POLYSACCHARIDE DEGRADATION

BY

MOULD ENZYMES

================================

Thesis for the Degree of Doctor of Philosophy
in
The University of Edinburgh

Presented by
MD. ABDUL MATIN BHUIYAN, M.Sc.(Dacca)

================================

Department of Applied Biochemistry
HERIOT-WATT COLLEGE
May, 1963
## CONTENTS

| Section I: Preliminary study of hemi-cellulase production by a strain of *Penicillium chrysogenum* | 22 |
| Section II: Comparative enzymolysis of barley $\beta$-glucan | 59 |
| Section III: Enzymolysis of araboxylan | 113 |
| General Discussion | 145 |
| Bibliography | 158 |
| Acknowledgements | 167 |
POLYSACCHARIDE DEGRADATION BY MOULD ENZYMES

GENERAL INTRODUCTION

The Substrates

The term hemicellulose was first used by Schulze (1891) for those substances which are present in plant cell-walls associated with cellulose, especially in lignified tissues, and it was originally restricted to substances soluble in alkali but not in water. These hemicelluloses yield primarily pentoses, hexoses and uronic acids on hydrolysis with mineral acids. The term hemicellulose, therefore, lacks precise chemical, structural and biological significance. Referring mainly to hemicelluloses from cereal sources, it may be noted that Tollens and Stone (1888) first isolated a substance of this type from brewers' spent grain and the product yielded arabinose and xylose on hydrolysis. Pentoses were also detected by Schulze (1892) in the hydrolysates of preparations from wheat and rye; Preece (1931), working with spent grains from barley malt, isolated along with a mainly hexosan product three principal substances, representing xylan, uronoxylan and urono-araban, which were similar to the hemicelluloses obtained from sawdust by
O'Dwyer (1923), who established the presence of uronic acid in such substances.

Early work on non-starchy hemicellulose-like water-soluble polysaccharides was carried out by O'Sullivan (1882), who isolated two products from barley grains. He named these two products α-amylan and β-amylan. The former name was given to a substance which was extracted with water at 40°C and the latter to that extractable at 20°C. As the substances isolated were later shown not to be related to starch, such naming was not appropriate.

Piratzky and Wiecha (1938) showed interest, after a long interval of fifty years, in O'Sullivan's materials which are now known as barley, or other cereal, gums, and they were able to precipitate these substances with Fehling's solution and alcohol. Their preparations were highly viscous in aqueous solution, and one of the gums gave only glucose on hydrolysis. The work included the detection of soluble pentosan materials in barley, malt, etc., and the fractionation of a polysaccharide into a series of fractions having increasing molecular weight and increasing viscosity.

In the field of fractionation technique, a major advance was made by Norris and Preece (1930). They used wheat bran as their starting
material. Pectic substances were removed by ammonium oxalate solution, and the lignin content of the material was reduced by alcoholic soda treatment. Alkaline extraction of the bran residue yielded a polysaccharide fraction A on acidification and two more fractions B and C were obtained from this acidified solution by adding acetone first to 33 per cent and then to 50 per cent concentration by volume. These three fractions were dissolved separately in alkaline solutions and A₁ and B₁ fractions were isolated with Fehling's solution and A₂, B₂ and C₂ fractions were isolated by adding acetone to the respective mother liquors. The polysaccharides could be separated from copper-complexes on acidification. After analysis of these fractions it was found that with the exception of B₂ fraction which was almost solely constituted by glucosan material, the fractions obtained were built up of either pentoses or urono-pentoses. Thus was provided a more detailed method for fractionating hemicelluloses.

This fractionation technique of Norris and Preece was criticised by Norman (1937). He claimed that the soda treatment at high temperature was not without effect upon the hemicelluloses. This claim was upheld by Preece (1941). Later Preece and Hobkirk (1954), omitting the
alcoholic soda treatment, isolated two types of hemicelluloses from barley, namely husk-type materials which gave low viscosity and high pentosan content, and endosperm-type materials which gave high viscosity and high glucosan content.

