

(ii) D - Galactose Methylphenylhydrazone

To a solution of cell wall D - galactose (8mg.) in 95% ethanol (0.2 ml.) were added 1 - methyl - 1 - phenyl hydrazine (0.01ml.) and 50% ($^v/_v$) aqueous acetic acid (0.01 ml.) in succession. The mixture was incubated at 37° for 6 hr. and then stored at 4° overnight. Crystals of D - galactose methylphenylhydrazone (10 mg., 80%) were filtered, washed successively with ethanol and ether and dried. The m.p. and mixed m.p. (188 - 189°) and infrared spectrum were identical with D - galactose methylphenylhydrazone, prepared from authentic D - galactose.

(iii) D - Galactose Oxidation with Specific D - Galactose Oxidase

To a solution of D - galactose (100 - 140 ug.) in water (1 ml.) was added Galactostat reagent (Worthington Biochemical Corp.) in distilled water (1 unit of galactose oxidase in 1 ml.) and the solution was incubated at 37° for 24 hr. The whole incubation mixture was chromatographed in Solvent c. after denaturation of enzyme at 100° for 5 min. and concentration under reduced pressure. The mobility of the dialdehyde formed, detected with spray reagents a and b, was identical to that formed from authentic D - galactose treated at the same time under identical conditions ($R_{Galactose}$ 0.33). An enzyme blank under identical condition did not give any spot.

D. L - Rhamnose

Free sugars, obtained from the distilled water washings of Amberlite IRA - 400 (formate) column in Expt. 22 (for identification of sugar phosphates), were chromatographed on Whatman 3MM paper using Solvent c. The band, having the same R_G value as rhamnose was eluted, as in the case of glucose. The eluate was concentrated to dryness under reduced pressure at 37° and dried over phosphorus pentoxide at room temperature in vacuo to constant wt. $[\alpha]_D$ + 8.2° at equilibrium (c 0.78, water). Authentic L - rhamnose had $[\alpha]_D$ + 8.7° at equilibrium (c 0.78, water).

E. D - Mannose

D - Mannose was isolated from the same hydrolysate and paper chromatogram used to isolate cell wall L - rhamnose (as above). The eluted band, having the same R_G value as mannose, was further purified twice on Whatman 540 paper using Solvent b in order to remove the last traces of glucose. The final eluate was worked up as in the case of rhamnose (as above). The product had $[\alpha]_D$ +14.0° at equilibrium (c 1.4, water). Authentic D - mannose had $[\alpha]_D$ + 14.4° at equilibrium (c 1.4, water).

E. Xylose

The bands having the same R_G value as xylose were eluted from the above chromatograms, combined, and examined by chromatography in Solvents a, b and c and by electrophoresis using Buffers 1 and 2. There was always a single spot corresponding to authentic xylose.

F. Arabinose

The bands having the same R_G value as arabinose were eluted from the above chromatograms and examined by chromatography and electrophoresis as for xylose. There was always a single spot corresponding to authentic arabinose.

Expt. 3. Purification of Nigeran

Nigeran (Koch - Light Laboratories, Biochemical Division, Colnbrook, Bucks.) contained material insoluble in hot water (100°) and was dark brown. It was purified by extracting with boiling water, filtering hot, and cooling to 25°. The precipitated nigeran was

washed with water (25°) and freeze dried. It had $[\alpha]_D$ + 294° (c 0.2, in N - sodium hydroxide) Barker, Bourne and Stacey (1953) recorded $[\alpha]_D$ + 283° (c 0.78, in N - sodium hydroxide) for nigeran.

Expt. 4 Attempted Removal of Amylase Activity from the Digestive Enzymes of the Snail, Helix Pomatia.

A freeze dried preparation of the digestive enzymes of the snail, Helix pomatia, was obtained from Industrie Biologique Francaise S.A., 35849, Quai Du Moulin De Cage, Gennevilliers (Seine), France.

A. Using Enzyme Substrate Complexing : The methods were based on those of (i) Duke and Nordin (1961) with defatted insuluble potato starch and (ii) Greenwood, MacGregor and Milne (1965) with glycogen, which were used for the purification of α- amylase.

(i) Using Insoluble Potato Starch Column: A solution of snail enzyme preparation (100 mg.) in 0.2% aqueous calcium acetate (5 ml.) at 2° was mixed with an equal volume of 60% ethanol, containing 2 g. calcium acetate/l., maintaining the temperature between 0 - 5°. The precipitate formed was removed by centrifugation (5,000g., 20 min.) and the supernatant was applied to a column of insoluble defatted potato starch (2 x 25 cm.).The enzymes, having reduced amylase activity, were eluted from the column using a 0.2% solution of calcium acetate in 30% ethanol. The eluted material was again passed through a fresh starch column of the same size and eluted in the same manner. The enzymes were then dialysed against 0.002 M - sodium phosphate buffer, pH 7.2, and freeze dried (Fraction 4A5).

The freeze dried enzyme preparation was tested for its activity towards starch and laminarin (G.M. 18 and 19).

(ii) Using Glycogen: Ethanol was added slowly with mixing to a solution of snail enzyme preparation (100 mg.) in water (6 ml.) at 2° to a concentration of 40% (v/v) ethanol. The precipitate formed was removed by centrifugation (5,000 g., 20 min.), and a mixture of 2% (w/v) aqueous glycogen solution (0.2ml.) and 0.2M - sodium phosphate buffer, pH 8.0,(0.5 ml.) was added to supernatant (10 ml.) The mixture

was stirred for 10 min. at 2° and then centrifuged (5,000 g; 20 min.). The latter treatment was repeated and the supernatant obtained was dialysed against 0.002 M - sodium phosphate buffer, pH 7.2, and then freeze dried (Fraction 4AG).

The freeze dried enzyme preparation was tested for its activity towards starch and laminarin (G.M. 18 and 19).

B. Using Differential Denaturation by Heating: Snail enzyme preparations with reduced amylolytic activity, obtained from starch treatment [Expt. 5a(1), 2 mg.], were dissolved in 0.05M - sodium acetate buffer, pH 4.8, (1 ml.), heated at temperatures of 45°, 50°, 55°, 60° and 65° for 30 min. and than cooled to 37°. 2% substrate solution (laminarin or amylose) in 0.05 M - sodium acetate buffer, pH 4.8, (0.5 ml.) was added to each enzyme solution (0.5 ml.) and the solutions were incubated at 37° for 18 hr. Aliquots (10 ul.) of each incubation mixture were chromatographed on T.L.C. plates, irrigated with Solvent b and developed with spray reagent a. The amounts of glucose liberated from laminarin and amylose were estimated by visual comparison of the intensity of the spots.

Expt. 5. Isolation and Purification of Endo - β- D - (1→3) - Glucanase from the Digestive Enzymes of the Snail, Helix Pomatia.

The method was based on that of Noble and Sturgeon (1968), using enzyme - substrate complexing on pachyman.
It was first necessary to purify pachyman from Poria cocos Wolf fungus, which contained a significant amount of starch.

A. Purification of Pachyman : Dry Poria cocos Wolf fungus (70 g.), (obtained from San AE Trading Co. Ltd., Chung - Ku, Seoul, South Korea.) was cleaned from barks etc. with a scalpel and cut into small pieces. It was then suspended in water (1 l.), autoclaved (121°, 15 atmos.) for 30 min. and left soaking under sterile conditions overnight. It was washed with water and three times with 0.02M - sodium phosphate buffer, pH 6.9, containing 0.0067 M - sodium chloride, and again sterilised in the same buffer (100 ml.). Saliva (50 ml.), diluted to 200ml. with the above buffer, was added to the above suspension of

sterile Poria cocos mycelium, after sterilisation using a MF Millipore filter (Type GS). After incubation for 48 hr at 37°, the suspension was filtered and the residue was washed thoroughly with water, suspended in N - sodium hydroxide (1 l.) and stirred under nitrogen for 3 hr. at room temperature. After centrifugation (15,000 g., 30min.) the residue was washed with N - sodium hydroxide (2 x 200 ml.). The supernatant and washings were filtered through a sintered glass funnel under reduced pressure and the filtrate was neutralised with glacial acetic acid to pH 7.0. The precipitate was collected by centrifugation, washed with water, dialysed against water and finally freeze dried to give pachyman (25 g.), $[\alpha]_D$ + 15° (c 2.0, in N - sodium hydroxide).

B. Enzyme - Substrate Complexing on Pachyman : (All operations were carried out at 0 - 4°.) . A mixture of freeze dried snail enzyme preparation (500 mg.; containing 0.3 units of β- D - (1→3) - glucanase/mg., G.M.19), in 0.1M - sodium citrate buffer, pH 4.8, (20 ml.) and 5% suspension of pachyman in the same buffer (20 ml.) was stirred for 30 min. and then centrifuged at 15,000 g. for 10 min. The residue was washed with fresh buffer (5 x 30 ml.) within a period of 2 hr. and then suspended in 0.1M - sodium borate buffer, pH 8.2, (30 ml.) and the mixture was stirred for 1 hr. and then centrifuged at 15,000 g. for 10 min. The residue was washed with the borate buffer (2 x 10 ml.), and the supernatant and washings were mixed and dialysed sequentially against water (1 x 5 l.), 0.002M - sodium phosphate buffer, pH 7.2, (1 x 5 l.) and finally water (1 x 1 l.) within a period of 24 hr. The preparation was assayed for β- D - (1→3) - glucanase activity (G.M.19) and then freeze dried.

The freeze dried enzyme was tested for its activity towards starch, cellulose, pustulan, pachyman and laminarin in 0.05 M - sodium acetate buffer, pH 4.8. (G.M.19)

Expt. 6. Preparation and Partial Purification of Exo - β- D - (1→3) - Glucanase from Basidiomycete QM 806

The growth of Basidiomycete QM 806 and preparation of the exo - β- D - (1→3) - glucanase was carried out essentially

according to the method of Huotari, Nelson, Smith and Kirkwood (1968). The method was modified as described below.

(a) Growth of Basidiomycete QM 806

The medium pH 6.2, contained per 10 l.; ammonium sulphate (14 g.), potassium dihydrogen phosphate (19 g.), urea (3 g.), calcium chloride (3 g.), magnesium sulphate heptahydrate (3 g.), yeast extract (1 g.), glucose or starch (100 g.), ferrous sulphate heptahydrate (50 mg.), manganese sulphate tetrahydrate (20.2 mg.), zinc sulphate heptahydrate (26 mg.), cobalt chloride hexahydrate (20.2 mg.) and water to 10 l.

Media at the seed stage (100 ml./500 ml. flask) were inoculated with Basidiomycete QM 806 spores from an agar slant and were then incubated at 24° on a shaker for 3 days. The seed stage cultures were quantitatively transferred into the second medium (100 ml. seeds/1.of second medium /4 l. flask). Incubation was continued at 24° on a shaker for a further 14 days. Samples of incubation mixture were withdrawn during the last four days of incubation and tested for starch (using 0.1N - iodine solution), pH and activity towards laminarin and starch.

The filtered culture medium (10 l., containing 1.3 g of protein, measured by G.M. 8b) was evaporated under reduced pressure at 37° on a rotary evaporator to approximately 100 ml. After centrifugation (10,000 g., 30 min.) the supernatant was dialysed against distilled water (3 x 5 l.) at 4° for 24 hr. and finally freeze dried to give the crude enzyme (1.3 g.), which had 13 units of β- D - (1→3) - glucanase activity/mg. (G.M.19) and 1.6 units of amylase activity/mg. (G.M.19).

(b) DEAE Cellulose Chromatography :

A DEAE cellulose (Whatman DE - 52) column (1.3 x 42 cm.) was equilibrated with 0.002 M - sodium phosphate buffer, pH 7.0 (G.M.26). Crude enzyme (50 mg.), dissolved in the same buffer (5 ml.), was applied to the column, and elution was begun with the phosphate buffer at 6 ml./hr. The effluent was collected in 1.5 ml. fractions and the absorbancies were measured at 260 and 280 nm. After the first protein peak had been eluted (66 ml.), elution was continued with a

linear gradient between the starting buffer (300 ml.) and 0.05 M - sodium phosphate buffer, pH 7.0, containing 0.2 M - sodium chloride (300 ml.). The whole process was carried out at 4°.

Activities of the fractions towards laminarin and starch were determined in 0.05 M - sodium acetate buffer, pH 4.8, (G.M.19).

(c) Adsorption of β- D - (1→3) - Glucanase Activity on Pachyman.

Operations were carried out at 0° unless otherwise stated. A solution of crude freeze dried enzyme (300 mg., containing 9 units of β- D - (1→3) - glucanase activity/mg. and 1.1 unit of amylase activity/mg. in water (8 ml.) and McIlvine (1921) citrate - phosphate buffer, pH 3.5, (16 ml.) was mixed with a 1% suspension of pachyman (Expt. 5A) in water (20 ml.) The mixture was stirred for 30 min. and then centrifuged at 15,000 g. for 10 min. The residue was washed three times by resuspension and centrifugation with the same buffer, diluted 1 : 2 with water, (3 x 15 ml.). The residue was resuspended in 0.05 M - sodium acetate buffer, pH 4.8, (15 ml.) and incubated at 37° for approximately 6 - 8 hr. (within that time almost all the pachyman had been hydrolysed to glucose and gentiobiose). The mixture was centrifuged at 15,000 g. for 10 min. and the supernatant, which contained 26% of the original β- D - (1→3) glucanase activity, was dialysed against 0.002 M - sodium phosphate buffer, pH -7.0, (2 x 5 l.) and finally freeze dried. Activities towards, starch, cellulose, pustulan and laminarin were determined as described in G.M.19.

Residual activity in the supernatant and washings of pachyman could be recovered by repeated complexing of the supernatants with the freeze dried pachyman, resulting in almost quantitative recovery of β1→3 glucanase activity.

Expt. 7. Preparation and Partial Purification of Pullulanase from Aerobactor aerogenes.

Pullulanase was prepared by a modification of the method of Bender and Wallenfels (1961), as described below.

(a) Growth of Aerobactor aerogenes :

Intermediate seed medium, pH 7.0, contained per 200 ml.:

beef extract (600 mg.), peptone (600 mg.), sodium chloride (140 mg.), di-potassium hydrogen phosphate (140 mg.), glucose (1.2 g.) and water to volume.

Culture medium, pH 6.8, contained per 2 l. : sodium nitrate (10 g.), potassium dihydrogen phosphate (3 g.), magnesium sulphate heptahydrate (1 g.), potassium chloride (1 g.), ferrous sulphate heptahydrate (0.02 g.), peptone (16 g.), maltose 10 g.) and water to volume.

Seed stage medium (100 ml./500 ml. flask) was inoculated with Aerobactor aerogenes (the strain isolated by Bender and Wallenfels (1961) and kindly given by Prof. W.J. Whelan) and incubated at 30° for 48 hr. The culture was quantitatively transferred into the second medium (100 ml. seeds/1. of second media/4 l. flask). Incubation was continued at 30° for a further 48 hr.

(b) Isolation of Crude Enzyme:

The cells were removed by centrifugation at 10,000 g. for 10 min. and the supernatant was cooled to 0°. The enzyme was precipitated with precooled acetone (-20°, 1.5 l. acetone/1. culture media). The precipitate was collected by centrifugation (1,000 g., 5 min.), washed with 66% aqueous acetone and dried in vacuo over calcium chloride at 4° (weight of dried precipitate 1.4 g.).

Precipitate (1 g.) was shaken with 0.02 M - sodium phosphate buffer, pH 6.8, (9.0 ml.) at 4° for 3 hr., and the insoluble residue, obtained on centrifugation for 30 min. at 10,000 g., was extracted with the same buffer (1 ml.) and then centrifuged as before. The combined extracts were dialysed against the same buffer (2 x 1 l.) for 24 hr. at 4° and then freeze dried to give the crude enzyme (200 mg.).

(c) DEAE - Cellulose Chromatography :

A solution of the crude enzyme (30 mg.) in 0.1% sodium lauryl sulphate (15 ml.) was applied to a Whatman DE-52 DEAE - cellulose column (1.3 x 15 cm.), equilibrated with 0.01 M - tris - hydrochloric acid buffer, pH 7.7, and eluted stepwise at 15 ml./hr with (a) 0.1 M - ammonium sulphate (20 ml.) and (b) 0.4 M ammonium

sulphate (25 ml.). 1 ml. fractions were collected and absorbancies measured at 280 nm.

10 µl. of each fraction was incubated with pullulan solution (G.M.17) and the incubation mixtures were examined by T.L.C. in solvent b, using a multiple ascending development technique, and sprayed with reagent a. On the basis of the chromatographic result the following fractions were combined : 14 to 17 (Fraction A), 18 to 24 (Fraction B) and 34 to 38 (Fraction C). Fractions A, B and C were dialysed separately against 0.002 M - sodium phosphate buffer, pH 7.2, (1 x 5 l.) for 8 hr. at 4° and then freeze dried. Yield, Fraction A, 10 mg.; Fraction B, 4 mg.; Fraction C, 2.5 mg.

The three fractions were tested for activity towards laminarin (G.M.19), amylose (G.M.18), and pullulan (G.M.17).

Expt. 8 Preparation and Partial Purification of Human Salivary α- Amylase

The preparation and purifaction of salivary α- amylase was carried out according to the method of Bernfeld (1955), omitting the crystallisation procedure. The preparation was tested for activity against amylose, amylopectin , starch, dextran, Nigeran, Laminarin, pustulan, cellulose, cellobiose and laminaribiose in 0.02 M - sodium phosphate buffer, pH 6.9, containing 0.0067 M - sodium chloride, at 37° (G.M.18).

Expt. 9 Action of Enzymes on the Intact Mycelium of Fusicoccum amygdali

(a) Action of Salivary α- Amylase: To a 3 day old mycelial culture grown in medium II (G.M.30) in shaken flasks (adjusted to 0.01 M -sodium chloride by the addition of sodium chloride), or mycelium (5 g. wet wt.), collected from a 40 l. fermenter (G.M.30) by centrifugation, and suspended in 0.02 M sodium phosphate buffer, pH 6.9, (100 ml.) containing 0.0067 M - sodium chloride, was added salivary α- amylase (100 units/100 ml. incubation mixture) and the mixture was incubated at 37° for 24 hr. Mycelium treated under

identical conditions omitting the enzyme was used as control. Small portions of treated and control mycelium were mixed with 0.1N - iodine solution and examined under the phase contrast microscope.

(b) Action of Basidiomycete QM806 Exo-β-D - (1→3)-Glucanase : The mycelial culture was adjusted to pH 4.8 with 0.1N - acetic acid or the centrifuged mycelium was suspended in 0.05 M - sodium acetate buffer, pH 4.8. Exo - β- D - (1→3)- glucanase (crude acetone precipitate) (100 units/100 ml. culture) was added and the mixture was incubated at 37° for 24 hr. Mycelium treated under identical conditions was used as control. The staining properties of the treated and control mycelium towards iodine solution and cotton blue solution were determined.

Expt. 10 Action of the Digestive Enzymes of the Snail, Helix Pomatia on the Total Cell Wall.

A mixture of a 1% suspension of cell wall (Expt. 1A) homogenate and 0.1% freeze dried snail enzyme preparation in 0.01 M - citrate - phosphate buffer, pH 5.8, was incubated at 37° with stirring for 96 hr. (Millbank and Macrae, 1964). An enzyme blank and a cell wall blank were incubated at the same time. Aliquots were withdrawn at different time intervals and the reaction was stopped by placing in a boiling water bath for 5 min. After centrifugation (15,000 g., 20 min.) the supernatants were assayed for total hexose and glucose (G.M.1 and 3), before and after N - sulphuric acid hydrolysis (G.M.15A), and N - acetyl - D - glucosamine (G.M.5).

Other sugar constituents in the supernatants were identified by paper chromatography (Solvents b and d).

After 96 hours' incubation the solutions were heated at 100° for 5 min., and centrifuged (30,000 g., 20 min.), and the supernatants were dialysed against distilled water (3 x 5 l. per 20 ml. of supernatant), freeze dried and weighed.

The insoluble residue was recovered by centrifugation (15,000 g., 20 min.), washed with water (4 x 45 ml. per 100 mg. of incubated cell walls) and lyophilised.

Residual cell wall (20 mg.) after snail enzyme treatment was suspended in N - sodium hydroxide (10 ml.), stirred under nitrogen for 1 hr. and then centrifuged (10,000 g., 10 min.). The residue was washed with N - sodium hydroxide (10 ml.). The combined supernatant and washings were neutralised with glacial acetic acid, dialysed against distilled water (3 x 5 l.) for 24 hr., and freeze dried. (Wt. 16 mg.)

Expt. 11 Action of Chitinase on the Total Cell Wall

Cell walls (Expt. 1A) were incubated with chitinase, as described in G.M. 21(c), including an enzyme blank and a cell wall blank. Aliquots were withdrawn at different time intervals and then heated at 100° for 5 min., cooled and centrifuged (15,000 g.,20 min.), and then supernatants were assayed for N - acetyl - D - glucosamine (G.M.5), hexose (G.M.1) and glucose (G.M.3).