In 1929, Norman realised that there is no essential difference between the so-called plant gums and hemicelluloses, but he did not explain the different physical characters of these substances. The relationship of the cereal gums to hemicelluloses was put on a firm basis as a result of the work of Preece and Ashworth (1950), who were, to some extent, extending the work of Brown et al. (1906). The former group found that upon prolonged contact between the barley gums and barley enzymes, a point was reached when attempted precipitation, by normal means, failed. Nevertheless, they did obtain a similar water-soluble polysaccharide from malt in increased yield, and this was taken as indication that a cytolytic system of enzymes had converted certain initially insoluble hemicelluloses to water-soluble hemicelluloses. They pointed out that these substances have sufficient chemical resemblances to outweigh the unimportant solubility character. Preece then declared that the change from the insoluble form to the soluble
form could be brought about by the action which in its early stages gave results not dissimilar to the effects of dilute alkali; later he described in some detail the degradation of barley gums by barley enzymes both in its early and later stages (Preece and Hoggan, 1956).

To prevent the enzymic activity, Preece and Ashworth (1950) used 85% alcohol for refluxing the ground barley before extraction of gums with water at 40°C. By employing the Norris and Preece (1930) fractionation scheme, they obtained the gum fractions B2 and C2 in which the former mainly consisted of glucan accompanied by 5-10% pentosan. Meredith, Bass and Anderson (1951) reported the isolation of gums from barley, malt and wort and they obtained higher yields than those obtained by Preece and Ashworth (1950). They did not inactivate the enzymes and because of the co-precipitation of enzymes which was later shown by Anderson (1952), these gums were unstable in solution. However, the hydrolytic products were the same in both cases. The viscosities of solutions of such gums from barley were later shown by Meredith and Anderson (1955) to be very much less than corresponding solutions of alcohol-inactivated gum preparations, indicating a large amount of enzymic degradation. Though Meredith et al. (1953,
1955) started to use enzyme inactivation prior to extraction of gum in their later works, they used papain at different concentrations to make the gum protein free and obtained much degraded gum. Preece (1955) criticised the use of papain and pointed out that papain may contain enzymes capable of attacking hemicelluloses.

Preece and Mackenzie (1952) evolved a method of preparing water-soluble gums from barley, and this method eliminated major factors responsible for the degradation of gums. The enzymes of the ground barley were inactivated according to the method of Preece and Ashworth (1950). The dried grain was then extracted with water at 40°C. The aqueous extract was centrifuged and filtered through a bed of celite. The water-bright extract thus obtained was free from starch, and by the addition of 20% ammonium sulphate to the concentrated extract, a homogeneous fraction was obtained. By repeated resolution and reprecipitation with ammonium sulphate and dialysis and acetone precipitation, a laevorotatory glucan was obtained which was free from pentosan material. By using the same technique, Preece and Hobkirk (1954) and later Preece and MacDougall (1958) isolated an almost pure arabinofuranose from rye at the 40% ammonium sulphate concentration level. The structural
analyses of these polysaccharides were carried out by several workers, by chemical and enzymic methods which will be described later on.

Using the principle of ammonium sulphate fractionation, Preece and Hobkirk (1953) investigated five cereals and showed the contrasting water-soluble gum contents in these cereals. Rye was exceptionally rich in pentosan and contamination of $\beta$-glucan was small. Barley was the richest source of $\beta$-glucan with very little pentosan. From the solubility characters of these gums, they suggested that pure pentosan could be isolated from rye.

The Enzymes

Brown and Morris (1890) observed that, during germination, the cell-walls of barley endosperm gradually dissolved, resulting in considerable softening of tissues. They also showed that the cytolytic enzyme was different from the amylases by the demonstration that the cytolytic enzyme was inactivated by heating at 60°C whereas the amylases survived at least in part. Lüers and Volkämer (1928) separated a cytolytic enzyme which was called cytase from the amylase by selective adsorption on alumina at pH 5.0, and showed it likewise to be inactivated by heating at 60°C, and undergoing almost complete
inactivation in kilned malt. It was earlier thought that much of the endosperm cell-wall was composed of cellulose, and that therefore an important part of cytolytic action must be contributed by cellulase, but Lüers and Loibl (1923) found that the raw-fibre of barley composed largely of cellulose remained unchanged during malting. Brown and Morris (1890) and later Reinitzer (1897) were unable to detect any attack by malt extract on native wood cellulose. However, Pringsheim and Baur (1929) found that malt extract weakly attacked soluble-cellulose, and Zeise (1931) showed that malt extract degraded hydroxyethyl cellulose. Such work led Sandegren and Enebo (1952) to use ethylhydroxyethyl cellulose to determine the enzymic activity at different stages of malting. They found increased activity but slightly during germination. Enebo et al. (1953) found that the cellulase activity in the finished malt was about seven to ten times as great as that of the barley. They also partially purified the green malt enzyme by precipitation with ammonium sulphate and by acetone and showed that its activity was decreased on addition of β-linked sugars, cellobiose and lactose. These sugars were not hydrolysed and it was thought that these sugars combined with the enzyme in competition with the
substrate. The activity of the enzyme was also stimulated by glucose and they suggested that there were two enzymes; one was responsible for break-down of β-polysaccharides and the other was responsible for transglucosidase action.

Later, MacLeod and Napier (1959) employing an experimental pearling machine to the barley grain, obtained 6 fractions successively representing progressively deeper layers of the grain and after analysing the cellulose content of these fractions, they found that the whole cellulose present was accounted for by the husk, embryo and aleurone. They suggested that the cell walls of the starchy endosperm might be free from cellulose and measurements of cellulase activity, carried out to assess the potential ease of modification of a barley sample, was rather ill-directed. This clearly implies that the carbohydrate enzymes important in modification are the hemicellulases.

Preece and Ashworth (1950) by using mixed gums established that there are two types of enzyme activity present in barley; one type is cytoclastic, which reduces the viscosity of a gum solution without liberating much reducing sugar, while another type which has very little effect on the viscosity of the gum solution sets free relatively large amounts of reducing sugars.
The work was confirmed by the Canadian workers, Bass et al. (1952, 1953). They also used mixed gums as substrate. Preece et al. (1953) showed from their results that artificial substrates cannot be used to compare the enzyme activity as they are not susceptible to enzymes in the way natural substrates are. This was also confirmed by van Roey and Hupé (1955) and Anderson (1955).

Knowledge of cereal hemicellulose degradation could not at first go very far for lack of pure substrates. The isolation of pure $\beta$-glucan by Preece and MacKenzie (1952) from barley opened a new field for study of the enzymology of cereal enzymes and even for enzymes obtained from some other sources. Immediately after the isolation of pure $\beta$-glucan, the study of enzymolysis of $\beta$-glucan was undertaken by Preece and his school in Britain and Meredith and collaborators in Canada. Preece, Aitken and Dick (1954) were able to detect endo-$\beta$-glucanase and exo-$\beta$-glucanase systems in barley enzymes, the former being initially cytoclastic but becoming cytolytic, whereas the latter is always cytolytic. A cellobiase system was also detected. Bass and Meredith (1955, 1956) and Meredith and Anderson (1955) also continued the study of $\beta$-glucan enzymolysis and supported the information obtained by Preece and his school.
Preece and Hoggan (1956) then started more detailed work on \( \beta \)-glucanase system. They were also able to get more concentrated enzyme preparation from barley using citric-acid-phosphate buffer (pH 4.5) per extraction. Differential inactivation of this enzyme preparation using phenyl mercuric salts allowed exo-\( \beta \)-glucanase activity to be eliminated without destroying endo-\( \beta \)-glucanase. \( \beta \)-glucanase activities of enzyme preparations from different cereals were determined. The work was gradually applied to give knowledge of events in malting at different stages.