After 24 hr. the incubation mixture was boiled for 5 min., centrifuged as above and the supernatant was examined by paper chromatography in solvent d, and then dialysed against water (3xl l.) per 10 ml. of supernatant) and lyophilised.

The residue was washed with water (3 x 45 ml. per 100 mg. of incubated cell walls), and lyophilised.

Expt. 12 Action of Basidiomycete QM 806 Exo -β- D - (1→3) - Glucanase on the Total Cell Wall

A suspension of cell walls (2 mg.) in 0.05 M -sodium acetate buffer, pH 4.8, (0.5 ml.) was homogenised and mixed with a solution of β- D - (1→3) - glucanase (0.2 mg.,crude acetone precipitate) in the same buffer (0.5 ml.) and incubated at 50°, including an enzyme blank and a cell wall blank. Aliquots were withdrawn at different time intervals, boiled for 5 min. and centrifuged (15,000 g., 20 min.), and the supernatant was assayed for total hexose and glucose (G.M.1 and 3). After 24 hr. the incubation mixture was boiled for 5 min., cooled, and centrifuged as above and the supernatant was deionised with a mixture of Amberlite 1R - 120 (H^+)

and Amberlite 1R - 4B (OH^-) resins and examined by paper chromatography (Solvents b and d). The residue was washed with water (3 x 45 ml. per 100 mg. of incubated cell walls) and lyophilised.

Expt. 13 Deproteinisation of Cell Walls

A. Using Pronase : A solution of pronase [2 mg., British Drug Houses, Poole, Dorset, 45,000 PUK units/gm. (One PUK unit of proteolytic activity is the amount of enzyme required to give an optical density reading of 1.0 at 660 nm. 40° and pH 7.4, as determined by the Casein Folin method; Nomoto and Narahashi, 1959)] in 0.03 M - sodium phosphate buffer, pH 7.4, (1.0 ml.) was added to a homogenised cell wall suspension (200 mg., 3rd Batch) in the same buffer (18.6 ml.), containing ethanol (0.4 ml.) and the mixture was incubated at 37° for 2 days, and then centrifuged (10,000 g., 20 min.). The residue was washed with distilled water (5 x 45 ml.) and then freeze dried. Recovery 174.4 mg. The supernatant was analysed for carbohydrate composition before and after hydrolysis with N - sulphuric acid (G.M.15A). Retreatment of residual cell wall had no effect.

B. Using - chymotrypsin : A solution of - chymotrypsin [2 mg., Boehringer, Cat. No. ECAA 15139, 10,000 units/mg. (One unit is that amount of enzyme which causes a decrease in the optical density of 0.001/min. under the assay conditions; TaKenaKa, 1955)] in 0.15 M - sodium phosphate buffer, pH 7.0, (5 ml.) was added to a cell wall suspension (200 mg., 3rd Batch) in the same buffer (15 ml.) and the mixture was inoubated at 37° for 2 days and then centrifuged (10,000 g., 20 min.). The residue was washed with water (5 x 45 ml.) and then freeze dried. Recovery 182.2 mg. The supernatant was analysed for total hexose (G.M.1) and free glucose (G.M. 3.)

C. Using 90% Phenol : Cell wall preparations (500 mg., 3rd Batch) were homogenised in 90% (w/v) phenol (45 ml.), heated at 65 - 68° for 15 min., and then centrifuged (10,000 g., 20 min.). The residue was re-extracted twice with 90% phenol (2 x 45 ml.) in the same manner, washed with distilled water (6 x 45 ml.) and freeze dried : Recovery 450 mg.

Expt. 14. Fractionation of Cell Walls with Potassium Hydroxide.

Alkali extractions were carried out in an atmosphere of nitrogen (oxygen free) at room temperature (20 - 25°) unless otherwise stated.

A. A suspension of cell walls (100 mg. 1st Batch) was stirred in water (12 ml.), containing sodium borohydride (10 mg.), for 18 hr., adjusting the pH to 8.0 with carbondioxide, and then adjusted to N - potassium hydroxide by addition of concentrated potassium hydroxide solution (final volume 20 ml.). The suspension was stirred for 3 hr., and centrifuged (10,000 g., 20 min.) and the residue was washed with N - potassium hydroxide (2 ml.). The residue was further extracted with N - potassium hydroxide (20 ml.) for successive periods of 6 hr., 15 hr., and 30 hr. by the same method. The final residue was washed with water (2 x 20 ml.), dialysed against water (3 x 1 l.) and freeze dried.

The supernatant and washings from the 3 hr., 6 hr., and 15 hr., extracts were neutralised separately with glacial acetic acid, dialysed exhaustively against water and freeze dried.

B. Cell walls (200 mg., 3rd Batch) were homogenised in water (20 ml.), sodium borohydride (20 mg.) was added, the pH was adjusted to 8 with carbon dioxide, and the mixture was stirred for 24 hr. 2 N - Potassium hydroxide (20 ml.) was added and the mixture was stirred for a further period of 24 hr., and then centrifuged (10,000 g., 20 min.) and the residue was washed twice with N - potassium hydroxide (2 x 20 ml.). The combined supernatant and washings were neutralised with acetic acid and then dialysed exhaustively against water. The resultant precipitate was centrifuged as above and the residue was washed with water (2 x 20 ml.), and freeze dried to give Fraction 14B B, and the combined supernatant and washings were freeze dried to give Fraction 14B A.

The residue remaining after N - potassium hydroxide extraction was neutralised, dialysed and freeze dried to give Fraction 14B C.

C. Cell walls (200 mg., 3rd Batch) were homogenised in 0.05M - sodium acetate buffer, pH 5.0, (40 ml.) and heated for 3 hr. at 75°. After

cooling the mixture was centrifuged (10,000 g., 20 min.) and the residue was washed with water and freeze dried.

The residue was fractionated with N - potassium hydroxide as described in Expt. 14 B to give : Fraction 14 C A (water soluble, alkali soluble); Fraction 14 C B (water insoluble, alkali soluble); Fraction 14 C C (water insoluble, alkali insoluble).

D. Cell walls were deproteinised with pronase, as described in Expt. 13 A, and then fractionated with N - potassium hydroxide, as described in Expt. 14 B, to give : Fraction 14 D A (water soluble, alkali soluble); Fraction 14 D B (water insoluble, alkali soluble); Fraction 14 D C (water insoluble, alkali insoluble).

E. Cell wall preparations (300 mg., 3rd Batch) were boiled under reflux with 0.53 N - hydrochloric acid (150 ml.) for 2 hr., cooled and then centrifuged (10,000 g., 30 min.), washed with water (5 x 45 ml.) and freeze dried to give Fraction 14 E U. The supernatant was analysed for total hexose (G.M. 1), glucose (G.M. 3) and examined by paper chromatography with Solvent b.

Fraction 14 E U (50 mg.) was reduced with sodium borohydride (50 mg.) in water (5 ml.) for 16 hr. The reduced suspension was then diluted with 2 N - potassium hydroxide (5 ml.), stirred for 3 hr., centrifuged (10,000 g., 20 min.) and washed with N - potassium hydroxide (2 x 10 ml.). The residue was stirred for a further 24 hr. with N - potassium hydroxide (10 ml.) and then centrifuged (10,000 g., 20 min.), washed with water until free from alkali, and freeze dried, to give Fraction 14EV (14.7 mg.).

The combined supernatant and washings from the 3 hr. alkali extraction were neutralised with glacial acetic acid, dialysed against water (6 x 5 l.) and freeze dried to give Fraction 14 EW.

The second alkali extraction (24 hr.) solubilised less than 1% anhydrohexose (G.M. 1).

F. Cell walls (112 g., 4th Batch) were suspended in water (9 l.), containing sodium borohydride (9 g.), stirred for 36 hr., centrifuged (10,000 g., 30 min.) and washed with water (2 x 2 l.).

(a) The combined supernatant and washings were neutralised with glacial acetic acid, evaporated under reduced pressure to ca. 500 ml., dialysed exhaustively against water and freeze dried, to give Fraction 14FA (1.8 g.).

(b) The residue was suspended in water (total volume 5 l.), containing sodium borohydride (9 g.), 2.25 N - potassium hydroxide (4 l.) was added and the mixture was stirred for 24 hr. and then centrifuged (20,000 g., 30 min.). The residue was washed with N - potassium hydroxide (1 l.), neutralised with glacial acetic acid, washed with water (18 l.) on a sintered funnel covered with nylon nettings, and freeze dried to give Fraction 14FB (60 g.).

(c) The combined supernatant and alkali washings from (b) ($[\alpha]_D$ + 132°) were adjusted to pH 11.5 with glacial acetic acid at 0°. As soon as the precipitate started to form the pH was adjusted to 13.5 by the addition of 10 N - potassium hydroxide and the mixture was stored at 0° for 2 hr. The resultant precipitate, $[\alpha]_D$ + 180°, was collected by centrifugation.

The pH of the supernatant was further adjusted to 12.5 with glacial acetic acid. After standing at 0° for 2 hr., the precipitate, $[\alpha]_D$ + 170°, was collected by centrifugation.

The pH of the supernatant was further adjusted to pH 7.0 with glacial acetic acid, and after standing at 0° for 1 hr. the precipitate formed, which had $[\alpha]_D$ +136°, was collected by centrifugation.

The supernatant, which had $[\alpha]_D$ +11.1°, was concentrated under reduced pressure to 2 l., cooled to 0° and an equal volume of 95% ethanol at 0° was added with stirring. After standing at 0° overnight, the precipitate was collected by centrifugation, dialysed against water (6 x 5 l) and freeze dried to give Fraction 14FCI (2.0 g.), $[\alpha]_D$ - 95°.

The supernatant was adjusted to 70% v/v ethanol and stored overnight at -20°. The resultant precipitate, was dialysed against distilled water (9 x 5 l.) and freeze dried to give Fraction 14FCII (0.7 g.,), $[\alpha]_D$ - 100°.

Individual fractions were further fractionated by the following general method. Fractions were dissolved in N - sodium

hydroxide to give a 1 to 5% solution, and cooled to below 15°, and glacial acetic acid (1/20 vol.) was added slowly keeping the temperature of the solution below 20° (no precipitation occurred at this stage). Further addition of acetic acid (3N) was carried out dropwise with stirring, until precipitation was induced as judged by the turbidity of the solution. After standing for at least one hr. at 0°, the precipitate was centrifuged off, and the supernatant was further neutralised with dilute acetic acid in the same manner and the precipitates formed were collected separately at the different pH's until the pH reached 7.0. The final fractions were dialysed exhaustively against water and freeze dried.

Expt. 15. Attempted Separation of Glucan and Heteroglycan Polymers

(a) Barium Hydroxide Precipitation (i) Saturated aqueous barium hydroxide (80 ml.) was added with mixing to a solution of cell wall Fraction 14BB (135 mg.) in N - sodium hydroxide (20 ml.) and after one hour the mixture was centrifuged (10,000 g., 30 min.). The supernatant was neutralised with glacial acetic acid and the resultant precipitate was collected by centrifugation (10,000 g., 20 min.), washed with water (2 x 45 ml.), dialysed against water (6 x 5 l.) and lyophilised. Recovery 97 mg.

The combined supernatant and washings were concentrated to 50 ml., centrifuged (10,000 g., 20 min.). The supernatant was dialysed (6 x 5 l.) and freeze dried. Recovery 20 mg.

(ii) Saturated aqueous barium hydroxide (50 ml.) was added with mixing to a solution of cell wall Fraction 14BA (83 mg.) in M - sodium - acetate (15 ml.) and after one hour the mixture was centrifuged (10,000 g., 20 min.). The supernatant was dialysed against water (6 x 5 l.), and freeze dried. Recovery 60 mg. The precipitate (5.3 mg.) was analysed for total hexose (G.M.1)

(b) Precipitation with Fehling Solution.

Reagents

A. Copper sulphate pentahydrate (17.5 g.) was dissolved in water (250 ml.), containing concentrated sulphuric acid (0.3 ml.).

B. Potassium sodium tartrate tetrahydrate (85 g.) and sodium hydroxide (25 g.) were dissolved in water (250 ml.).

Before use 1 volume of Solution A was mixed with 1 volume of Solution B.

Procedure

Fehling solution (4 ml.) was added dropwise with mixing to a solution of cell wall Fraction 14BB (333 mg.) in N - potassium hydroxide (33 ml.) at 0°. No precipitate was observed. After one hour the solution was centrifuged (10,000 g., 20 min.) and the pH of the supernatant was adjusted to 3.0 with conc. hydrochloric acid at 0°. After 2 hr. at 0° the suspension was centrifuged (10,000 g., 20 min.) and the residue was washed with 0.1 N - hydrochloric acid (2 x 45 ml.), dialysed against water (6 x 5 l.) and lyophilised. Recovery 218 mg.

The combined supernatant and washings were concentrated to 40 ml., neutralised, and dialysed against water (9 x 5 l.). The resultant precipitate was centrifuged (10,000 g., 20 min.) and both supernatant and precipitate were dialysed and lyophilised. Recovery 5 mg. and 87 mg. respectively.

(c) Extraction with 8 M - Urea. Cell wall Fraction 14BB (50 mg.) was homogenised in 8 M - urea, pH 10.0, (5 ml.) and then centrifuged at 20,000 g..for 20 min. The residue was washed with 8 M - urea (2 x 5ml.) and then stirred in 8 M - urea (10 ml.) overnight at room temperature. The insoluble residue was collected by centrifugation (20,000 g., 20 min.), washed with water (5 x 45 ml.) and freeze dried. Recovery 37 mg.

Polysaccharides were precipitated from the combined extracts and washings by addition of ethanol to 70% (v/v) final concentration. The precipitate was washed with ethanol and freeze dried. Recovery 8.5mg.

(d) Ultrasonication : Cell wall Fraction 14BB (50 mg.) was suspended in water (10 ml.) and sonicated for 1 min. (MSE 100 W, 20 kHz ultrasonic disintegrator) at an amplitude of 8 micron from peak to peak at 0 - 4°. After centrifugation (10,000g., 20 min.) the residue was washed with water 2 x 10 ml.), and freeze dried. Recovery

45 mg.

The combined supernatant and washings were freeze dried. Recovery 3 mg.

Expt. 16 Dimethyl Sulphoxide Fractionation

Cell wall preparations (100 mg. 3rd Batch) were stirred for 24 hr. or 5 days at room temperature, or 3 hr. at 60° in anhydrous dimethyl sulphoxide (20 ml.). The mixture was then centrifuged at 10,000 g. for 30 min. and washed twice with anhydrous dimethyl sulphoxide (2 x 5 ml.). The combined washings and supernatant were diluted with water, dialysed exhaustively against water and freeze dried. The residue was dialysed against water and freeze dried.

Expt. 17 Ethylene Diamine Fractionation

Cell wall preparations (200 mg. 3rd Batch) were stirred for 24 hr. at room temperature in anhydrous ethylene diamine (200 ml.). The mixture was then centrifuged at 10,000 g. for 30 min. and the residue was re-extracted with fresh anhydrous ethylene diamine (20 ml.) as before for a further 24 hr. After centrifugation the residue was washed twice with ethylene diamine (2 x 10 ml.). The combined supernatants and washings were evaporated under reduced pressure to 20 ml., diluted with water (20 ml.) and dialysed exhaustively against water. The resultant precipitate was centrifuged off, washed with water and freeze dried to give Fraction 17B . The combined supernatant and washings were freeze dried to give Fraction 17A.

The residue insoluble in ethylene diamine was dialysed, and freeze dried to give Fraction 17C .

Expt. 18 Chloral Hydrate Fractionation

Cell wall preparations (100 mg., 3rd Batch) were homogenised in 33% (W/v) aqueous chloral hydrate (10 ml.), heated at 100° for 5 hr., and centrifuged hot at 16,000 g. for 10 min. The

residue was washed with hot 33% (w/v) aqueous chloral hydrate (1 x 10 ml.). The combined supernatant and washings were dialysed exhaustively against water and freeze dried. The residue was dialysed and freeze dried as above.

Expt. 19 Test for Cell Wall Chitosan

The method was based on that of Bartnicki-Garcia and Nickerson (1962), used for chitosan isolation from Mucor rouxii cell walls.

Cell wall preparations (500 mg., 3rd Batch) were heated at 100° for 30 min. with N - hydrochloric acid (250 ml.) or N - acetic acid (250 ml.) respectively, and then cooled and centrifuged (10,000 g., 20 min.). The supernatants were neutralised and since no precipitate of chitosan formed, evaporated under reduced pressure to 50 ml., dialysed exhaustively against water and freeze dried, to give an extract (10 mg. and 15 mg. respectively), soluble in both N - sodium hydroxide and 0.1 N - acetic acid.

Expt. 20 Chemical Isolation of Cell Wall Chitin.

A. Cell wall preparation (1.0 g., 2nd Batch) was homogenised in distilled water (50 ml.), 6% sodium hydroxide (50 ml.) was added and the mixture was heated in a nitrogen atmosphere at 60° for 30 min., and then centrifuged (10,000 g., 20 min.). The residue was washed with 3% sodium hydroxide (2 x 50 ml.) and then with water until free from alkali and was freeze dried (Fraction 20 AC).

The supernatant and washings were neutralised with glacial acetic acid. The precipitate formed was collected by centrifugation as above, washed with water (1 x 100 ml.) and then dialysed exhaustively against water and freeze dried (Fraction 20AB). The supernatant and washings from Fraction 20AB were dailysed and freeze dried (Fraction 20AA).

Fraction 20AC was homogenised in 2% hydrochloric acid (50 ml.) and heated under reflux for 2 hr. at 116 - 120°. After cooling and centrifugation (10,000 g., 20 min.) the residue was washed with water

until free from acid, and freeze dried (Fraction 20AD). The supernatant was tested for chitosan and other free sugars.

Fraction 20AD (100 mg.) was re-extracted with 3% sodium hydroxide as above and the residue (Fraction $20AD_I$) and the supernatant (Fraction $20AD_{II}$) were freeze dried.

Fraction $20AD_I$ (12 mg.) was stirred with 36% hydrochloric acid (4 ml.) at 4° for 20 min. and after centrifugation the residue was washed with water and freeze dried (Fraction $20AD_Ia$). The combined supernatant and washings were neutralised with N - sodium hydroxide, dialysed, and freeze dried (Fraction $20AD_Ib$).

B. Cell wall preparations (1.5 g., 2nd Batch) were stirred at 4° with fuming hydrochloric acid (50 ml.) for 20 min. and then centrifuged at 11,000 g. for 15 min. The supernatant was poured into cold 50% (v/v) ethanol (100 ml.) with stirring and ethanol (50 ml.) was added. The resultant precipitate was collected by centrifugation, washed with water, dialysed and freeze dried (Fraction 20BA).

The residue from the hydrochloric acid extraction was stirred with fuming hydrochloric acid (20 ml.) for 2 hr at 4°, the suspension was centrifuged and the supernatant was worked up as above.(Fraction 20BB). The residue was washed with water until free from acid and freeze dried (Fraction 20BC).

Expt. 21 Preparation of Chitosan from Cell Wall Chitin

A. Cell wall Fraction 14BC (300 mg.) was homogenised in 10 M - sodium hydroxide (20 ml.) and heated under reflux at 115° under nitrogen for 6 hr. The resultant mixture was centrifuged (10,000 g., 20 min.) and after neutralisation with N - hydrochloric acid, the residue was heated with 0.1 N - hydrochloric acid (5 ml.) at 80° for 10 min. After cooling and centrifugation (10,000 g., 20 min.) the supernatant was neutralised with sodium hydroxide and the resultant precipitate was centrifuged, washed with 70% (v/v) ethanol (5 x 4 ml.), and freeze dried to give a white and fluffy solid Fraction 21AA (1.5 mg.)

B. Preparation of Chitosan from Crustacean Chitin.

The yield of chitosan from crustacean chitin using the method described in Expt. 21A was less than 1%. A good yield was obtained with the following method.

Crustacean Chitin (British Drug Houses, Poole, Dorset, England, 500 mg.) was suspended in 90% potassium hydroxide (10 ml.) and heated in a nickel crucible under nitrogen at 160° for 30 min. The resultant semisolid material was poured cautiously into ethanol and the precipitate was centrifuged, and washed with ethanol and water until free from alkali and then shaken with 10% acetic acid (20 ml.). After centrifugation (10,000 g., 20 min.), the supernatant was neutralised with 10 N - sodium hydroxide. After two more precipitations from 10% acetic acid solution, the final precipitate was washed with water (5 x 45 ml.) and freeze dried to give chitosan, Fraction 21BA (350 mg., 70%).

van Wisselingh Test for Chitosan (Tracey, 1955). A drop of 0.2% iodine in 20% potassium iodide solution was mixed with a small portion of the chitosan preparation (Fraction 21AA) on a slide. The preparation became brown and turned dark red-violet on addition of a drop of 1% sulphuric acid. Authentic chitosan preparation Fraction 21BA under identical conditions of staining gave the same colour transformation.