Following the study of the properties of \( \beta \)-glucanase systems by Preece and Hoggan (1956), Preece and Garg (1961) investigated further the structural implications of the types of oligosaccharide obtained from \( \beta \)-glucan by enzymolysis. Aspinall and Telfer (1954) had shown by chemical methods that, in the \( \beta \)-glucan molecule, anhydro-\( \beta \)-D-glucopyranose residues are joined by equal numbers of \( \beta \)-1,4- and \( \beta \)-1,3-linkages. Gilles et al. (1952) concluded from their work that these linkages are arranged alternately which was contrary to the detection of laminaritriose (enzymic hydrolysis) and cellotriose (acid hydrolysis) by Preece, Garg and Hoggan (1960) by chromatography. Preece and Hoggan (1956)
explained the composition of barley $\beta$-glucan on the assumption that the $\beta$-1,3- and $\beta$-1,4-linkages are each present in small groups and these groups may be randomly disposed throughout the molecule. There are three possibilities in the distribution of the two types of linkages in the molecule. These are (a) a regular alternation of the linkages; (b) blocks of $\beta$-1,3-linkages of moderate dimensions alternating with blocks of $\beta$-1,4; and (c) a more random arrangement of short and variable runs of $\beta$-1,4- and $\beta$-1,3-linkages. Possibility (a) was excluded from the work of Preece and Hoggan (1956), though possibility (b) was in accord with the results of Aitken et al. (1956); (c) seemed very likely from the work of Preece, Garg and Hoggan (1960). They showed that in the $\beta$-glucan molecule, sequences of up to at least three $\beta$-1,3-linkages must be present to account for the appearance of the oligosaccharides produced during enzymolysis which were characterised by chromatographic and electrophoretic methods. Preece and Garg (1961) using different substrates, compared enzymolysis of $\beta$-glucan with different types of enzymes and concluded that the barley enzyme preparation contained endo-1,4-ase, endo-1,3-ase, exo-1,4-ase, laminaribiose and a general $\beta$-glucosidase.
A water-soluble araboxylan from rye was isolated comparatively free from β-glucan by Preece and MacDougall (1958) using pancreatic α-amylase followed by the fractionation technique of Preece and Hobkirk (1953). By using this as substrate they compared the pentosanase activities of malt at different stages. They also compared the pentosanase activities of different cereals. Araboxylan enzymolysis revealed that arabinose appeared from the start at different rates with enzymes from all cereal sources, indicating the presence of arabinosidase activities in them. The enzyme preparations also contained exo-xylanase, endo-xylanase and xylobiase activities in different proportions from different sources. There were very close quantitative and physical resemblances between the endo-activities of β-glucanase and araboxylanase systems. Cereals rich in endo-β-glucanase were also rich in endo-xylanase. Both exo-β-glucanase and exo-xylanase systems were susceptible to heat and phenylmercuric nitrate inactivation, whereas both endo-β-glucanase and endo-xylanase were resistant to these effects. 

β-glucanase systems can also be obtained from sources other than cereals. Duncan, Manners and Ross (1956) investigated extracts of different sea-weeds, and found that these
extracts contained essentially similar complements of carbohydrates including α-glucosidase, β-glucosidase, amylase, β-1,3- and β-1,4-glucanase and xylanase. The carbohydrate complements were very similar to those of cereal extracts, but relative hydrolytic activities were much slower. They also showed that two distinct endo-type enzymes were present in the extracts of *Cladophora rupestris*; one was endo-β-1,3-glucanase which was inactivated after heating at 60°C whereas another enzyme, endo-β-1,4-glucanase was only partly inactivated.

Aitken et al. (1956) used cell-free culture filtrate of *Myrothecium verrucaria* to degrade β-glucan, and showed that cellobiose, laminaribiose and glucose were produced during enzymolysis. The culture filtrate had also laminarinase activity and that activity could be destroyed after heating the culture filtrate at 60°C for 10 min., but laminaribiose activity remained unimpaired. More recently Parrish et al. (1960) showed the activities of "laminarinase" from *Rhizopus arrhizus* and "cellulase" from a *Streptomyces* species on β-glucan. It was found that cellulase degraded β-glucan to a tri-saccharide and two tetrasaccharides, whereas laminarinase degraded β-glucan to a trisaccharide and a tetrasaccharide. The structures of the tri- and tetrasaccharides were different according to the sources. Reese and Mandels
(1959) made a survey of production of \( \beta-1,3 \)-glucanases in culture filtrates of fungi using different carbon sources. They found \( \beta-1,3 \)-glucanases in most of the culture filtrates of fungi and these were constitutive, whereas the cellulase, chitinase and xylanase were adaptive in nature. Very little is known about the occurrence of \( \beta \)-glucanase in bacteria except in \textit{Bacillus subtilis}. Moscatelli et al. (1961) showed that a commercial amylase preparation derived from \textit{B. subtilis} contained a hydrolysing enzyme that could degrade \( \beta \)-glucan. After purification of the enzyme, \( \beta \)-glucanase was heat stable and could produce chiefly tri-saccharide and tetrasaccharides with some glucose, laminaribiose and laminaritriose but no cellobiose.