Expt. 22 Isolation and Identification of Sugar Phosphates in the Cell Wall Fraction Solubilised by Pronase

Freeze dried supernatant (240 mg.), obtained after pronase treatment on the cell walls (Expt. 13A), was suspended in 2 N - hydrochloric acid (100 ml.), heated at 100° for 75 min. and then neutralised with Amberlite LA-2 in petroleum ether (b.p.100 - 120°) and evaporated to dryness under reduced pressure at 37°. The residue was taken up in water (10 ml.) and applied to an Amberlite IR-120 (H^+) column (G.M.6). The sugar phosphates and neutral sugars were collected by eluting the column with water (10 ml.). The combined effluent and washings were concentrated to 5 ml. and applied to an

Amberlite IRA - 400 ($HCOO^-$) column (1 x 10 cm.). The column was eluted with water (50 ml.) to remove neutral sugars, and the sugar phosphates were then eluted with N - formic acid (100 ml.). The formic acid eluate was extracted with ether (10 x 100 ml.) and the aqueous phase was evaporated under reduced pressure to 1 ml. The mixture was chromatographed on Whatman 3MM paper in solvent k, and sugar phosphates and inorganic orthophosphate were detected by spraying strips cut from the edges of the chromatogram with reagents a, b or d. The bands with R_{Pi} values 1.15 (Fraction $22X_1$) and 1.3 (Fraction $22X_2$) were eluted separately with water (2 ml.) as described in Expt. 2B (i).

Periodate Oxidation. Aliquots (1 ml.) of Fractions $22X_1$ and $22X_2$ were mixed with a solution of 0.025 M - sodium metaperiodate in water (4 ml.) and stored in the dark at 4° for 24 hr. Redistilled ethylene glycol (0.1 ml.) was added and after 5 min. 0.025 M - barium chloride (4 ml.). The precipitate was removed by centrifugation and the supernatant concentrated (0.5 ml.) and chromatographed in Solvent k. Both fractions revealed one main component with R_{Pi} value 1.74 and a trace amount of material with R_{Pi} 1.15, both detectable with spray reagents b and d. The band with R_{Pi} value 1.74 was separated by preparative chromatography on Whatman 3MM paper and after elution with water was incubated with acid phosphatase (G.M.21d) for 18 hr. Sodium borohydride (5 mg.) was then added and after 2 hr. the solution was acidified with acetic acid. Borate was removed as methyl borate by repeated evaporation from methanol and sodium ions were removed with Amberlite IR - 120 (H^+). Paper chromatography in solvent e revealed (spray reagent a) a single component with R_G value 2.54.

Expt. 23 Action of Enzymes on Cell Wall Fraction 14FJ

(a) α- Amylase: A mixture of cell wall Fraction 14FJ (100 mg.), salivary α- amylase (150 units, as prepared in Expt. 8), and erythritol (1.5 mg.) in 0.02 M - sodium phosphate buffer, pH 6.9 (5 ml.), containing 0.0067 M - sodium chloride was incubated at 37°

for 24 hr. After 3 hr. the preparations ceases to stain with 0.1 N iodine solution. After centrifugation (20,000 g. 30 min.), the residue was washed with water (5 x 10 ml.) and freeze dried (86 mg.). A portion of the residue was retreated with salivary α- amylase under the same conditions.

The supernatant was analysed for total hexose (G.M.1), reducing sugars (G.M.2) and glucose (G.M.3) and was examined by paper chromatography (Solvent b) with detection by reagents a and b. Components with the same R_G values as maltose and maltotriose were separated by chromatography on Whatman 3MM paper (G.M.22), eluted with water and aliquots (0.1 ml., containg 100 - 500 µg. of sugars) were incubated with emulsin (G.M.21a., 0.1 mg.) and β-amylase (G.M.16, 25 units). The enzyme incubation mixtures were deionised with Amberlite IR-120 (H^+) and Amberlite IR-4B (OH^-) and examined by paper chromatography (Solvent b, spray reagents a and b). Standard maltose and maltotriose were treated under identical conditions.

An essentially similar experiment was carried out using hog pancreatic α-amylase (Worthington Biochemical Corporation, 100 units).

(b) β - Amylase: Cell wall Fraction 14FJ (20 mg.) was digested in β- amylase - buffer incubation mixture [2 ml., containing human serum albumin (1 mg.); glutathion (0.3 mg.); sodium citrate buffer, pH 5.0, 25 u mole); erythritol (5 m moles); β- amylase (1,140 units)] at 37° for 24 hr. under a layer of toluene. A substrate and an enzyme blank were used as controls. The mixture was centrifuged (20,000 g.,30 min.) and the residue was washed with water (5 x 5 ml.) and freeze dried (16.0 mg.). A portion was reincubated under the same conditions.

The supernatant was analysed for total hexose (G.M.1), reducing sugars (G.M.2) and glucose (G.M.3) and was examined by paper chromatography (Solvent b; spray reagents a & b).

(c) Pullulanase: Cell wall Fraction 14FJ (10 mg.), before and after treatment with α-amylase (Expt. 23a.) or β - amylase (Expt. 23b.), was treated with pullulanase (1 unit, G.M.17) in 0.02 M citrate-phosphate buffer, pH 5.0, (2 ml.) at 37° for 24 hr. A substrate

blank of appropriate cell wall fraction and pullulan, and an enzyme blank were used as controls. The supernatant obtained after centrifugation (20,000 g., 30 min.) was chromatographed in Solvent b after deionisation with a mixture of Amberlite IR-4B (OH^-) & IR-120(H+) resins. Sugars were detected by spray reagents a and b .

(d) Basidiomycete QM806 β- D - (1→3) - Glucanase : Cell wall Fraction 14FJ (10 mg.), before and after treatment with α- amylase (Expt. 23a), was incubated with crude or purified (Expt. 6a & c) Basidiomycete QM806 β- D - (1→3) glucanase (10 units) in 0.05 M-sodium acetate, pH 4.8, (1 ml.) at 37° for 24 hr. A substrate blank and an enzyme blank were used as controls. After diluting with water to 10 ml. the mixture was centrifuged (20,000 g., 30 min.) and the residue was washed with water (5 x 10 ml.) and freeze dried (7.2 - 7.8 mg.).

The supernatant was analysed for total hexose (G.M.1) and glucose (G.M.3) and was examined by paper chromatography (Solvent b, spray reagent a and b).

(e) Fractionated Snail Enzyme : Cell wall fractions (10 mg.) were treated with snail enzyme Fraction 4AS (1 mg.) using the conditions described in Expt.. 23d.

(g) Glucanylase : Cell wall fractions were treated with glucanylase for 24 hr. as described in G.M.21(b).

Expt. 24(a) Action of Basidiomycete QM806 Exo -β- D - (1→3) - Glucanase on Cell Wall Fraction 14FB

A mixture of cell wall Fraction 14FB (200 mg.), purified Basidiomycete QM806 exo -β- D - (1→3) glucanase (50 units, Expt. 6c) and D gluconolactone (6 mg.) in 0.05 M - sodium acetate buffer, pH 4.8, (25 ml.) was incubated at 48° for 48 hr. After centrifugation (20,000 g., 30 min.), the residue was washed with water (5 x 45 ml.) and freeze dried (100 mg.). (Reincubation of the residue under identical conditions liberated only traces of glucose).

The supernatant was analysed for total hexose (G.M.1), reducing

sugars (G.M.2) and glucose (G.M.3) and was examined by paper chromatography (Solvent b, spray reagents a and b).

(b) Fractionation of Residual Cell Wall with N - Potassium Hydroxide

Residual cell wall (100 mg.) from above was stirred with N - potassium hydroxide (100 ml.), containing sodium borohydride (5 mg.), for 3 hr. at room temperature under nitrogen. After centrifugation (20,000 g., 30 min.) the residue was washed with N - potassium hydroxide (2 x 10 ml.) and then with water until free from alkali. The washed residue was freeze dried to give Fraction 24X (22 mg.).

The supernatant was neutralised with acetic acid, dialysed against water and freeze dried to give Fraction 24Y (72 mg.), $[\alpha]_D$ + 238°.

Expt. 25 Search for Nigeran in the Cell Wall Glucan Fraction 14FJ

Cell wall glucan Fraction 14FJ (100 mg.) was heated with water (20 ml.) on a boiling water bath for 10 min. and filtered hot through a sintered glass funnel under reduced pressure. The residue was treated as above for a further 4 times. The residue and the combined extracts were freeze dried.

Wt. of extract 0.2 mg.

Wt. of residue 95 mg.

The recovery was not quantitative because of losses due to absorption on the sintered glass funnel.

Expt. 26 Fractionation of Cell Wall Fractions 14FCI and CII with Iodine

Cell walls Fraction 14FCI and CII were combined (2.5 g.) and dissolved in N - sodium hydroxide (80 ml.) and the pH was adjusted to 7 by dropwise additon of glacial acetic acid. After adjusting the volume to 400 ml. with water 0.1N - iodine solution (4 ml.) was added and after 1 hr. the precipitate was centrifuged off. 0.1N - iodine solution (4 ml.) was added to the supernatant and after

1 hr. the precipitate was centrifuged off. The precipitates were combined and washed with water (200 ml.), containing 0.1N - iodione (2 ml.) and ethanol (10 x 90 ml.) and freeze dried, to give Fraction 26U (800 mg.) $[\alpha]_D$+ 40°.

The combined supernatant and washings were concentrated under reduced pressure at below 30° to a volume of 400 ml. 0.1N - iodine solution (8 ml.) was added, and the resultant precipitate was centrifuged off, washed with water (2 x 10 ml.) and ethanol (5 x 40 ml.), suspended in water and freeze dried to give Fraction 26V (80 mg.), $[\alpha]_D$+ 38°.

Ethanol (2 vols.) was added to the combined supernatant and washings and the precipitates was centrifuged off, washed with ethanol, suspended in water and freeze dried to give Fraction 26W (1.012 g.) $[\alpha]_D$- 110°.

Expt. 27 Separation of the Products of α- Amylase Action on Cell Wall Fraction 14 FJ.

Cell wall Fraction 14 FJ (3.6 g.) was dissolved in N - sodium hydroxide (200 ml.) and precipitated quantitatively by neutralisation with glacial acetic acid. The precipitate was washed sequentially with water (5 x 200 ml.) and 0.02M - sodium phosphate buffer, pH 6.9, containing 0.0067M - sodium chloride, (2 x 200 ml.) and was then suspended in the same buffer (350 ml.), containing erythritol (120 mg.), and sterilised by autoclaving at 121° and 15 atmospheres for 10 min. Hog panereatic α- amylase (Worthington Biochemicals, 5 mg., 586 units/mg. - manufacturers specification) was taken up in water (5 ml.), filtered through a MF-Millipore filter type GS of pore size 0.22 μ and mixed with the cell wall suspension under sterile conditions. The mixture was incubated at 37° for 18 hr. and then centrifuged at 20,000 g. for 20 min. After incubation the enzyme in the supernatant was found to be still very active (G.M.18).

The residue was washed with water (5 x 400 ml.) and then dissolved in N - sodium hydroxide (400 ml.) under nitrogen. Glacial

acetic acid was added slowly with stirring until the pH fall to 6.9. The precipitate was centrifuged off at 20,000 g. for 30 min. and washed with water (2 x 100 ml.). It had $[\alpha]_D$ + 270°. The combined supernatant and washings were concentrated under reduced pressure at 37° to 100 ml. & 300 ml. of ethanol were added. The precipitate was collected by centrifugation, washed with 75% ethanol (6 x 100 ml.), suspended in water and freeze dried, to give β- D - Glucan Fraction 27K (0.40 g.), $[\alpha]_D$ + 16°.

The neutralised precipitate from above was washed with water (4 x 500 ml.) followed by 0.05 M - sodium acetate buffer, pH 4.8, (350 ml.) and then suspended in the same buffer (370 ml.). 350 units of purified Basidiomycete QM806 exo-β- D - (1→3) glucanase (Expt. 6c) were added and the mixture was incubated at 37° for 18 hr. The residue was collected by centrifugation at 20,000 g. for 30 min. and then dissolved in N - sodium hydroxide (400 ml.) and the pH was adjusted to 10 by addition of glacial acetic acid. The resultant precipitate was washed with water until free from alkali and salts (10 x 400 ml.) and finally freeze dried to give α- D - Glucan, Fraction 27 O (2.4 g.), $[\alpha]_D$ + 290°.

Expt. 28 Action of Enzymes on β- D - Glucan Fraction 27K

(a) Basidiomycete QM806 Exo - β- D - (1→3)- Glucanase : A suspension of β- D - Glucan Fraction 27K (5 mg.), in 0.05M - sodium acetate buffer, pH 4.8, (1 ml.), containing purified exo - β- D - (1→3) glucanase (5 units, Expt. 6c) and D - gluconolactone (0.5 mg.) was incubated for 24 hr. at 37°. After diluting with water to 10 ml. the mixture was centrifuged (20,000 g., 30 min.). The supernatant was analysed for hexose (G.M.1), reducing sugars (G.M.2) and glucose (G.M.3) and was examined by paper chromatography (Solvent b, Spray reagents a & b).

(b) Action of Helix Pomatia Endo -β- D - (1→3) - Glucanase: A suspension of β- D - Glucan Fraction 27 K (3.5 mg.), in 0.005M - citrate phosphate buffer, pH 5.8, (0.15 ml.) containing

Helix pomatia endo -β- D - (1→3) glucanase (Expt. 5, 0.1 units) was incubated at 37° for 16 hr. After centrifugation the supernatant was deionised with a 1:1 mixture of Amberlite IR-120 (H^+) and Amberlite IR-4B (OH^-) resins and then spotted in a corner of a piece of Whatman No.1. chromatography paper (46 x 57 cm.). After chromatography in the first dimension in Solvent b for 72 hr., the chromatogram was air dried overnight. The oligosaccharide zone was sprayed lightly with 10 units/ml. purified Basidiomycete QM806 exo -β D - (1→3) glucanase (Expt. 6c.) in 0.005 M - sodium acetate buffer, pH 4.8, and left at room temperature to dry (ca. 20 min.). Chromatography in the second dimension was carried out with Solvent b or f for 48 hr. using glucose, laminaribiose (gift from Dr. A.G. Dickerson), gentiobiose and laminaritriose (gift from Prof. W.J. Whelan) as standards. After chromatography the papers were sprayed with reagent a.

Pachyman and laminarin treated under identical condition were used as references.

Expt. 29 Permethylation of β- D - Glucan Fraction 27K

This glucan was methylated essentially according to the method of Anderson and Creo (1966). The method was modified as described below.

To a solution of β- D - Glucan Fraction 27K (80 mg.) in dry dimethyl sulphoxide (5 ml.) in an oxygen free dry nitrogen atmosphere was added with stirring powdered sodium hydride (200 mg.) in small portions over a period of 4 hr. After stirring overnight, methyl iodide (5 ml.) was added in 1 ml. portions every 2 hr. Excess methyl iodide was removed by evaporation under reduced pressure at 37° and then water (100 ml.) was added. The mixture was dialysed against water (6 x 5 l.) over a period of 48 hr. and freeze dried. The product (95 mg.) still had absorption at 3500 $cm.^{-1}$ in its infrared spectrum (Nujolmull), indicate the presence of free hydroxyl-groups.

Renethylation of the above partially methylated β-D- glucan once more by the same method gave a fully methylated product (100.5 mg.). Its infrared spectrum (5% solution in carbontetrachloride) showed no hydroxyl band at 3500 cm.$^{-1}$.

The hydrolysis of the methylated β- D - glucan and the identification and quantitative determination of the hydrolysis products was carried out using the methods described in Expt. 35 for α- D - Glucan Fraction 27 O.

Expt. 30(a) Periodate Oxidation of β-D- Glucan Fraction 27K

Periodate oxidations of β-D- Glucan Fraction 27K (50 mg.) were carried out in 0.0025 M - sodium metaperiodate (20 ml.) with continuous magnetic stirring at 0 - 4° in the dark. At different time intervals samples were withdrawn for periodate uptake (G.M.31), formic acid production (G.M.33) and formaldehyde determination (G.M.32).

After 240 hr., the final mixture was treated with an excess of ethylene glycol to destroy the excess periodate, dialysed against water (6 x 5 l.) and freeze dried. Yield 90% of original glucan.

The freeze dried residue (60 mg.) was reduced with sodium borohydride (100 mg.) in water (20 ml.) at room temperature for 24 hr. Excess borohydride was destroyed with N - acetic acid and the reduced product was dialysed against water (6 x 5 l.), and freeze dried Wt. 58 mg.

Composition of periodate oxidised, sodium borohydride reduced β- D - glucan, Smith degradation and analysis of the products formed were carried out using the methods described in Expt. 37.

(b) Second Smith Degradation of β- D - (1→3) - Glucan Fraction 27K

Once Smith degraded β- D - (1→3) Glucan Fraction 27K (Expt. 30a, 6 mg.) was stirred with 0.025 M - sodium metaperiodate (5 ml.) at 4°, in the dark for 2 days. The uptake of periodate after 2 days was measured (G.M.31). The excess periodate was destroyed by addition of ethylene glycol (0.1 ml.) and after dialysis against water (1 x 5 l.), the oxidised glucan was reduced with sodium borohydride

(10 mg.) overnight. Excess borohydride was destroyed with N - acetic acid and the suspension was dialysed against water (2 x 5 l.) for 16 hr. and freeze dried. Wt. 4 mg. This material was stirred with 0.1N - sulphuric acid (1 ml.) for 24 hr. at room temperature and the suspension was centrifuged (20,000 g., 30 min.) The residue was washed with water (3 x 10 ml.) and freeze dried. Wt. 3.8 mg.

Expt. 31 Action of Basidiomycete QM806 Exo - β- D - (1→3) Glucanase on Smith Degraded β- D - Glucan Fraction 27K

A suspension of once (or twice) Smith degraded β - D - Glucan Fraction 27 K (Expt. 30a and b, 2.5 mg.) in 0.05M - sodium acetate buffer, pH 4.8, (0.5 ml.) containing purified exo -β-D- (1→3) glucanase (Expt. 6c, 2.5 units) and D gluconolactone (0.25 mg.), was incubated at 37°for 24 hr. and then centrifuged (20,000 g., 30 min.). The supernatant and residue were analysed as described in Expt. 28(a).

Expt. 32 Action of Enzymes on α- D - Glucan Fraction 27 O.

(a) Aspergillus niger Glucanylase : Cell wall α - D - Glucan Fraction 27 O (10 mg.) was incubated with purified A. niger glucanylase as described in G.M.21(b). An enzyme blank, a substrate blank and a 1% amylose solution containing the enzyme (0.1%) was incubated under the same condition.

(b) Trichoderma viride Cellulase preparation (Worthington Biochemical, 1 mg. liberates /0.1 mg. of glucose /hr. from pretreated or modified cellulose): The incubation with the T.viride enzyme preparation with the α- D - Glucan Fraction 27O before and after Smith degradation was carried out as follows.

Cell wall fractions (10 mg.) were incubated with the freeze dried T.viride cellulase enzyme preparation (1 mg.) in 0.005 M - citrate - phosphate buffer, pH 5.8, (1 ml.) at 37° for 24 hr. The supernatant was chromatographed on T.L.C. in Solvent b and sprayed with reagent a.

Expt. 33 Partial Acid Hydrolysis of α- D - Glucan Fraction 27 O

Partial hydrolysis of α- D - Glucan Fraction 27 O was carried out essentially according to the method of Johnston (1965).

α- D - Glucan Fraction 27 O (25 mg.) was stirred in 98% formic acid (0.2 ml.) and then heated for 10 min. at 100°. 0.44N - sulphuric acid (2 ml.) was added and the heating at 100° continued. The rate of hydrolysis was followed by withdrawing samples at 15 min. intervals for estimation of total hexose (G.M.1), reducing sugar (G.M.2) and glucose (G.M.3).

Identification of oligomers were done on the samples obtained in a separate hydrolysis for 40 min. after the addition of 0.44N - sulphuric acid. The cooled hydrolysate was extracted with diethyl ether to remove formic acid, neutralised with Amberlite-1R 4B (OH^-) resin and chromatographed in Solvents g and h. with detection by spray reagent a.

Other types of hydrolysis tried were 90% formic acid instead of 98% formic acid (Peat, Whelan and Edwards, 1958) and according to G.M.15A with N - sulphuric acid hydrolysis for 15 min. instead 4 hr.

Expt. 34 Permethylation of α- D-Glucan Fraction 27 O

Initial methylation of α- D - Glucan Fraction 27 O was carried out essentially according to the Haworth method, modified by Wallenfels, Bechtler, Kuhn, Trischmann and Egge (1963). The method was modified as follows.

To a solution of α- D - Glucan Fraction 27 O (200 mg.) in N - sodium hydroxide (15 ml.) were added 43% sodium hydroxide (35 ml.) and dimethyl sulphate (17 ml.) simultaneously and dropwise at 20° with stirring under nitrogen over a period of 6 hr. After stirring for a further 18 hr. the mixture was stirred at 37° for 4 hr. and finally at 100° for 20 min. to destroy the excess dimethyl sulphate. After cooling the reaction mixture was neutralised with glacial acetic acid, dialysed against water (9 x 5 l.) and freeze dried to give the

partially methylated glucan (225 mg.), which still had absorption at 3500 $cm.^{-1}$ in its infrared spectrum (Nujolmull).

The partially methylated α- D - Glucan, Fraction 27 O (200 mg.) was stirred with dry dimethyl sulphoxide (15 ml.) under nitrogen and powdered sodium hydride (300 mg.) was added in small portions over a period of 8 hr. Stirring was continued for a further 18 hr. and then methyl iodide (5 ml.) was added in 1 ml. portions every 2 hr. Excess methyl iodide was removed by evaporation under reduced pressure at 37° and then water (100 ml.) was added and the mixture was dialysed against distilled water (6 x 5 l.) and finally freeze dried. The product (224 mg.) still had absorption at 3500 cm^{-1} in its infrared spectrum.

However a second methylation by this method gave a product (233 mg.), which was completely methylated as indicated by lack of absorption at 3500 cm^{-1} in its infrared sprectrum (Nujolmull).

Expt. 35 Hydrolysis of the Methylated α- D - Glucan Fraction 27 O Identification and Quantitative Determination of the Hydrolysis Products

Methylated α- D - Glucan Fraction 27 O was hydrolysed by N sulphuric acid as described in G.M.15 A. The hydrolysate was neutralised with barium hydroxide (G.M.15A (ii)), and examined by paper chromatography in Solvent h (detection with reagent b).

For the quantitative determination and identification of the hydrolysis products the neutralised solution was reduced with sodium borohydride (0.5 - 5 mg. of methylated sugars in 0.5 - 2 ml. water containing 10 mg. sodium borohydride) for 2 hr. at room temperature and the excess borohydride was destroyed with an excess of acetic acid. After evaporation to dryness under reduced pressure below 45°, the residue was evaporated to dryness five times with fresh methanol (5 x 2 ml.) containing one drop of glacial acetic acid to remove borate. The residue was dissolved in acetic anhydride (10 mg. of methylated sugars/ml. of acetic anhydride) and heated in a flask,

sealed with a glass stopper at 135 - 140° for 2 hr. Samples (1 - 10 µl.) of this reaction mixture were injected directly to the gaschromatographic colums at 180° (G.M.24) for the separation and identification by Mass spectrometry. Quantitative analysis was done from the measurement of the area of each peak by cutting out the peak area from the chromatography chart paper and weighing. Peak areas using the flame ionisation detector for different tri- and tetra methyl D - glucitol acetates do not vary more than ±2% with equivalant molarities. Methylated glucitol acetates were identified on the basis of their retention times related to that of 1,5 - di - O - acetyl - 2, 3, 4, 6 - tetra - O - methyl - D - glucitol.

Expt. 36 Periodate Oxidation of α- D - Glucan Fraction 27 O

Periodate oxidation of α- D - Glucan Fraction 27 O (72 mg. and 162 mg.) were carried out in 0.025 M - sodium metaperiodate (20 ml. each), with continuous magnetic stirring at 0 - 4° in the dark. At different time intervals samples were withdrawn for periodate uptake (G.M.31), formic acid production (G.M.33) and formaldehyde determination (G.M.32).

After 240 hr., the final mixture was treated with an excess of ethylene glycol to destroy the excess periodate, dialysed against water (6 x 5 l.) and freeze dried. Yield 96% of original glucan.

The freeze dried residue (100 mg.) was reduced with sodium borohydride (150 mg.) in water (20 ml.) at room temperature for 24 hr. Excess borohydride was destroyed with N - acetic acid and the reduced product was dialysed against water (6 x 5 l.), and freeze dried. Wt. 98 mg.

Expt. 37(a) Composition of the Periodate Oxidised and Reduced α- D - Glucan Fraction 27 O

Periodate-oxidised and reduced α- D - Glucan Fraction 27 O (Expt. 36) was hydrolysed with N - sulphuric acid, and neutralised with barium hydroxide (15 A (ii)). The neutralised solution

was examined by paper and thin layer chromatography in Solvent e with detection by spray reagent a. For quantitative analysis the neutralised solution was reduced with sodium borohydride and acetylated as described in Expt. 35. Samples (1 - 10 µl.) of the acetylation mixture were injected directly onto the gas chromatography column (G.M.24) at 100° and the mixture was analysed according to the method G.M. 24.

(b) Smith Degradation of α- D - Glucan Fraction 27 Q and Analysis of the Products : Smith degradation was carried out as described by Buck, Chen, Dickerson and Chain (1968).

Periodate oxidised and sodium borohydride reduced α- D - Glucan (Expt. 36, 50 mg.) was suspended in 0.1 N - sulphuric acid (15 ml.) and stirred at room temperature for 24 hr. After centrifugation (20,000 g., 30 min.) the residue was washed with distilled water and freeze dried. Yield 44.2 mg.

Supernatant and washings were neutralised with barium hydroxide (G.M. 15A (ii)) and divided into two equal fractions.

(i) The first fraction was chromatographed in Solvent e with silvernitrate (G.M. 22a) detection. Products with the same R_G values as glycerol were eluted with water (Expt. 2) acetylated using sodium acetate and acetic anhydride (Expt. 2B (ii)) at a temperature 135 - 140° for 2 hr., and then examined by gas liquid chromatography as described in G.M.24.

(ii) The second fraction was mixed with glucose (300 µg.) and the mixture was reduced with sodium borohydride as described in Expt. 35 and acetylated. Aliquots were examined by gas liquid chromatography and the products determined quantitatively as described in Expt. 35.

The Smith degraded residue was hydrolysed with N - sulphuric acid and the products were reduced and acetylated prior to examination by gas - liquid chromatography as described in Expt. 37(a).

Expt. 38 Action of a Mixture of Chitinase and Snail Enzymes on Fraction 24 X and 39 X

Preliminary experiments indicated that hydrolysis of Fraction 24 X and 39 X with snail enzymes (Expt. 10) or chitinase (Expt. 11) alone was incomplete after 24 hr. (50% and 70% was released as N - acetyl hexosamine by the respective enzyme). However a mixture of both in equal amount hydrolysed each fraction almost completely.

A suspension of Fraction 24 X or 39 X (1.2 mg.) in 0.05 M - phosphate buffer, pH 6.3, (1 ml.), containing 0.05% chitinase and 0.05% Helix pomatia enzymes was incubated at 37° for 24 hr. After centrifugation (20,000 g., 30 min.) the supernatant was analysed for N - acetyl - D - glucosamine, total hexose and glucose (G.M.5,1,3) and examined by paper chromatography (Solvent d, spray reagents a & c).

Expt. 39 Estimation of the Polysaccharide Linkage Composition of Total Cell Walls

A suspension of cell wall preparation (500 mg.) in 0.02 M - sodium phosphate buffer pH 6.9, containing 0.0067 M - sodium chloride, (40 ml.) was incubated with an α- amylase (Worthington Biochemical, Hog pancreatic) suspension (0.1 ml., ca. 3,000 units) at 37° for 24 hr. under toluene with slow magnetic stirring. After centrifugation (20,000 g., 20 min.), the supernatant was analysed for glucose (G.M.3), reducing sugars (G.M.2) and total hexose (G.M.1). The residue was washed with water (5 x 40 ml.) by resuspension and centrifugation and freeze dried. (Retreatment with α-amylase under the same condition did not liberate any soluble sugars).

A suspension of residual cell walls after α- amylase treatment (Ca. 200 mg.) in 0.05 M - sodium acetate buffer, pH 4.8, (40 ml.) containing gluconolactone (20 mg.) and purified Basidiomycete QM806 β- D - (1→3) - glucanase (Expt.6c, 130 units) was incubated at 37° for 24 hr. under toluene with slow magnetic stirring. After centrifugation (20,000 g., 20 min.) the supernatant was analysed for hexose (G.M.1), reducing sugars (G.M.2) and glucose (G.M.3). The

residue was washed with water (5 x 16 ml.) as above and freeze dried. [Retreatment with the exo - β - D - (1⟶3) glucanase under the same conditions did not liberate any soluble sugars].

A portion of the residual cell walls after α amylase and exo - β - D - (1⟶3)- glucanase action (55 mg.) was stirred with N - sodium hydroxide (40 ml.) containing sodium borohydride (5 mg.) under nitrogen for 24 hr. at room temperature. (It was later found that 3 hr. was sufficient). After centrifugation (20,000 g.,20 min.) the residue was washed with water (2 x 10 ml.) as above, dialysed and freeze dried to give Fraction 39 X. (This fraction was completely insoluble in N - sodium hydroxide).

The combined supernatant and washings were neutralised with glacial acetic acid and dialysed against water (6 x 5 l.) over a period of 48 hr. The resultant precipitate was centrifuged off (20,000 g., 20 min.) and the supernatant and precipitate were freeze dried to give Fraction 39 Q and 39 P respectively.

The calculation of the polysaccharide linkage composition of two batches of cell walls is described in the Result Section (p. 119).

Expt. 40 Isolation and Properties of Extracellular Polysaccharides

3½ Day old cultures grown on medium II (a) were filtered through Whatman GF/A glass paper, and the filtrate (200 ml.) was dialysed against water (200 ml.). Ethanol (2 volumes) was added to the dialysate. The resultant precipitate was centrifuged off (10,000g. 20 min.), washed with 70% v/v ethanol (5 x 40 ml.) and taken up into water (50 ml.) and freeze dried. (Fraction 40 I).

The contents of the dialysis bag were concentrated to 50 ml. by evaporation under reduced pressure and dialysed against water (6 x 5 l.) at 4°. The resultant precipitate was centrifuged off (20,000 g., 30 min.) and freeze dried (Fraction 40 III).

The non-dialysable supernatant was freeze dried (Fraction 40 II)..

GENERAL METHODS

Reagents were obtained from the British Drug Houses, Poole, Dorset, unless otherwise stated and were the highest purity available.

Optical Densities were measured with a Unicam SP-500 or SP-600 spectrophotometer.

Homogenisations of cell walls and cell wall fractions prior to chemical or enzymic reactions, were carried out in the appropriate buffer using an MSE Homogeniser (Catalogue No. 7700-A) at full speed for 5 min.

The water used throughout was glass distilled.

Room temperature was 20 - 25°

The term in vacuo refers to reduced pressure obtained using a water pump ($<$15mm. Hg.)

The pH of solutions was measured using an E.I.L.pH meter, equipped with a glass electrode, calomel reference and temperature compensator.

Visking tubing (The Scientific Instrument Centre Ltd.) was used for dialysis. All dialyses were carried out for at least 8 hr.

G.M.I. Determination of Total Hexoses

Hexoses were determined essentially according to the phenol-sulphuric acid method of Dubois, Gilles, Hamilton, Rebers and Smith (1956). The method was modified as described below.

Procedure

98% Sulphuric acid (2.5ml.) was added rapidly with mixing to a mixture of an aqueous solution (0.5ml.), containing 10 - 70 µg. of hexose/ml., and 5% aqueous phenol (0.5ml.) using a graduated pipette, the tip of which had been sawn off to allow rapid flow. After standing at room temperature for 30 min. the optical density of the yellow-orange colour, which was stable for several hours, was measured at 490 nm. Blanks were prepared with water (0.5ml.) in place of the hexose solution. Hexose was determined by reference to a standard curve prepared for the particular sugar being assayed. A linear relationship between optical density at 490 nm. and concentration in the range 10 - 70 µg./ml was observed. Optical densities obtained with a concentration of 50 µg./ml. hexose were as follows: Glucose - 0.55; Galactose - 0.45 and Glucosamine - 0.007.

G.M.2. Determination of Total Reducing Sugars (Nelson - Somogyi Method)

Reducing sugars were determined essentially according to the method of Nelson (1944), modified by Somogyi (1952). The method was further modified as described below.

Reagents

A. Alkaline Copper Reagent

Solution I Anhydrous sodium sulphate (180 g.) in water (500ml.), heated to expel air and then cooled, was mixed with a solution of Rochelle salt (15 g) and anhydrous sodium carbonate (30 g.) in water (300ml.), containing sodium bicarbonate (20 g.), and diluted to 1 l. with water.

Solution II A solution of copper sulphate pentahydrate (5g.) and anhydrous sodium sulphate (45 g.) diluted to 250ml. with water.

Immediately prior to use, 4 volumes of Solution I were mixed with 1 volume of Solution II.

B. Arsenomolybdate Reagent

Solution (a). A solution of ammonium molybdate (50 g.) in water (900 ml.) and 98% sulphuric acid (42 ml.) was mixed with a solution of sodium arsenite heptahydrate (6 g.) in water (50 ml.) and diluted to 1 l. with water. The solution was incubated for 48 hr. at 37° in the dark and then stored in a glass-stoppered brown bottle. Stable for at least one year.

Solution (b) Immediately prior to use 1 volume of Solution (a) was mixed with 2 volumes of 1.5 N - sulphuric acid.

Procedure

A mixture of an aqueous solution (0.5 ml.), containing between 0.05 and 0.5 μ mole of reducing sugars/ml. and alkaline copper reagent (0.5 ml.) in a glass stoppered test tube was heated for 20 min. in a boiling water bath and then cooled to room temperature. Diluted arsenomolybdate reagent [0.5 ml. solution (b)] and distilled water (1.5 ml.) were added sequentially with mixing. The optical density of the blue colour, which was stable for several hours, was measured at 525 nm. after 1 hr. Blanks were prepared as above with distilled water in place of the reducing sugar solution. The reagent was calibrated with glucose and maltose. Optical densities obtained with a concentration of 0.25 μ mole/ml. reducing sugars were as follows: Glucose - 0.385; Maltose - 0.385.

G.M.3. Determination of D - Glucose (Glucose Oxidase Method)

D- Glucose was determined according to the method of Mansford (1965). The method was modified as described below.

Reagents

Enzyme Dye Reagent

Glucose oxidase (10mg.)- Analytical Grade (The Boehringer Corporation Ltd., London) Catalogue No. 15423EGAB. The enzyme preparation was found

to have α- glucosidase and / or α- glucanase activity, as had been observed by others (Lloyd and Whelan, 1969).

o - dianisidine (10 mg.) in ethanol (1 ml.). (twice recrystallised from ethanol and stored in a dark bottle).

Peroxidase (1 mg.) - Analytical Grade (The Boehringer Corporation Ltd., London). Catalogue No. 15301EPAA. The above constituents were mixed and diluted to 100 ml. with tris - glycerol buffer. Stored at 4° in a dark glass stoppered bottle. Stable for several months.

Tris - Glycerol buffer

Tris (hydroxymethyl) aminomethane (61 g.) and 5 N - Hydrochloric acid (85 ml.) were mixed and diluted with distilled water to 1 l. To the above solution glycerol (660 ml.) was added and the pH was adjusted to 7 with 5 N - sodium hydroxide or hydrochloric acid. Stored at 4°.

Protein Precipitation Reagent

5% (w/v) aqueous Zinc sulphate.

0.3N - aqueous Barium hydroxide.

Procedure

(a) Protein precipitation. (Required only when the protein concentration was higher than 1% of the glucose concentration).

To a mixture of the glucose solution (0.5 ml.) and 5% aqueous zinc sulphate (1 ml.) was added 0.3 N - barium hydroxide (1 ml.). After shaking well the mixture was centrifuged at 5,000 g. for 10 min. The supernatant was used for glucose determination.

(b) Glucose determination

A mixture of the glucose solution (0.5 ml.) (adjusted when necessary to pH 5.5 - 7.0), containing 10 - 70 µg glucose/ml. and enzyme dye reagent (1 ml.) was incubated at 37° for 30 min. 5 N-sulphuric acid (2 ml.) was added and mixed well. The optical density of the pink colour, which was stable for several hours, was measured at 525 nm.

against a blank prepared as above with distilled water in place of glucose solution. Glucose concentrations were determined by reference to a standard curve prepared from standard glucose solutions. A linear relationship between optical density at 525 nm. and glucose concentration was obtained in the range 10 - 70 µg./ml. glucose. The optical density with a concentration of 50 µg./ml. glucose was 0.45.

G.M.4a. Determination of D- Galactose (D- Galactose Dehydrogenase Method).

D- Galactose was determined according to the method of Dondoroff, Contopoulon, and Burns (1958). The method was modified as described below.

Reagents

Galactose UV - Test Kit (The Boehringer Corporation Ltd., London) containing:

1.0 M- Tris buffer, pH 8.6

NAD (33 mg.) dissolved in water (3.3 ml.) (Freshly prepared).

β- D- Galactose dehydrogenase enzyme suspension (5 mg./ml.)

Procedure

To a mixture of D- galactose solution (1.0 ml.), containing 3 - 21 µg. of D- galactose/ml., and tris buffer (0.1 ml.) was added, with mixing, 1% aqueous NAD (33 µl.) and the enzyme suspension (7 - 10 µl). After 40 min. at room temperature the optical density of the NADH formed, which was stable for several days, was measured at 340 nm. Blanks were prepared as above with distilled water in place of the D- galactose solution. For cell wall hydrolysates a substrate blank was included and its optical density was subtracted from the optical density obtained from the substrate, NAD and enzyme incubation mixture. Galactose concentrations were determined by reference to a standard curve prepared from standard D- galactose solutions. A linear relationship between optical density at 340 nm. and galactose concentration was obtained in the range 3 - 21 µg./ml. D-galactose. Optical density with a concentration of 15 µg./ml. D- galactose was 0.45. A standard solution of galactose was always included with each determination.

G.M.4b. Determination of D- Galactose (D- Galactose Oxidase Method.)

D- Galactose was determined essentially according to the method of Avigad, Amaral, Asensio and Horecker (1962), modified by Sempere, Gancedo, and Asensio (1965). The method was further modified as described below.

Enzyme Dye Reagent

D- Galactose oxidase (1.5 mg.) [12 units/mg. ex Dactylium dendroides obtained from Koch - Light Laboratories, Biochemical Division, Colnworth, Bucks. One unit of galactose oxidase activity is equal to a change in absorbance of 1.0 per min. under the conditions of the reactions of the manufacturer, in a coupled peroxidase - dianisidine system, using galactose as substrate.].

o - Tolidine (2.5mg.) in methanol (0.5 ml. twice recrystallised from methanol and stored in a dark bottle).

Peroxidase (2.5 mg.) Analytical Grade (The Boehringer Corporation Ltd., London).

The above constituents were mixed and diluted to 50 ml. with 0.15 M - sodium phosphate buffer pH 7.0 and stored at 4° in a dark glass stoppered bottle. The reagent was used within two days of preparation.

Procedure

A mixture of D - galactose solution (0.5 ml.), containing 10 - 70 µg. D - galactose/ml. and enzyme dye reagent (0.5 ml.) was incubated at 37° for 1 hr. 0.5 M - sodium EDTA (pH 9.7) (60 - 70 µl.) was added and mixed well. The optical density of the yellowish colour, which was stable for several hours, was measured at 425 nm. Blanks were prepared as above with distilled water in place of the D- galaotose solution. D- Galactose concentrations were obtained from a standard curve, prepared in parallel with the test

G.M.5. Determination of N - Acetyl - D - Glucosamine

N - Acetyl D - glucosamine was determined essentially according to the method of Reissig, Strominger and Leloir (1955). The

method was modified as described below.

Reagents

A. 0.8 M - Potassium tetraborate, pH 9.2

B. 10% (w/v) p - dimethylaminobenzaldehyde (twice recrystallised from ethanol) in 100 ml. of ethanol containing 11 ml. of 11.4 N - hydrochloric acid. Stored at 4°; stable for a month. Immediately prior to use, 1 volume of Reagent B was diluted with 9 volumes of glacial acetic acid.

Procedure

A mixture of an aqueous solution (0.5 ml., containing between 10 and 70 µg of N - acetyl D - glucosamine/ml.) and 0.8 M - potassium tetraborate (0.1 ml.) was heated in a boiling water bath for exactly 3 min. and then cooled in tap water. Diluted p- dimethylaminobenzaldehyde (3 ml.) was added, mixed and immediately placed in a water bath at 37°. After exactly 20 min. it was placed in an ice bath and immediately the optical density of the pink colour was measured at 544 and 585 nm. against a blank prepared as above with distilled water in place of the N - acetyl D - glucosamine solution. The optical density with a concentration of 50 µg/ml. N - acetyl D - glucosamine was 0.55 (544 nm.) and 0.62 (585 nm.)

G.M.6. Determination of Glucosamine

Glucosamine in cell wall hydrolysates was determined by a modified method of Boas (1953) as described below.

Reagents

4% (v/v) Acetyl acetone (redistilled) in 1.25 N - sodium carbonate

2.6% (w/v) N, N - dimethyl - p - aminobenzaldehyde (twice recrystallised from 80% ethanol) in a mixture of ethanol and conc. hydrochloric acid (1:1 by volume).