Several investigations have been made of pentosanase systems from sources other than cereals. Ehrenstein (1926) investigated the decomposition of wheat-xylan by an enzyme preparation from snail and found that the initial degradation was followed by a much slower decomposition stage. Duncan et al. (1956) have shown the presence of a xylanase system in marine algae. Voss and Butter (1938) investigated decomposition of xylans by using enzymes from snails, fungi and barley malts, and xylose and
uralic-acid were detected in the products of hydrolysis. Walker and Hopgood (1961) purified an enzyme from sheep rumen microflora that hydrolysed largely insoluble preparations of wheat-hay hemicellulose to xylose, xylobiose, xylotriose and higher oligosaccharides, together with glucose and arabinose; it did not hydrolyse starch or cellulose. Recently Bailey, Clarke and Wright (1962) reported the extraction of hemicellulase from a rumen ciliate, Epidinium ecandatum, in water after the disruption of cells. The enzymes involved were different from those of Butyrivibrio and Bacteroides amylogenes isolated by Howard et al. (1960). They hydrolysed wheat-flour xylan with the initial release of arabinose, without much accompanying xylose or xylobiose. Howard (1957) observed similar early release of arabinose from wheat-flour xylan by rumen bacteria. Sørensen (1953) investigated the decomposition of xylans by soil micro-organisms. Simpson (1954) made a survey of micro-organisms for the production of enzymes that attack the pentosans of wheat flour. The water-soluble pentosan substrate contained 78 to 82% pentosan and 0.5-1.0% protein. Several fungi, Streptomyces and bacteria were found to produce extracellular enzymes that hydrolysed the pentosan. They also found that the fungi and
Streptomyces possessed adaptive pentosanases, whereas 40% of the active bacteria had constitutive pentosanases. Simpson (1956) also reported the production of pentosanases by Bacillus pumilus and B. subtilis which were used to hydrolyse the pentosans of "squeegee starch" of wheat. Gascoigne and Gascoigne (1960) investigated the xylanase activity of 13 fungi and found Fusarium roseum as best producer of xylanase. The extracellular enzyme produced by the organism was fractionated by ethanol precipitation, and one of the fractions had no action on cellulose; xylanase; transpentosylation activities were also studied. Structural analyses of xylans from different sources have been made by different workers (Whitaker, 1953; Bishop, 1956; Aspinall et al., 1960) by using enzymes from microbial sources which will be described later.

Such information as is available on the structure of β-glucan can be described as follows. Preece and Garg (1961) concluded from the enzymolysis of barley β-glucan and from the knowledge of the oligosaccharides (Preece, Garg and Hoggan, 1960) characterised by chromatographic and electrophoretic methods that the β-glucan molecule of barley, already known to consist of a chain of D-glucose residues containing both β-1,3- and β-1,4-linkages, has
these linkages disposed at random with short and variable runs in the molecule. Peat, Whelan and Roberts (1957) reported the characterisation of cellobiose, laminaribiose, cellotriose and two trisaccharides containing mixed linkages as products of partial acid hydrolysis of oat β-glucan but they obtained no laminaritriose and proposed a structure in which isolated β-1,3-linked and D-glucopyranose residues were separated by two or three β-1,4-linked units. Parrish, Perlín and Reese (1960) degraded both oat and barley β-glucans with "cellulase" and "laminarinase" and after characterisation of oligosaccharides supported the structure earlier proposed by Peat et al. Recently Smith and Sorger-Domenigg (1960) by degrading the periodate-oxidised oat β-glucan followed by reduction and mild acid hydrolysis and characterisation of the oligosaccharides from the products, showed that the polysaccharide contained blocks of two and three contiguous β-1,3-linked D-glucose residues in addition to isolated units. This was in accord with the proposal of Preece and Garg (1961).