Amberlite IR - 120 (H^+) ion exchange column

Ion exchange columns measuring 20 - 25 cm. in length were made

from 10 mm. pyrex glass tubing; the lower ends were tapered to an opening of 1 to 2 mm., which were then connected to pieces of polyethylene tubing (2mm. diameter) with a clip to check the flow of liquids. Amberlite IR - 120 resin (5 ml.) was added slowly to the columns filled with water and small amounts of pyrex glass wool at the bottom of the columns to hold the resin beds. Glass paper circle were placed on the top of the resin beds. The resins were regenerated twice by using 2N - sodium hydroxide (10 ml.) and 2N - hydrochloric acid (20 ml.) and distilled water in that order until the effluent was neutral.

(After each experiment the column was regenerated once as above and stored in 2 N - hydrochloric acid when not in use.).

Procedure

Resin Separation of Hexosamines - 5.7 N - Hydrochloric acid hydrolysate was diluted with water to a normality of below 0.29 N. An aliquot of the diluted hydrolysate (5 to 10 ml.), containing 0.05 to 0.10 mg. of hexosamine, was transferred to the ion exchange column. The column was washed with distilled water (15 ml. at a flow rate of 10 ml./hr.). The effluent of solutions and water containing the interfering substances (neutral sugars, etc.) were discarded. The hexosamine absorbed on the resin of the column was eluted with 2N - hydrochloric acid (15 ml.) into a 25 ml. volumetric flask.

The 2 N - hydrochloric acid effluent was neutralised with 4 N - sodium hydroxide in presence of a drop of 0.05% phenolphthalein in ethanol as the indicator until the solutions just turn red. 0.29 N - hydrochloric acid was then added drop by drop until the indicator colour just disappears. The final volume was adjusted to 25 ml. with distilled water.

Hexosamine Determination - A mixture of the above solution (2 ml.) and 4% (W/v) acetylacetone in 1.25 N - Sodium carbonate (1 ml.) was heated at 90° for 1 hr. in a water bath and then cooled. Ethanol (8 ml.) was added followed by the 2.6% (W/v) p - dimethylaminobenzaldehyde solution (1 ml.) with mixing. The optical density of the red colour was measured

at 530 nm. against a blank prepared under identical conditions from the blank eluate of the column. Glucosamine solutions containing an equal amount of sodium chloride as the unknown were used to prepare a standard curve. Standard glucosamine hydrochloride (0.1 mg.) was eluted from the column and measured under identical condition as the unknown was used for the determination of accuracy.

(N.B. Recovery from the column was 90% ± 4 and the correction was made accordingly.)

G.M.7. Determination of Neutral Sugars by Paper Chromatography

Quantitative determination of individual neutral sugars in a mixture was carried out according to the method of Wilson (1959). The method was modified as described below.

Reagents

A. Aniline hydrogen phthalate reagent

o - phthalic acid (1.66 g) and aniline (redistilled from zinc) (0.91 ml.) were dissolved in a mixture of n - butanol (48 ml.), diethyl ether (48 ml.) and water (4 ml.). Stored at 4° and used within 14 days.

B. Eluting agent

0.7 N - Hydrochloric acid in 80% (v/v) ethanol.

Procedure

The sugar solution containing between 10 - 100 µg. of each individual sugar was spotted on Whatman No - 1 paper together with standards at an interval of at least 4.0 cm. The sugars were paper chromatographed with a suitable solvent (G.M.22) and the paper was air dried overnight. It was then drawn uniformly through the aniline hydrogen phthalate reagent in a polypropylene tank, air dried and then heated at 105° for 10 min. in a forced air oven to develop the spots.

The individual spots were cut out in rectangular areas which were equal for all the spots of one sugar. The paper was then cut into smaller strips and placed in a glass stoppered test tube. Eluting reagent

(4 ml.) was added, and the tubes were inverted three or four times during 1 hr. after which time the elution was complete. The optical densities of the solutions were determined at 390 nm. for hexoses and rhamnose and at 360 nm. for pentoses using fresh eluting reagent as blank.

G.M.8a. Determination of Total Cell Wall Protein

Total cell wall protein was determined by a modification of the method of Bartnicki - Garcia and Nickerson (1962) and is described below.

Reagents

A. Saturated Solution of Trisodium Phosphate and Sodium Tetraborate

Sodium tetraborate decahydrate (4 g.) was dissolved in saturated trisodium phosphate (100 ml.).

B. Ninhydrin - Hydrindantin Solution.

Ninhydrin (2g.) and hydrindantin (0.3 g.) were dissolved in methyl cellosolve (75 ml.) by gentle stirring with a magnetic stirrer in a dark glass reagent bottle under nitrogen atmosphere, taking care not to incorporate air bubbles into the solution. 4 N - sodium acetate buffer, pH 5.5 (25 ml.) was added into the solution followed by stirring under nitrogen. Stored at 4° under nitrogen. Stable for one weak.

N.B. The methyl cellosolve used was peroxides free as judged by the reaction with freshly prepared 4% aqueous potassium iodide (1 ml.) addition to methyl cellosolve (2 ml.). No colour was observed.

Procedure

Deamination of 2 - aminosugars: Samples of 6.0 N - hydrochloric acid hydrolysates (G.M. 15B.) were adjusted to pH 5 to 6 with N - sodium hydroxide. Aliquots (0.5 ml.) were mixed with saturated phosphate - borate solution (0.5 ml.) and heated in a boiling water bath for 30 min., and subsequently evaporated to dryness at 50° on a rotary evaporator in vacuo; the pH was adjusted to 5.5 by the addition of the calculated amount of 1.2 N - acetic acid and distilled water was added to give a

protein concentration of between 5.0 and 25.0 µg./ml.

Aliquots (0.5 ml.) of this solution, were mixed with the ninhydrin - hydrindantin reagent (0.5 ml.), heated in stoppered test tubes for exactly 15 min. in a covered boiling water bath, and cooled quickly to below 30° in front of an electric fan. The mixture was diluted with 50% (W/v) aqueous ethanol (2.5 ml.), and mixed thoroughly to oxidise the excess hydrindantin. The optical density was measured at 570 nm. Protein concentrations were measured directly from a standard curve obtained from a hydrolysate of crystalline bovine serum albumin (B.D.H.) subjected to the same treatment.

A glucosamine solution (1.0 mg/ml.) when subjected to the same treatment was found to be completely deaminated as it gave no colour when subjected to the same assay.

G.M.8b. Determination of Total Soluble Protein.

Total soluble protein was determined essentially according to the method of Lowry, Rosebrough, Farr and Randall (1951), modified by Miller (1959). The method was modified as described below.

Reagents

A. Alkaline Copper Reagent

10% sodium carbonate (anhydrous) in 0.5 N - sodium hydroxide (10 ml.) was mixed with 0.5% (W/v) copper sulphate pentahydrate in 1% (W/v) aqueous sodium potassium tartrate (1 ml.) just before use.

B. Folin Phenol Reagent

This reagent was obtained from British Drug Houses Ltd., Poole, Dorset and diluted 10 times with distilled water.

Procedure

Protein solutions (1.0 ml.), containing between 40 to 200 ug. of soluble protein, and alkaline copper reagent (1.0 ml.) were mixed and stored at room temperature for 10 min. Diluted Folin phenol reagent (3 ml.) was added rapidly with mixing and the mixture was incubated

for 10 min. at 50°. The optical density was measured at 650 nm. against a blank prepared as above with distilled water in place of the protein solution. Protein concentrations were determined by reference to a standard curve prepared using bovine serum albumin. Optical density with a concentration of 100 µg./ml. bovine serum albumin was 0.40.

G.M.8c. Amino Acid Analysis

The analysis was carried out by Dr. A. J. Thomas, according to the method of Thomas (1970), after hydrolysis (G.M.15B).

G.M.9. Determination of Cell Wall Lipids

Cell wall Lipids (readily extractable and bound) were determined essentially according to the method of Bartnicki - Garcia and Nickerson (1962). The method was modified as described below.

Reagents

Redistilled ethanol, ethyl ether and chloroform.

Procedure

Cell walls (100 mg.) were shaken with a mixture of ethanol and diethyl ether (50 ml., 1:1 by volume) for 24 hr. at 30° . The solvent phase containing lipid was separated by filtering through a sintered glass funnel (No AG 38 x 3). The extraction was repeated two more times with fresh solvent mixture, followed by two extractions with chloroform (50 ml.). All extracts were combined and the solvents were removed in vacuo. Lipids were extracted from the concentrate with ethyl ether (5 x 5 ml.), dried with anhydrous sodium sulphate and the ether evaporated. The residue was dried under reduced pressure (15 mm Hg.) at 45 - 50° to constant weight. This fraction constitutes the readily extracted Lipids.

Residual cell walls obtained from the above treatment were incubated in 1:1 (v/v) ethanol - diethyl ether, acidified by addition of 1% concentrated hydrochloric acid (50 ml.) , at 50° for 5 hr. with occasional shaking, followed by two extractions with 1:1 (v/v) ethanol-diethyl ether (50 ml.) and one with chloroform (50 ml.); each extraction

lasted 24 hr. The extracts were combined and treated as described for the "readily extracted lipids" fraction. This fraction constitutes the "bound lipids". Solvent blanks were carried out in both the procedures under identical conditions.

G.M.10. Determination of Total Phosphorus

Total phosphorus was determined essentially according to the method of Fiske and SubbaRow (1925), modified by Bartlett (1959). The method was modified as described below.

Reagents

Fiske and SubbaRow Reagent

1 - amino - 2 - naphthol - 4 - sulphonic acid (50 mg.) and anhydrous sodium sulfite (100 mg.) dissolved in freshly prepared 15% (W/v) aqueous anhydrous sodium bisulfite (20ml.). The solution was filtered and prepared daily.

Procedure

(a) Hydrolysis of Organic Phosphates - A cell wall preparation or standard phosphorus solution containing between 0.5 - 4 µg. of phosphorus was boiled with 10 N - sulphuric acid (0.5 ml.) in a Kjeldal flask using a microburner (150 - 160°) for 3 hr. 30% hydrogen peroxide (phosphorus free) (0.05 - 0.1 ml.) was added and the solution was heated again for at least 1.5 hr. to complete combustion and to decompose all the peroxide.

(b) Determination of Inorganic Phosphate - The digest was quantitatively transferred into a graduated glass stoppered test tube and the volume was adjusted with distilled water to 4.6 ml. 5% (W/v) Ammonium molybdate (0.2 ml.) and Fiske - SubbaRow reagent (0.2 ml.) were added and the solution was heated in a boiling water bath for 7 min. The optical density of the blue colour, which was stable for several hours, was measured at 830 nm. against a blank prepared as above omitting the phosphorus containing sample. Standards were prepared with anhydrous potassium dihydrogen phosphate. The optical

density obtained with 3 μg. of phosphorus was 0.56.

G.M.11. Determination of Acid Labile Phosphate

Acid labile phosphate was determined by a modified method of Harold (1962) as described below.

Procedure

A mixture of cell wall preparation containing 0.5 - 4 μg. of acid labile phosphate and N - hydrochloric acid (0.5 ml.) was heated in a boiling water bath for 15 min. and then cooled. Inorganic phosphate liberated was then determined as described in G.M.10(b).

G.M.12. Determination of Ash.

Ash was determined by combustion of the cell walls (50 - 100 mg.) in a 1¼" diameter porcelain crucible with a lid. The crucible containing the cell walls with the lid on was heated directly with a Bunsen burner (oxidising flame) until soot free. Heating was continued for a further 2 hr. Cooled in a desiccator over phosphorus pentoxide followed by weighing of the crucible containing the residue and the lid. Heating, cooling and weighing process was repeated (2 x 2hr.), to make sure that the combustion of organic matter was complete.

G.M.13. Determination of Optical Rotation

Optical rotations were determined using a photoelectric polarimeter provided with the yellow light from a sodium vapour lamp (Hilger and Watts M511.2/60205) and a polarimeter tube of 1 decimeter with a volume of 1 ml.

For polysaccharides, determinations were carried out in N - sodium hydroxide at 20 - 25° and concentrations were determined by the phenol-sulphuric acid method (G.M.1). In other cases rotations are based on dry weights.

G.M.14. Infrared Spectroscopy.

Infrared spectra were recorded on a Unicam SP-200G

Infrared Spectrophotometer.

Procedure

Three types of medium were used to support the compound before taking the spectrum.

(1) Nujol mulls - The material (1 - 5 mg.) was ground in a small agate mortar and then mixed with Nujol (0.05 - 0.1 ml.). The mull obtained was enclosed as a film between two sodium chloride cylindrical plates.

(2) Potassium Bromide discs - The material (0.5 - 2mg.) was mixed with dry potassium bromide crystals and ground in an agate mortar. The disc was made in an hydraulic press (30 Ton Press C-30, Research and Industrial Instrument Co., London.) in vacuo (below 0.01 mm Hg.) at upto 20 tons/sq. in. A reference disc was prepared under identical conditions using potassium bromide (100 mg.) alone.

(3) Solution spectra were obtained in cells having sodium chloride windows and 0.1 mm path length. Cells containing the solvent only were used in the reference beam.

G.M.15. Total Acid Hydrolysis of Cell Wall Preparations

A. N - Sulphuric Acid Hydrolysis (Buck, Chen, Dickerson, and Chain, 1968) - A solution of cell wall preparations (or cell wall fractions) (upto 10 mg.) in 72% (v/v) aqueous sulphuric acid (0.1 ml.) was stirred in an ice bath for 1 hr. Water (2.5 ml.) was added, and the solution was boiled under reflux for 4 hr. and then cooled and neutralised according to one of the methods described below.

(i) Sodium Hydroxide Neutralisation - (For the quantitative determination of D - Glucose with glucose oxidase (G.M.3), D - Galactose with galactose dehydrogenase (G.M.4a), in the presence of other hexose and pentose.).

The acid hydrolysate was diluted with distilled water to a measured volume, a portion of it was pipetted out and diluted further in a volumetric flask, which was then cooled in an ice bath to below 4°. The calculated amount of ice cooled 0.1 - 1.0 N - sodium hydroxide

solution was added dropwise with shaking to pH 5 - 6 (using pH paper) and the final volume was adjusted with distilled water.

(ii) Barium Hydroxide Neutralisation - (For the quantitative determination of D - galactose with galactose dehydrogenase (G.M.4a) and galactose oxidase (G.M.4b), separation of sugars by paper chromatography and their quantitative estimation, gas - liquid chromatography).

The acid hydrolysate was cooled in an ice bath to below 4° and neutralised by dropwise addition of saturated aqueous barium hydroxide with magnetic stirring to pH 5 to 6. The barium sulphate formed was removed by centrifugation and filtration.

(iii) Amberlite IR - 4B (hydroxide form) Neutralisation - (For paper chromatography, when the amount of neutral sugars in the hydrolysate was less than 1mg.).

The resin used (British Drug Houses Ltd., Poole, Dorset) was always regenerated using 2% solution of sodium carbonate followed by thorough washing with distilled water according to the booklet "Ion Exchange Resins" published by The British Drug Houses Ltd, Poole, Dorset.

Amberlite IR - 4B (OH^-) (1 ml. rinsed resin) was added into the cooled acid hydrolysate (1 ml. of 1N) and left to neutralise (pH - paper). Resin were removed from the solution by filtration.

(IV) Barium Carbonate Neutralisation - (For as in ii)

A small excess of barium carbonate was added to the solution for the neutralisation. Insoluble barium sulphate and barium carbonate were removed by centrifugation (3,000 g. for 10 min.) and filtration. Soluble barium carbonate left in the solution was removed with Amberlite IR - 120 (H^+) resin.

B. Constant Boiling Hydrochloric Acid Hydrolysis - (For the determination of cell wall amino acids and protein).

A suspention of cell walls (5 - 10 mg.) in 6.09N - hydrochloric acid (5 to 10 ml.) in a sealed tube was heated in a boiling water bath

for different length of times (24, 48 and 72 hr.). After cooling the solution was neutralised according to the following method.

(i) Removal of Hydrochloric acid by repeated evaporation - (For quantitative determination of amino acids by amino acid analyser).

The hydrolysate was filtered and evaporated on a rotary evaporator under reduced pressure at below 37°. The evaporation was repeated by addition of distilled water until free from acids (4 or 5 times).

C. 5.7 N - Hydrochloric Acid Hydrolyses - (for the determination of glucosamine and its isolation).

A suspension of cell walls (5 to 10 mg. for determination and 500 mg for isolation) in 5.7 N - hydrochloric acid (2 ml. for determination and 100 ml. for isolation.) in a sealed tube or in a conical flask (for isolation and characterisation) was heated at 100° for 18 hr., and then cooled and neutralised as described below.

(i) Removal of Hydrochloric Acid in a Vacuum Desiccator over Phosphorus Pentoxide and Potassium Hydroxide - (For isolation of glucosamine).

The hydrolysate was filtered and hydrochloric acid was removed by evaporation in a vacuum desiccator over phosphorus pentoxide and potassium hydroxide.

D. 70% Perchloric Acid Hydrolysis (Marshak and Vogel, 1951 and Bartnicki-Garcia and Nikerson, 1962) (For the identification of purine and pyrimidine bases by paper chromatography).

The cell wall preparation (20 mg.) was digested with 70% perchloric acid (0.1 ml.) at 100° for 1 hr. in an open tube . Cooled and diluted with water, filtered and then evaporated to dryness on a rotary evaporator under reduced pressure at 37°. The residue was chromatographed in Solvent m.

G.M.16. Determination of β- Amylase Activity.

β- amylase activity was measured as by Hobson, Whelan

and Peat (1950). The method was modified as described below.

Procedure

Enzyme solution (Ca. 0.95 units/ml. according to the supplier, Worthington Biochemicals Ltd., 0.3 ml.) was added to a mixture of 0.6 % (W/v) aqueous soluble starch (8.3 ml.) and 0.2 M - sodium acetate buffer pH4.8 (1 ml.) at 30° and the volume was adjusted to 10 ml. with distilled water. The mixture was incubated at 30° and after 30 min. samples (0.5 ml.) were assayed for maltose by Nelson's method (G.M.2) using the substrate buffer incubation mixture, without the enzyme, as blank.

The activity was calculated according to the definition that 1 unit of enzyme catalysed the release of 1 μ mole of maltose/min./ml. of digest at pH 4.8 and 30°.

G.M.17. Determination of Pullulanase Activity

Pullulanase activity was determined according to the method of Abdullah, Catley, Lee, Robyt, Wallenfels and Whelan (1966).

Procedure

A mixture of 5% (W/v) aqueous pullulan (Kindly given by Prof. W.J. Whelan) solution (0.2 ml.) and 0.02 M phosphate - citrate buffer, pH 5.0 (0.7 ml.) was incubated at 30° . Pullulanase solution (0.1 ml.) was added and then incubation continued noting the time of enzyme addition. Samples (0.1ml.) were withdrawn after 30 min, and diluted into a mixture of water (0.9 ml.) and Nelson reagent (1 ml.). The reducing power was then estimated by Nelson's method (G.M.2). The reagent was calibrated against maltotriose and the buffer substrate incubation mixture was used as blank. A unit of pullulanase activity is defined by international standard (Commission on Enzymes, International Union of Biochemistry, 1961) and is the amount of enzyme catalysing the release of 1 μ mole of maltotriose/min./ml. of digest.

G.M.18. Determination of α- Amylase Activity

Reagents

1% (W/v) aqueous soluble starch in 0.02 M - sodium phosphate buffer pH 6.9 containing 0.0067 M - sodium chloride.

α- Amylase solution in the same buffer as above.

Procedure

A mixture of 1% starch solution (1 ml.) and α- amylase solution (1 ml.) was incubated at 37° for 15 min. The reducing power was immediately determined by Nelson's method (G.M.2).

One unit of enzyme is defined as that amount catalyzing the formation of 1 μ mole of reducing sugars/min./ml. of digest under the conditions described.

G.M.19. Determination of Basidiomycete QM806 Exo-β-D-(1→3)-Glucanase Activity

Exo-β-D-(1→3)-glucanase activity was determined essentially according to the method of Huotari, Nelson, Smith and Kirkwood (1968). The method was modified as described below.

Reagents

0.05% (W/v) soluble laminarin in 0.05 M - sodium acetate buffer, pH 4.8, containing 0.025% gelatin.

Procedure

0.05% soluble laminarin solution in the buffer (1 ml.) was incubated at 37° . Enzyme solution (10 μl.) was added and then incubation continued for a further 15 min. The reaction was stopped either by heating in a boiling water bath for 5 min. for the determination of glucose (G.M.3.), or by the additon of Nelson reagent for the determination of total reducing power as glucose (G.M.2.).

A unit of enzyme activity is defined as that amount catalyzing the formation of 1 μ mole of glucose/min. under the conditions described.

G.M.20. Preparation of Buffers

Buffers were prepared according to Gomeri (1955) and the pH was adjusted during mixing of the two component of buffer using a pH meter. All the buffers prepared were expressed in actual molarity,

except the citrate phosphate or phosphate citrate buffer, where the molarity was based on the concentration of the first component (except the McIlvaine buffer, 1921).

G.M.21. Enzyme Incubation Conditions.

(a) β- Glucosidase (Emulsin) from Almonds (2.5 units/mg., where one unit frees 1 μ mole of glucose/min. at 37° from salicin, according to manufacturer Worthington Biochemical Corporation).

Incubation with the enzyme was carried out in 0.01 M - sodium acetate buffer, pH 5.0 with a concentration of enzyme 0.01 - 0.1% and substrate concentration of 1% at 37°.

(b) Glucamylase (Aspergillus niger) - (10 units/mg., where one unit frees 1 μ mole of glucose/min. at 37° from soluble starch, according to Dr. B.E. Ryman, who kindly gave the enzyme preparation).

Incubation with the enzyme was carried out in 0.04 M - sodium acetate buffer, pH 4.8 with a concentration of enzyme 0.01 - 0.1% and substrate concentration of 1% at 37° under toluene.

(c) Chitinase from Streptomyces griseus - (40 units/mg., where one unit frees 1 μ mole of N - acetyl - D - glucosamine/min. at 37° from colloidal chitin according to the manufacturer Worthington Biochemical Corporation).

Incubation was carried out as follows: 0.5% homogenised cell walls and 0.05% enzyme in 0.05 M - sodium phosphate buffer, pH 6.3 was incubated at 37°.

(d) Acid Phosphatase from Potato (2 units/mg, where one unit frees 1 μ mole of phosphate/min. at 37° from p - nitrophenyl phosphate according to manufacturer The Boehringer Corporation Ltd., London).

Incubation with the enzyme was carried out in 0.01M - sodium acetate buffer, pH 4.8 with a concentration of enzyme 0.01 - 0.1% and substrate concentration of upto 1% at 37°.

G.M.22. Chromatographic Procedures.

Compounds were separated by descending chromatography on paper (Whatman No. 1, 540 and 3 M M , size 46 x 57 cm.) and by ascending chromatography on thin-layer plates covered with silica gel G (E. Merk AG, Darmstadt), or Whatman CC41 cellulose powder (thickness 1 mm., size of plates 20 x 20 cm. and 20 x 5 cm.).

A. Solvents Used for the Separation of Sugars - (a) ethyl acetate + pyridine + water + acetic acid (5 + 5 + 3 + 1); (b) butan-1 - ol + pyridine + water + benzene (5 + 3 + 3 + 1); (c) ethyl acetate + pyridine + water (3.6 + 1 + 1.5); (d) ethyl acetate + pyridine + water (8 + 2 + 1); (e) pyridine + ethyl acetate + water (2 + 5 + 7); (f) propan - 1 - ol + ethyl acetate + water (7 + 1 + 2); (g) butan - 1 - ol + pyridine + water (6 + 4 + 3); (h) butan - 1 - ol + ethanol + water (4 + 1 + 1.9); (j) propan - 1 - ol + ethyl acetate + water (6 + 1 + 3). All by volume.

B. Solvents Used for the Separation of Sugar Phosphates and Inorganic Orthophosphate - (k) propan - 1 - ol + ammonia (specific gravity 0.88)+ water (6 + 3 + 1 by vol.); (l) t - butanol + picric acid + water (80 ml. + 2g. + 20 ml.)

C. Solvent Used for the Separation of Purine and Pyrimidine Bases - (m) 95% isopropanol (68 ml.) + hydrochloric acid (sp.gr. 1.18) (17.2 ml.) - diluted to 100 ml. with water.

Table G.M.22 A, B and C shows the R_G, R_{Pi}, and R_F values respectively of standards, which were always run in parallel with the material under investigation.

The atmosphere of the large paper chromatography tanks was always presaturated with the solvents system at least 48 hr. before the irrigation of papers for separation and the chromatography papers were suspended in the presaturated tank for 2 hr. prior to irrigation. In the case of two phase systems the aqueous phase was used for the saturation of the tanks' atmosphere and the organic phase was used for the irrigation. For thin-layer chromatography the saturation and irrigation both were carried out with the organic phase. For better and quicker saturation of thin-layer plates, paper wicks were used

Table G.M.22(A) R_G Values of Sugars

Sugar	Solvent System and RG Values						
	a	b	c	d	e	f	g
Glycerol					2.54		
Erythritol					1.66		
Threitol					1.66		
2-Deoxy Ribose				5.25			
Ribose	1.30	1.79		3.70			1.36
Arabinose	1.10	1.21	1.62	2.00	1.28	1.21	1.16
Xylose	1.20	1.50	2.12	2.60	1.66	1.40	1.21
Rhamnose	1.39	2.05	3.90	4.20	2.25	1.83	
Mannose		1.29	1.30	1.45		1.21	1.16
Glucose	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Galactose	0.93	0.85	0.8	0.88	0.81	0.94	0.95
Glucosamine	0.73	0.75					
N-Acetyl Glucosamine		1.35		1.30			

Table G.M.22(A) (Continued)

Sugar	Solvent System and R_G Values			
	b	f	g	j
Laminaribiose	0.76	0.61	0.80	
Laminaritriose	0.51	0.30		
Laminaritetraose	0.34			
Laminaripentaose	0.23			
Laminarihexaose	0.15			
Laminariheptaose	0.10			
Nigerobiose			0.76	0.82
Nigerotriose			0.59	0.67
Nigerotetraose			0.44	0.51
Nigeropentaose			0.33	0.42
Nigerohexaose			0.24	0.31
Maltose	0.65	0.46	0.70	
Maltotriose	0.40	0.22		
Panose		0.20		
Isomaltose	0.23	0.13		
Isomaltotriose	0.09			
Gentiobiose	0.44	0.35	0.54	
Cellobiose	0.59	0.44		

Table G.M.22 (B) R_{Pi} Values of Sugar Phosphates

Compound	Solvent System and R_{Pi} Values	
	k	l
Orthophosphate	1.00	1.00
Glyceraldehyde-3-phosphate	1.74	
Mannose-6-phosphate	1.15	0.52
Glucose-6-phosphate	1.11	0.42
Galactose-6-phosphate	1.09	0.38

Table G.M.22 (C) R_F Values of Purine and Pyrimidine Bases

Bases	Solvent System and R_F Values
	m
Guanine	0.20
Adenine	0.33
Cytosine	0.45
Uracil	0.67
Thymine	0.78

surrounding the inside of the tank.

For long runs the paper was serrated, to allow the irrigating solvents to drip and maintain an even solvent flow.

After irrigation with a suitable solvent the paper or the thin-layer plate was air dried and sprayed with one of the reagents described below.

Spray Reagents

(a) Silver Nitrate/Sodium Hydroxide Reagent (Trevelyan, Proctor and Harrison, 1950) - A saturated solution of silver nitrate (0.5ml.) was diluted with acetone (99.5 ml.) and the precipitate redissolved by the addition of a few drops of water.

Sodium hydroxide (4 g.) was dissolved in water (8 - 10 ml.) and the solution made up to 200 ml. with ethanol.

The chromatogram was first sprayed or drawn through silver nitrate reagent, dried in a current of air and then sprayed with the sodium hydroxide reagent. The sugar zones showed up as dark-brown spots against a light-brown background. To remove the background and preserve the chromatogram it was sprayed with 10% (W/v) aqueous sodium thiosulphate, thoroughly rinsed with water, and then dried at room temperature.

(b) Aniline Hydrogen Phthalate Reagent (Wilson, 1959) - o - phthalic acid (1.66 g.) and aniline (redistilled from zinc) (0.91 ml.) were dissolved in a mixture of butan - 1 - ol (48 ml.), diethyl ether (48 ml.), and water (4 ml.) The reagent was stable for at least 2 weeks when stored in the refrigerator.

The paper was sprayed or drawn through the reagent, dried in a current of air and then heated at 105° for 10 min. in a forced air oven. Reducing hexose sugars showed up as dark-brown spots, reducing pentoses as pink spots and 2 - deoxy pentoses as yellow spots.

(c) Elson - Morgan and Morgan - Elson Reagents (Partridge and Westall, 1948)

(i) Acetylacetone Reagent. Solution (1): Acetylacetone (0.5 ml.) dissolved in butan - 1 - ol (50 ml.). Solution (2): 50% (W/V) aqueous potassium hydroxide (5 ml.) and ethanol (20 ml.). Solution (2) (0.5ml.)

was added to solution (1) (10 ml.) immediately before use. The reagent was made up daily from distilled acetylacetone (stored at 4°).

(ii) p - Dimethylaminobenzaldehyde Reagent - p - Dimethylaminobenzaldehyde (1 g., recrystallised from aqueous ethanol) was dissolved in ethanol (30 ml.) and conc. hydrochloric acid (30 ml.) was added. The solution was diluted with redistilled butan - 1 - ol (180 ml.) immediatley before use.

The chromatogram was sprayed with Solution (i) and heated in the oven for 5 min. at 105°. The dry chromatogram was then sprayed with Solution (ii) and returned to the oven for a further short heat treatment (5 min.) at 90°. Under the conditions free hexosamines gave a cherry-red colour, and N - acetyl glucosamine gave a strong purple-violet colour. Both colours were stable for several days. N - acetyl glucosamine could be distinguished from glucosamine by spraying with only solution (ii). Under the above condition N - acetyl glucosamine gave a strong violet colour but the glucosamine gave no colour.

(d) Molybdate Spray for Sugar Phosphates and Inorganic Phosphate (Hanes and Isherwood, 1949) - 4% (W/V) aqueous ammonium molybdate (25 ml.) was mixed with 60% (W/W) perchloric acid (5 ml.) and N - hydrochloric acid (10 ml.), and diluted with water to 100 ml.

The chromatogram was sprayed with the reagent and then air dried. The air dried chromatogram was then irradiated with UV light (Hanovia Model 16) for 5 - 10 min. Inorganic phosphate shows up first yellow and on standing at room temperature for several hours to several days it changes to blue. Hexose phosphates, pentose phosphates and triose phosphates always gave blue colours.

(e) p - anisidine/Trichloroacetic acid Spray Reagent (Bielaski and Young, 1963) - p - anisidine (1 g.) and trichloroacetic acid (3 g.) were dissolved in 70% ethanol (100 ml.)

The chromatogram was sprayed with the reagent and then air dried. The air dried paper was then heated for 5 min. at 105°.

(f) p - anisidine/Sulphuric Acid Spray Reagent (Hough, Jones and Wadman, 1950) (for polysaccharides on glass paper) - p - anisidine

(1 g.) and 98% sulphuric acid (2 ml.) were dissolved in moist butanol (100 ml.)

The chromatogram was sprayed with the reagent and then air dried. The air dried paper was then heated for 15 min. at 100°.

(g) Vanillin /Sulphuric Acid Spray Reagent ("Chromatography", E. Merck AG, Darmstadt, 2nd odn., p - 30) (for any organic compound on a silica gel thin layer chromatogram).-5% Vanillin in ethanol (10 ml.) was mixed with 98% sulphuric acid (0.5 ml.).

The chromatogram was sprayed with the reagent and then air dried. The air dried chromatogram was then heated for 10 min. at 100°. Black spots were produced against a gray background.

(h) 0.1N Iodine Solution (for α- D - (1→4) linked glucan).

The chromatogram was sprayed with the reagent. Amylaceous polysaccharides gave blue to black spots. When dried in the air thoroughly the colours of the spot gradually dissappear but reappear when sprayed with distilled water.

G.M.23. Paper Electrophoresis.

Electrophoretic separations were carried out on Whatman 3MM cellulose paper or Whatman GF 81 glass fiber paper (46 x 57 cm.) using a Miles Hivolt electrophoresis apparatus. (Miles Hivolt Ltd., Shoreham, Sussex)

The buffers employed were (1) 0.05 M - sodium tetraborate, pH 9.3; (2) 0.05 M - sodium germinate, pH 10.7; (3) 0.05 M - sodium tetraborate in 0.5 N sodium hydroxide.

After electrophoresis papers were air dired and sprayed with a suitable reagent (G.M.22).

M_G values were defined as the mobility relative to the mobility of glucose, taking the mobility of 2, 3, 4, 6 tetra - O - methyl D - glucose as zero.

Table G.M.23 shows the M_G values.

GM.24. Gas - Liquid Chromatography

Table G.M.23. Relative mobilities of sugars on electrophoresis

Sugar	M_G Values			
	Whatman 3MM Cellulose Paper		Whatman GF 81 Glass Paper	
	0.05M-Borate pH 9.3	0.05M-Germanate pH 10.7	0.05M-Borate pH 9.3	0.05M-Germanate pH 10.7
2-deoxy Ribose	0.34			
Arabinose	0.92	1.60		
Xylose	1.0	1.45		
Rhamnose	0.5	1.3		
Mannose	0.7	1.42	0.67	
Glucose	1.0	1.00	1.00	1.00
Galactose	1.0	1.20	0.91	1.00
Tetramethyl D-Glucopyranose	0.00	0.00	0.00	0.00
Glycogen			0.34	0.62
Laminarin			0.39	0.61

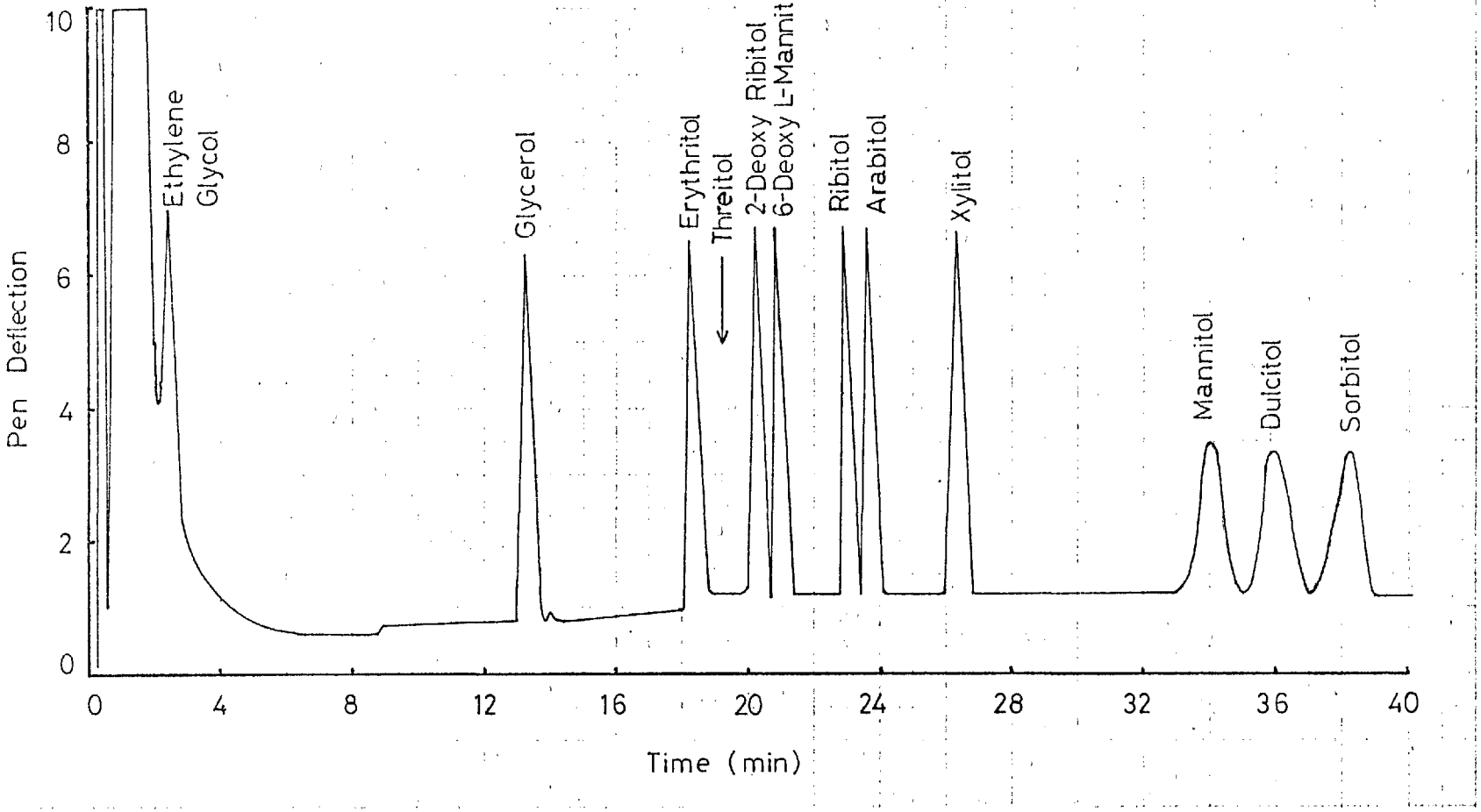


Fig. G.M.24. Gas-Liquid Chromatography of alditols acetates on ECNSS-M column using temperature programming as described in G.M.24.

The methods were based on those described by Bjorndal, Lindberg, Svensson (1967a and 1967b).

Material

Column Packing - 3% (w/w) of a copolymer of ethylene glycol succinate polyester and a nitrile silicone polymer (ECNSS - M) on Gas Chrom Q (100 - 120 mesh).

Column Used (1) Glass column (5ft x 0.4 cm inside diameter) used on Pye series 104 gas chromatograph .

(2) Stainless steel column (200 x 0.22 cm. inside diameter) used on a Perkin Elmer Model 270 Combined gas chromatograph-mass spectrometer.

Equilibration of the Column - Nitrogen (oxygen free) (40 ml./min.) for column (1) and helium for column (2) was used as carrier gas throughout. The temperature of the column was raised in steps:

(a) Quickly raised to 150°,
then (b) 5° per hour upto 190°, and
kept at (c) 190° for 16 hr. prior to use.

When the column was used at a higher temperature, then the column was baked for at least 8 hr. at an elevated temperature of 10° above the required temperature. The columns were never used at above 230°.

Column Temperature for Different Procedures

(1) Methylated sugars were separated for quantitative determination on Column (1) at a fixed temperature of 180°.

(2) Alditol acetates from periodate oxidation products were separated for quantitative determination on column (1) using the temperature programmer with a starting temperature of 100° for 5 min. and then 8°/min. to 200°. The column was left at 200° until sorbitol hexa-acetate was eluted from the column (45 min.)

(3) Methylated sugars were separated for identification by mass spectroscopy on Column (2) at a fixed temperature of 190°.

Fig. G.M.24 shows the elution profile of authentic alditol acetates on Column (1).

G.M.25. Preparation and Packing of Sephadex Column.

The method used for the swelling of sephadex and the packing of columns were based on those described in the manufacturers booklet "Sephadex - gel filtration in theory and practice" (Pharmacia G.B. Ltd., Sinclair House, The Avenue, West Ealing, London, W.13.).

Preparation of Sephadex Gel

(a) In 0.5 N - Sodium Hydroxide

Dry sephadex G - 200 (1.5 - 2g.) was suspended in water (200 ml.), containing sodium borohydride (50 mg.) and stirred for 2 days at room temperature. Excess borohydride was destroyed with glacial acetic acid, washed by decantation with distilled water (10 x 100 ml. or more) during a period of 24 hr.

The swollen gel was mixed with an equal volume of N - sodium hydroxide and then washed by decantation with 0.5 N - sodium hydroxide (5 x 100 ml.) during a period of 8 hr.

(b) In 0.3 - 0.5 M - Sodium Chloride or Distilled water

The swelling was exactly according to the booklet of the manufacturer.

Packing of the Sephadex Column (using sephadex precision chromatographic column) : The column was packed according to the manufacturers booklet (page - 34, 1966) and the column was equilibrated by running eluting solvent through the column for 7 days at a low pressure (5 to 6 cm. of water pressure) using a reservoir. The pressure was then increased upto 15 cm. so that the flow rate was inbetween 3 to 4 ml./ cm^2./hr.

The homogeneity and the void volume of the column were determined by running "Blue Dextran - 2000" , which gave a sharp coloured zone throughout its movement in the bed and a recovery of approximately 75% within 3 ml. of effluent for a column of 1.5 x 30 cm; sephadex G - 200, with an applied sample volume of 0.5 ml. (maximum) of 0.5% (maximum) blue dextran.

Sample Application: The eluant was withdrawn from above the gel bed (by using pipette), taking care not to shrink the bed or run it dry

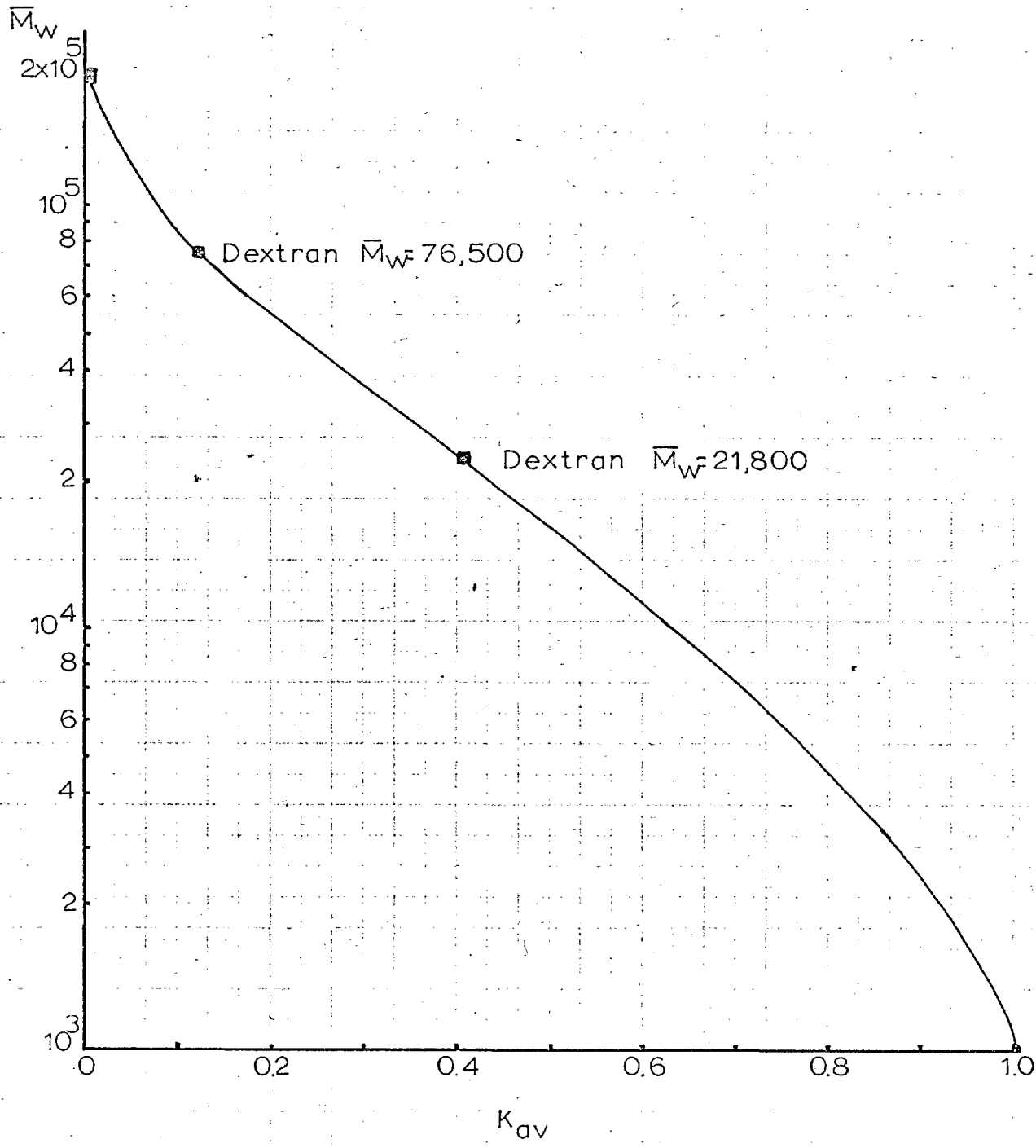


Fig. G.M.27. The Calibration curve for Dextran on Sephadex G-200.

and the sample was applied according to the manufacturers booklet (page 37, 1966).

G.M.26. Preparation and Packing of DEAE Cellulose Column

Equilibration of DEAE cellulose and column packing was carried out according to the methods described in the manufacturers booklet "Whatman advanced ion - exchange cellulose laboratory manual" (Wand R Balston Ltd., Maidstone, Kent.).

G.M.27 Molecular Weight Distribution Analysis by Gel Chromatography on Sephadex G-200

The determination was based on the method of Granath and Kvist (1966) and was modified as follows.

Solutions (0.5% W/v) of different fractions of cell walls, Smith degraded cell wall residues and dextrans of known molecular weight were passed individually and under identical conditions through the same column (1.4 x 29 cm.) of Sephadex G-200, packed and eluted with 0.5N - sodium hydroxide (G.M.25). Dextrans were first reduced with sodium borohydride, neutralised with glacial acetic acid and then taken up in sodium hydroxide. Eluant were collected immediately following the application of sample solution. Fractions of 0.05 to 0.1 ml. were collected as soon as the polysaccharides starts coming out. Total hexose (G.M.1) in each fraction was determined and the elution profile was obtained by plotting the concentration of anhydrohexose against elution volume (Ve).

The calibration graph (Fig. G.M.27), obtained by plotting the K_{av} value of different dextrans against $\log_{10}$ of molecular weight, was used for the determination of the molecular weight distribution of other polymers of unknown molecular weight.

Where $K_{av} = \frac{Ve - V_o}{Vt - V_o}$; Ve = elution volume of each dextran upto the peak; V_o = void volume (the elution volume of a solute that was completely excluded from the gel e.g. blue dextran); V_t = total volume of the gel bed.

Dextrans used for calibration (obtained from Pharmacia G.B.Ltd.) were dextran 2000 (blue dextran; $\bar{M}_w$, 2 x 10^6), dextran 70 ($\bar{M}_w$ 7 x 10^4) and dextran 20 ($\bar{M}_w$, 2 x 10^4).

G.M.28 X - ray Analysis

X-ray diffraction patterns were kindly determined by Mr. P.L. Bird, Analytical Services Laboratory, Imperial College.

Debye - Scherrer Powder Diffraction Photograph.

Samples were compacted in a 1 mm. glass capillary by using two closely fitted plungers on either side of the capillary with application of pressure by hand on the introduced samples. The pellets (2 - 3 mm. in lenght) were transferred to thin walled Lindeman glass capillaries, which were mounted in the Philips Debye-Scherrer powder diffraction camera, (diameter 114.83 mm.). Nickel filtered Cukα (λ = 1.5418A°) radiation was obtained from a Philips PW1310 X-ray generator operation at 50 KV, 36mA. Photographs were obtained on an Ilford Industrial G film with exposure time of 18 minutes.

d spacings were calculated from Bragg's equation 2 d Sin$\theta = \lambda$ where λ =1.5418A°. The specimen to film distance was such that 1 mm. on film = 1° ; diameter of each circle in mm. = 4θ in°.

G.M.29 Analytical Ultracentrifugation and Determination of Sedimentation value

Analytical ultracentrifugation was carried out on a Beckman Model E Analytical Ultracentrifuge, provided with a Schlieren optical system.

The centrifugation was carried out at 48,000 r.p.m.(160,000 g.), with solutions of the polysaccharides in N - sodium hydroxide at 22 - 23°, using a 12 mm. Kel F 4° single sector cell with quartz windows. Photographs were taken automatically at intervals of 1 hr. 4min., after the centrifuge had attained full speed and the first sedimenting boundary was observed, using an exposure time of 10 sec., and Kodak 0.250 backed rapid metallographic plates.

After developing the plate individual peak distances were calculated by measuring on a projescope (G.K.N. Shardlow Metrology Ltd., Sheffield.) with reference to the distance of the inner and outer reference holes from the centre of rotation (5.70 cm. and 7.30 cm. respectively - Manufacturers specification). Sedimentation constants of individual concentration were determined from the slope of the straight line obtained by plotting the $\log_e$ of the distance of individual peaks from the centre of the rotar in cm. against time in secs. using the formula S = slope $/W^2$ (where W = angular velocity in radians/sec.). S°_{20} values were obtained by determining S_{20} values at four different concentrations and extrapolating to infinite dilution and were expressed in Svedbergs (S), where $1S = 10^{-13}$ sec^{-1}.

G.M.30 Microbiological Procedures

Organism : Fusicoccum amygdali Del. culture was obtained from Dr. A. Graniti (Istituto di Patologia Vegetale, University of Bari, Italy).

Maintenance of the Cultures : (1) Master Slopes were prepared on potato glucose medium (see below), containing 1.5% ($^{W}/_{V}$) agar, and kept under oil after incubation for one week at 24°.

(2) Stock Slopes were prepared on the above medium by inoculation from the master slope and were used within 30 days for the inoculation of the primary stage (see below).

Media

The media used were as follows.

Medium I for seed production: a potato infusion was made by boiling 30 g. of whole potatoes in 50 ml. water for 15 min.; after cooling the suspension was filtered through muslin and brought to pH 7.0 before addition of 2 g. of glucose and making upto 1 l.

Medium II : glucose monohydrate, 3.300% ; sodium nitrate, 0.33%; potassium dihydrogen phosphate, 0.100% ; potassium chloride, 0.050% ; magnesium sulphate heptahydrate, 0.050% ; ferrous sulphate heptahydrate, 0.001% and (a) yeast autolysate, 0.1% or (b) soyabean meal (milled) 0.2% ($^{W}/_{V}$).

Submerged Culture Procedures

Primary Stage: Medium I was inoculated from a stock slope culture and was then incubated at 24° on a rotary shaker for 4 days.

Secondary Stage : The primary stage cultures were mixed with medium I (10 vols) and incubated at 24° for 2 days.

Production Stage : (a) Fermentations in Shake Flasks

Medium II was inoculated with the secondary stage culture and was then incubated at 24° for 3½ days on a rotary shaker (200 rev./min., eccentric throw 10 cm.)

(b) Fermentations in Stirred Fermenters: Medium II, was sterilised at 121° for 20 min. and inoculated with 10% (v/v) of inoculum from the secondary stage culture (40 l. fermenters) or the previous batch (400 l. fermenters).

Cultures were grown in 40 and 400 l. fermenters in the "Pilot plant" of the Biochemistry Department, Imperial College of Science and Technology. Cultures were incubated at 24° on an agitator of shaft speed 61.3 r.p.m. The air flow rate was 200 l./min. for 0 - 12 hr. and 400 l./min. thereafter pressure was 15 p.s.i.

Experimental variables : Antifoam added : Silicone R.D. (Midland Silicones Ltd.) as required.

Mycelium was harvested after 3½ days, washed with water and stored at -12°, if not processed immediately.

G.M.31 Determination of Periodate Consumption

To a mixture of 0.1$\underline{\underline{N}}$ - sodium arsenite (1 ml.) and sodium bicarbonate (1.5 g.) was added, with shaking a 1 ml. aliquot of periodate oxidation mixture, followed by 20% (w/v) potassium iodide (1 ml.). After 20 min. the excess sodium arsenite was titrated with 0.1$\underline{\underline{N}}$ - iodine solution using thyodene indicator (Purkis, Williams Ltd.).

0.025$\underline{\underline{M}}$ periodate (1 ml.) ≡ 0.05$\underline{\underline{M}}$ (N/10) sodium arsenite (0.5 ml.)
≡ 0.1$\underline{\underline{N}}$ iodine (0.5 ml.)

G.M.32 Determination of Formaldehyde

Formaldehyde was determined essentially according to the method of Hay, Lewis, Smith and Unrau (1965). The method was modified as described below.

Reagents

A. 40% $^{w}/_{v}$ aqueous lead acetate trihydrate.

B. Chromotropic acid reagent

The disodium salts of chromotropic acid (1 g.) in water (100ml) was diluted to 500 ml. with 66% sulphuric acid.

Procedure

An aliquot (10 ml.) of the periodate oxidation mixture was mixed with the lead acetate solution (10 ml.) and the resultant precipitate was removed by centrifugation (10,000 g., 30 min.). The supernatant was placed in a test tube and a length of dialysis tubing (boiled several times with water and finally washed with cold water) containing water (5 ml.) was introduced. After equilibration overnight, aliquots (1 ml.) of the contents of the dialysis bag were mixed with the chromotropic acid reagent (10 ml.) and centrifuged at 3,000 g. for 3 min. to remove the lead sulphate precipitate. The supernatant was transferred to a glass stoppered test tube, heated for 30 min. in a boiling water bath, protected from light, and cooled. The optical density was determined at 570 nm. against a periodate blank treated precisely as above but without the polysaccharide substrate. Standards were prepared using the formaldehyde liberated from standard solution of erythritol by periodate oxidation under the conditions described for periodate oxidation of polysaccharides in the Experimental Section. A linear relationship was obtained between optical density and formaldehyde concentration in the range 2 - 16 µg. formaldehyde.

G.M.33 Determination of Formic Acid

Formic acid was determined essentially according to the method of Barker and Somers (1966). The method was modified as described below.

Reagents

10% v/v Aqueous ethylene glycol. Prepared using redistilled ethylene glycol.

5% w/v Sodium borohydride in 0.05M - sodium tetraborate - hydrochloric acid buffer, pH 8.0.

8N - Sulphuric acid.

6.25% w/v Thiobarbituric acid. The pH was adjusted to 5.4 with 4N - sodium hydroxide before diluting to volume.

Redistilled n - butyl alcohol containing 5% v/v hydrochloric acid (11.4N).

Procedure

The periodate oxidation mixture or standard sodium formate solution (1.0 ml.), containing between 20 and 200 µg. equivalant of formic acid, in a glass stoppered test tube (1.2 x 10 cm.) was incubated with 10% v/v aqueous ethylene glycol (0.05 ml.) for 5 min. 5% w/v sodium borohydride solution (0.1 ml.) was added and after 5 min. excess borohydride was destroyed by addition of 8 N - sulphuric acid (0.05 ml.). 6.25% w/v Thiobarbiturate (0.4 ml.) was added and the mixture was heated for 20 min. at 100°. After cooling to room temperature the colour developed was quantitatively extracted into acidic butanol (1.5 ml.). After clarification by centrifugation at 3,000 g. for 3 min. the optical density was determined at 450 nm. against a blank prepared from distilled water under identical conditions. A linear relationship between optical density and concentration was obtained in the range 20 - 200 µg. formic acid. The optical density obtained with a concentration of 100 µg./ml. of formic acid was 0.26.

APPENDICES

Table 1. Quantitative amino acid analysis of the total cell walls

Amino acid	Time of hydrolysis (hr.) with 6.09N hydrochloric acid (G.M.15B)					
	Anhydro amino acid as wt.% of total cell wall			Molar % of total amino acids		
	24 hr.	48 hr.	72 hr.	24 hr.	48 hr.	72 hr.
Alanine	0.23	0.24	0.25	11.24	11.81	12.90
Arginine	0.55	0.40	0.25	12.32	9.13	5.84
Aspartic acid	0.32	0.34	0.38	9.76	10.64	12.14
Glutamic acid	0.33	0.32	0.33	9.12	8.96	9.44
Glycine	0.18	0.18	0.19	10.84	11.06	11.41
Histidine	0.07	0.08	0.12	1.68	1.93	3.06
Isoleucine	0.10	0.11	0.14	3.13	3.43	4.65
Leucine	0.26	0.20	0.25	7.98	6.20	8.20
Lysine	0.11	0.11	0.11	3.00	3.01	3.23
Phenylalanine	0.19	0.27	0.18	4.48	6.37	4.46
Serine	0.23	0.28	0.22	9.22	9.72	9.35
Threonine	0.18	0.20	0.18	6.40	6.95	6.65
Tyrosine	0.12	0.04	0.00	2.52	0.75	0.01
Valine	0.22	0.25	0.23	7.84	8.88	8.32

Table 2. d-Spacings obtained from X-ray diffraction patterns of cell wall Fractions.

α-D-Glucan, Fraction 270 $[\alpha]_D$ + 290° (p. 94 & 98)			Total cell wall α-D-Glucan, Fraction 39P $[\alpha]_D$ + 290° (p. 114)			Johnston's α-D-Glucan, (A. Niger, Fraction IVR) $[\alpha]_D$ +260°		
9.46	M (broad)	(d)	9.46	S	(d)	9.56	S	(d)
6.17	M	(s)	6.17	M	(s)			
5.00	W	(s)	5.00	W	(s)	5.00	M	(s)
4.55	S	(s)	4.55	S	(s)			
4.09	V.S	(s)	4.13	V.S	(s)	4.18	V.S	(s)
3.75	V.W	(s)						
						3.63	V.W	(s)
						3.22	V.W	(s)
			3.19	V.W	(s)			
3.08	V.W	(s)						
			2.91	V.W	(s)	2.91	V.W	(s)
2.76	V.W	(s)	2.74	V.W	(s)	2.72	W	(s)
2.53	V.W	(s)	2.53	V.W	(s)			
2.34	V.W	(s)						
						2.09	V.W	(s)
			1.99	W	(s)	1.99	W	(s)

V.S = very strong
S = strong
M = medium
W = weak

V.W = very weak
(d) = diffuse
(s) = sharp

Table 2. (continued)

β-D-Glucan, Fraction 27K $[\alpha]_D$ + 16°		Purified pachyman $[\alpha]_D$ + 15°	
10.40 - 9.31 S (oval)	(d)		
		4.55 W (broad)	(d)
4.44 S (broad)	(d)		
2.76 W (broad)	(d)	2.76 V.W	(d)
2.00 W	(s)	2.00 W	(s)

Table (continued)

Chitosan prepared from Total cell walls (p. 74)		Chitosan prepared from crustacean chitin (p. 74)	
		8.50 M	(d)
4.50 V.S	(s)	4.48 V.S	(s)
3.99 W	(s)	3.99 M	(d)
		3.63 W	(s)
		3.24 W	(s)
		3.11 W	(s)
		2.93 W	(s)
		2.70 W	(s)
		2.56 W	(s)
		2.23 W	(s)
		2.09 W	(s)
		1.99 W	(s)

V.V.S = very very strong
V.S = very strong
S = strong
M = medium
W = weak
V.W = very weak
V.V.W= very very weak
(d) = diffuse
(s) = sharp

Table 2. (continued)

Cell wall chitin prepared enzymically Fraction 24 & 39X p. 75, 96, 119.		Cell wall chitin prepared according to Houwink and Kreger Fraction 20ADIb p. 69.		Powdered crustacean chitin (B.D.H)	
		9.56 S	(d)		
9.46 V.S	(d)			9.46 V.S	(s)
6.81 W	(s)	6.81 W	(s)	6.81 W	(s)
4.55 V.V.S	(s)	4.55 V.V.S	(s)	4.55 V.V.S	(s)
				4.23 V.W	(d)
				3.75 V.W	(d)
3.33 S	(s)	3.33 S	(s)	3.33 S	(s)
		3.21 W	(s)		
				3.03 V.W	(d)
2.74 V.W	(d)	2.78 W	(s)	2.74 V.W	(s)
2.53 W	(s)	2.56 V.W	(s)	2.53 W	(s)
2.26 V.W	(d)			2.28 V.W	(d)
1.99 W	(s)	1.96 W	(s)		
				1.87 V.V.W	(d)
		1.68 W	(s)		

V.V.S = very very strong
V.S = very strong
S = strong
M = medium
W = weak
V.W = very weak

V.V.W = very very weak
(d) = diffuse
(s) = sharp

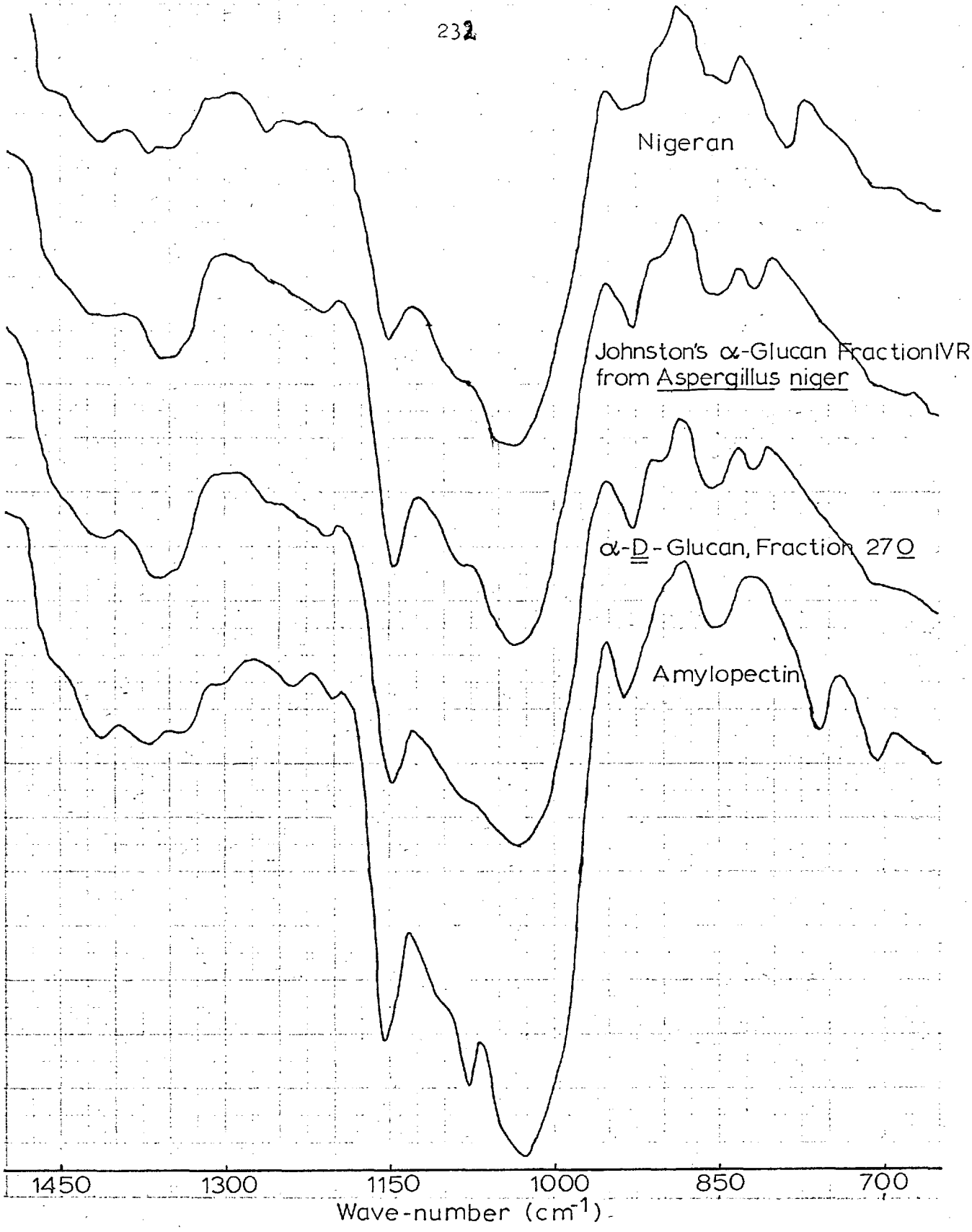


Fig. 1. Infrared Spectra of Cell Wall Fractions and Related Polysaccharides. (in KBr discs)

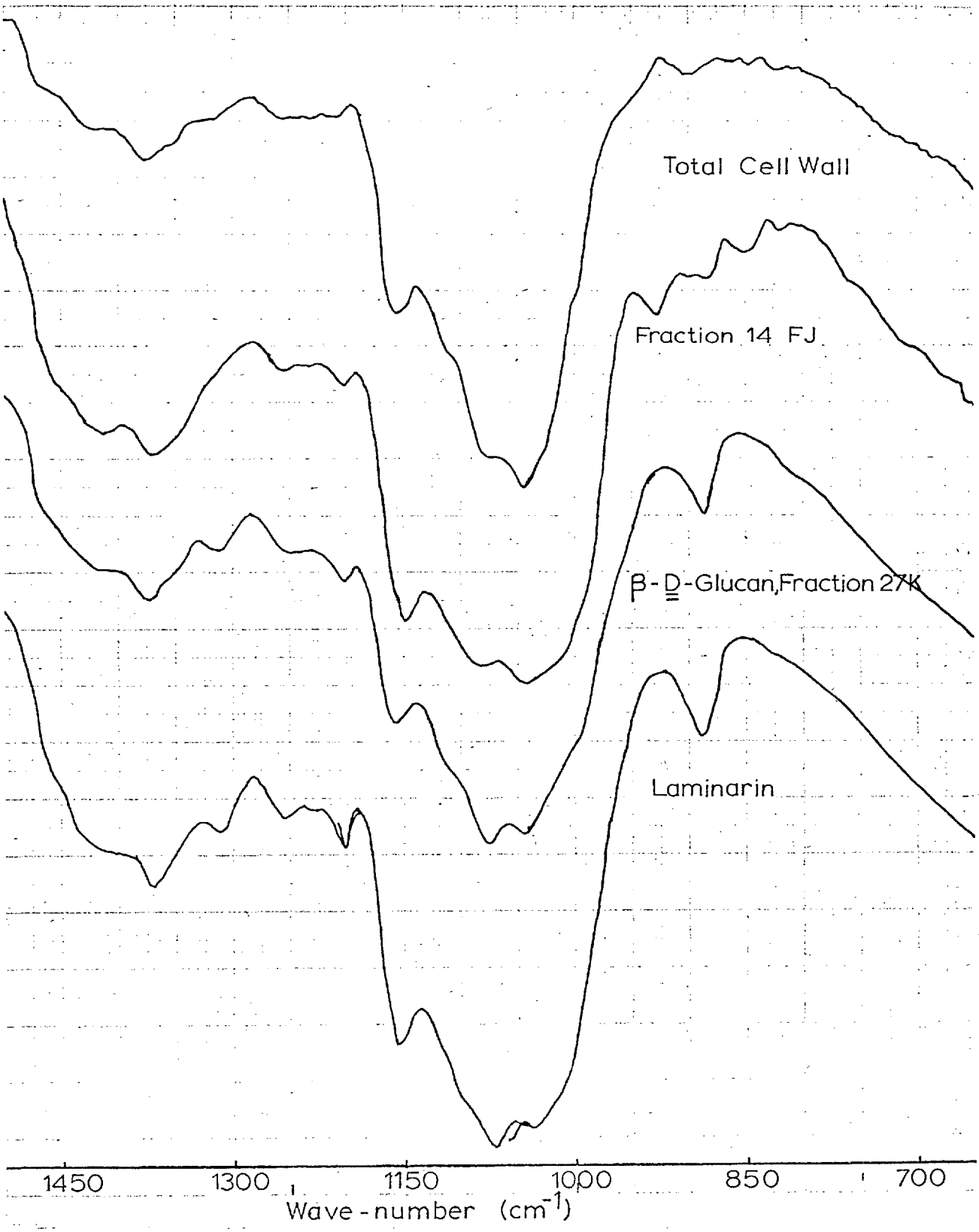


Fig. 1(contd.) Infrared Spectra of Cell Wall Fractions and Related Polysaccharides. (in KBr discs)

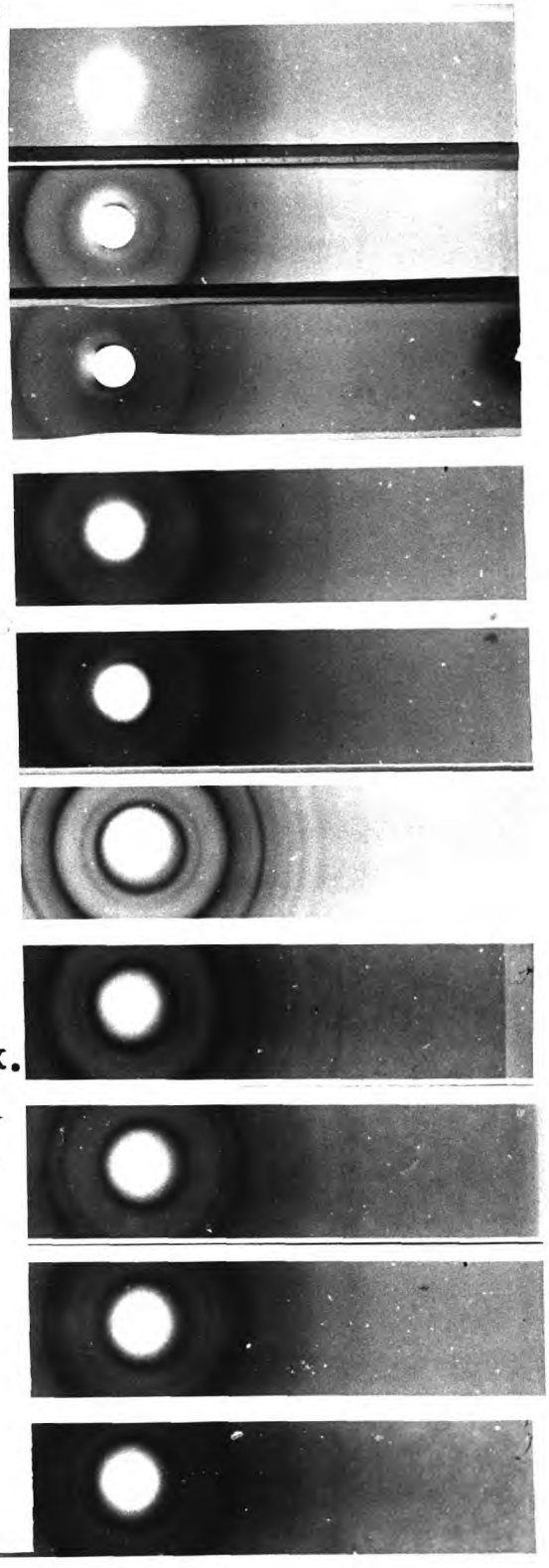


Fig. 2. X-ray Diffraction Photographs of Cell Wall Fractions and Related Polysaccharides.

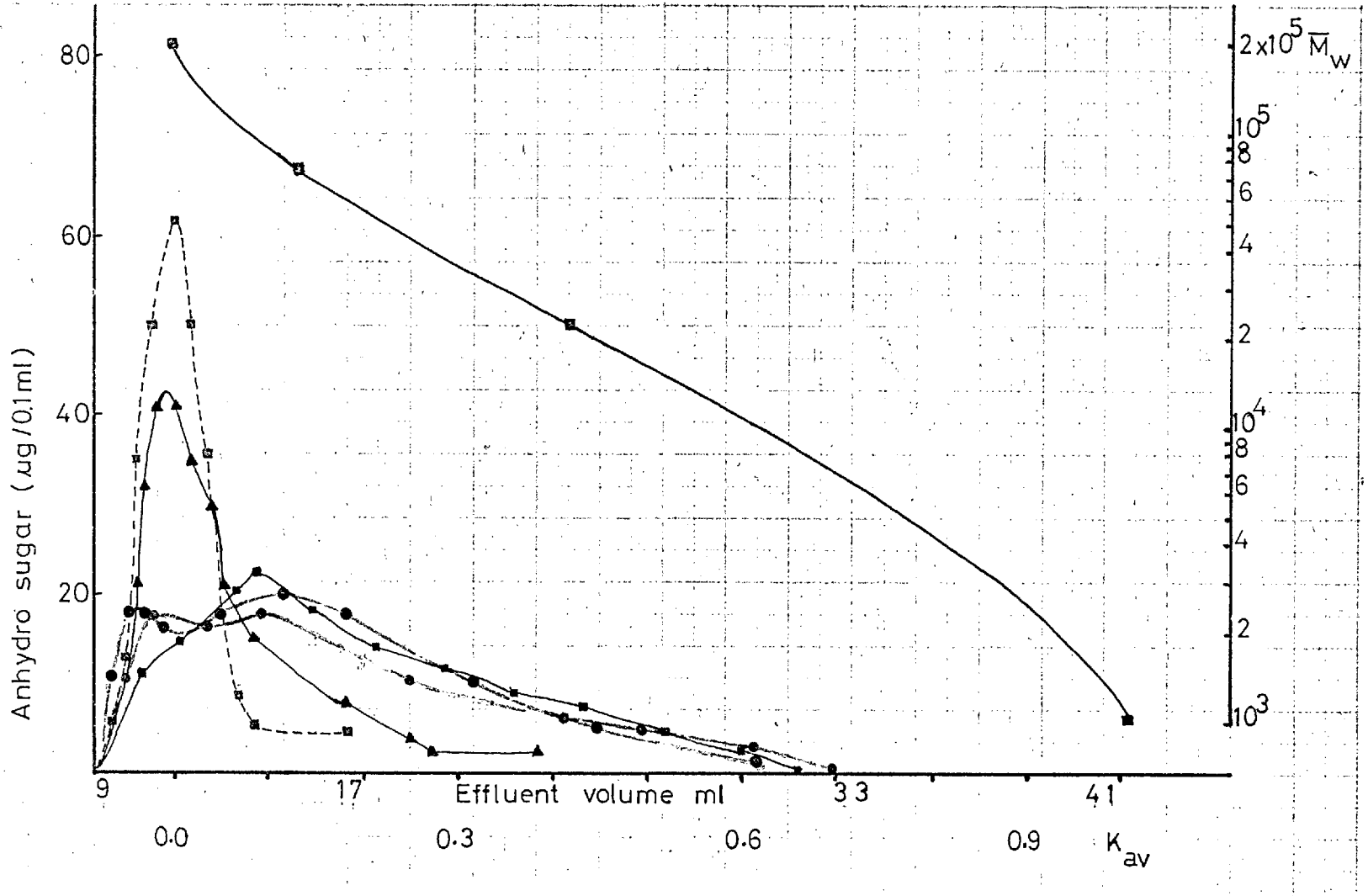


Fig. 3. Elution Profiles and Molecular Weight Distribution Analysis on Sephadex G-200. —▣—. Molecular weight calibration curve; - - ▣ - -, Blue dextran 2,000; —▲— Cell wall Fraction 14FJ; —■—, Fraction 14FJ after α-amylase action; —●—, Fraction 14FJ after exo-β-1,3-glucanase action; —●—, Fraction 14FJ after snail enzymes action.

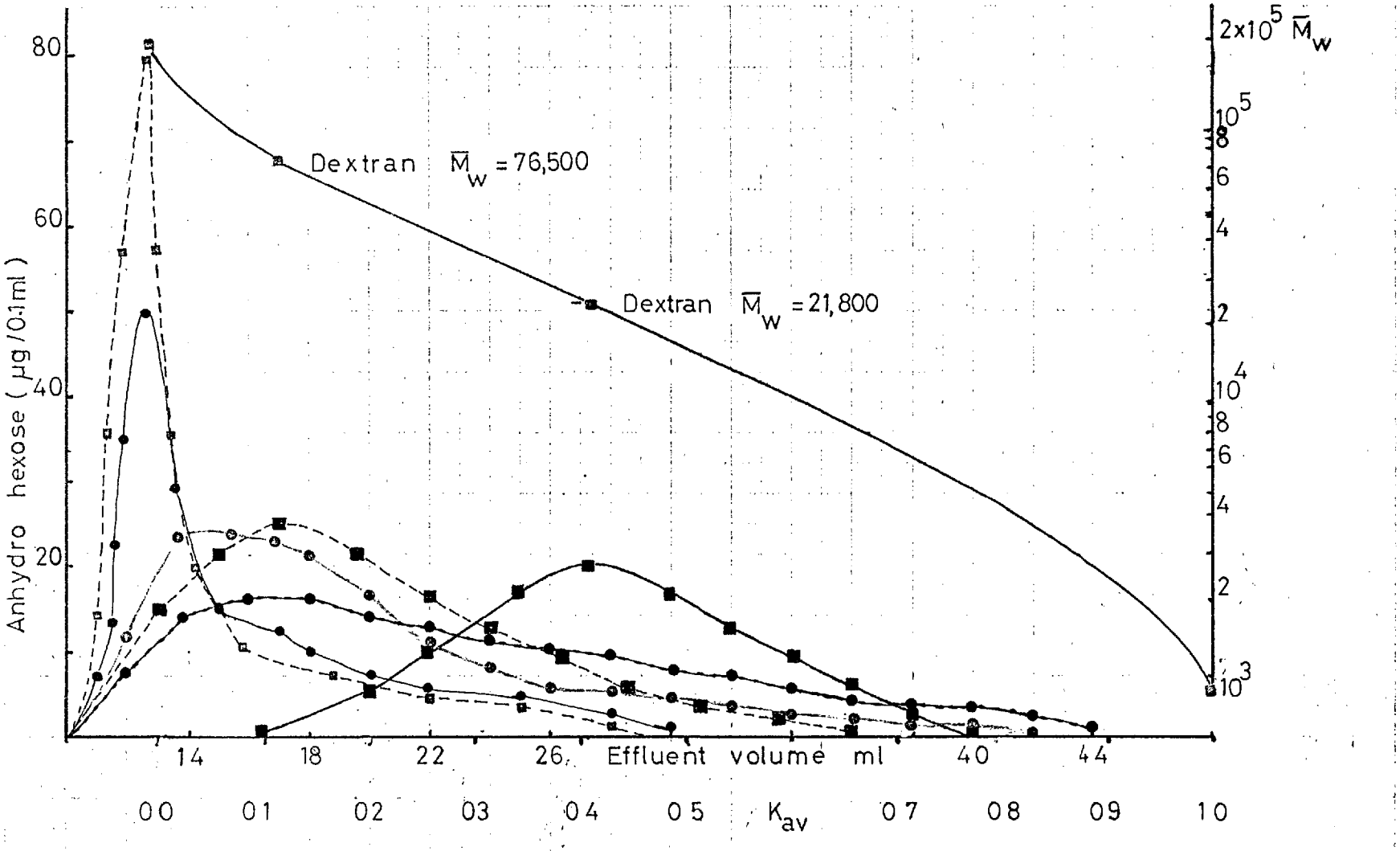


Fig. 4. Elution Profiles and Molecular Weight Distribution Analysis on Sephadex G-200. —■—, Molecular weight calibration curve; ---■---, Blue dextran 2,000; —●—, β-D-Glucan, Fraction 27K; —●—, α-D-Glucan, Fraction 27O; —●—, α-D-Glucan, Fraction 27O after Smith degradation; --■--, Dextran 70; —■—, Dextran 20;

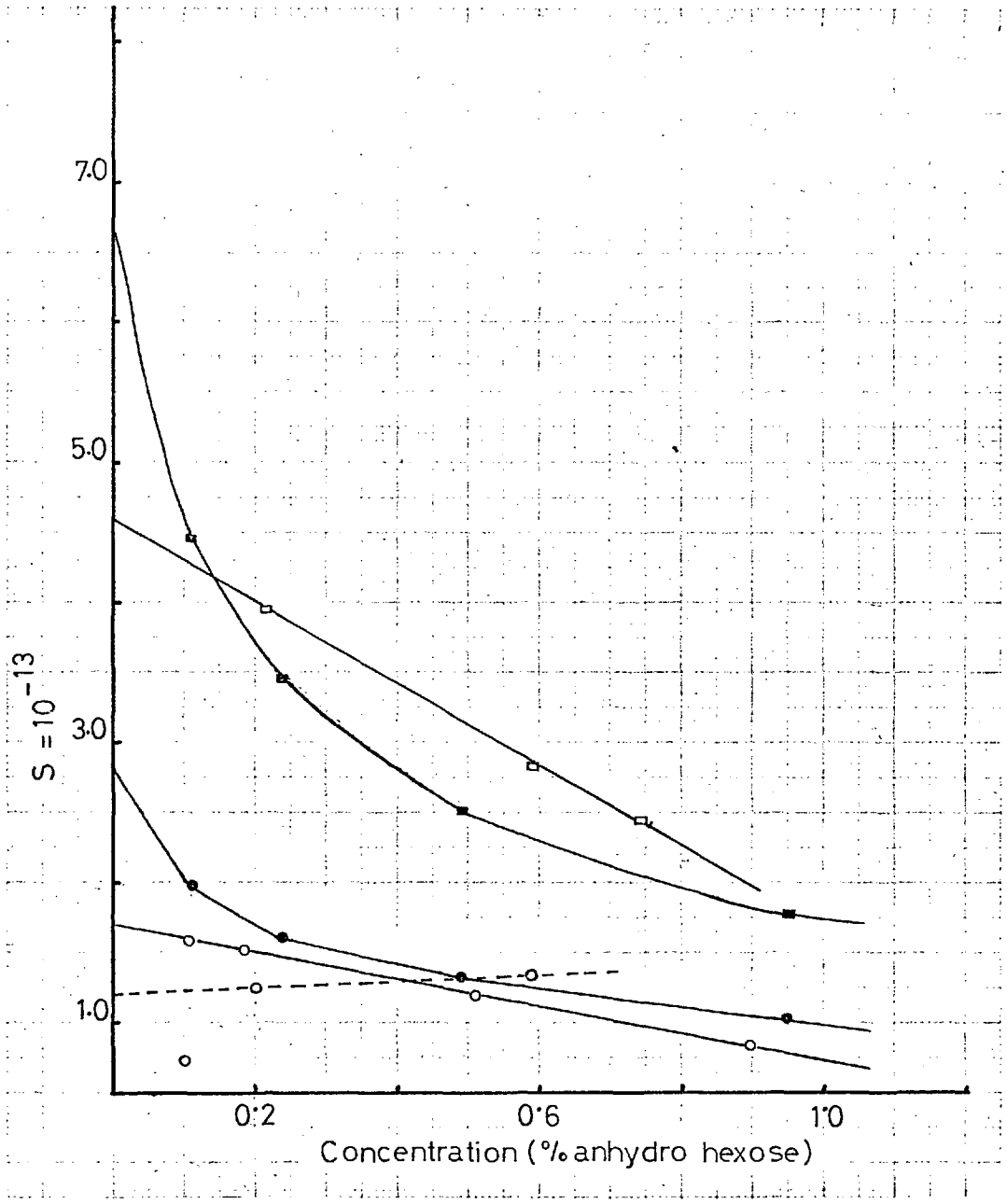


Fig. 5. S Values of Cell Wall Fractions at Different Concentrations.
Fraction 14FJ —●— , fast moving component;
—●—, slow moving component;
β-D-Glucan, Fraction 27K, —□— ; α-D-Glucan,
Fraction 27O, —○—; Smith degraded α-D-Glucan,
Fraction 27O, - - - ○ - - -.

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