Preece and MacDougall (1958) used araboxylan from rye for enzymolyses with different cereal enzymes. They found that the enzymes preferentially removed arabofuranose from side-chains.
They characterised the oligosaccharides produced during enzymic break-down, and these included xylolbiose, xylotriose, xylotetraose and higher oligosaccharides with mixed arabinose and xylose residues. They did not suggest the disposition of arabinose residues in the xylose linked chain. From the chemical and enzymic degradation, it has been shown that the arabinoxylan molecule is a chain composed of D-xylose residues linked with \( \beta-1,4 \)-linkages in which L-arabinofuranose residues are linked as side chains by \( 1,3 \)- and \( 1,2 \)-linkages to the xylose residues. The \( 1,3 \)-linked side chains are predominant. Little is known about the disposition of these side-chains. Ewald and Perlin (1959) degraded periodate-oxidised wheat-flour arabinoxylan by modified Barry's method and also degraded periodate-oxidised rye-flour arabinoxylan by Smith's method. After the isolation of degraded products with one, two and three xylose units, it was concluded that in these polysaccharides, side-chains are attached to isolated D-xylose residues and to adjacent D-xylose residues, less frequently to three, but not four or more contiguous D-xylose residues along the chain. More similar experiments are needed to establish this conclusion firmly.

From this it is evident that the fine
structure of these polysaccharides, \( \beta \)-glucan and arabinoxylan are not yet known conclusively. More work is needed for this purpose and this can be done in various ways. The use of enzymes in structural analysis of polysaccharides has already been fruitful. The structure found by chemical methods can be substantiated by enzymic methods, and the use of different enzymes for the degradation of the same substrate, not only provides knowledge for the structure of the substrate, but also the comparative properties of the enzymes used for this purpose.

It is known that the process of \( \beta \)-glucan degradation by cereal enzymes is not smooth; a wide variety of oligosaccharides is produced together with abundant glucose, leaving a resistant fraction of intermediate complexity (Preece, Garg and Hoggan, 1960). It is also known that the barley enzymes contain a \( \beta \)-glucanase system consisting of at least endo-\( \beta \)-1,3-ase, endo-\( \beta \)-1,4-ase, exo-\( \beta \)-1,4-ase, laminaribiase and a general \( \beta \)-glucosidase, and other cereal enzymes contain apparently the same complements but in different proportions for which the immediate patterns of \( \beta \)-glucan breakdown are different (Preece and Hoggan, 1956; Preece and Garg, 1961). The moulds are a well-recognised source of hemicellulases, and it
becomes of interest to compare the mode of degradation by mould and barley enzymes under identical conditions.
SECTION I
PRELIMINARY STUDY OF HEMICELLULASE PRODUCTION
BY A STRAIN OF Penicillium chrysogenum

INTRODUCTION

Carbon compounds, especially carbohydrates, serve two essential functions in the metabolism of fungi, as in all heterotrophic organisms. Firstly they are used for the synthesis of the compounds which go to build up the living cell—protein, nucleic acids, cell-wall materials, reserve foods, etc. In a typical fungus about 50 per cent of the dry weight is carbon. Secondly, the sole source of appreciable amounts of energy is the oxidation of carbon compounds, which may account for half or more of the carbon supplied to a culture.

Knowledge of carbon metabolism, therefore, is very important for an understanding of the physiology of fungi. The results required for confident generalisation are still lacking and many of the data available are based on experiments open to criticism on the ground of method.

The concept of utilisation may have more than one meaning. The most usual way of undertaking an experiment involves placing a small inoculum in a medium containing one major source of carbon and all other necessary growth factors; the organism must initiate and maintain growth at
the expense of the test compound. A second meaning of utilisation is the ability of the viable active mycelium to make further growth at the expense of test compounds. Here, the design of the experiments usually involves providing a small amount of a utilisable carbon source and measuring the additional, if any, growth obtained from a second compound. The materials which are not utilised as single source are often utilised in this type of mixed source experiment; examples which are available for particular fungi include lactose (Moyer and Coghill, 1946), sugar alcohols (Perlman, 1948), and cellobiose (Norkrans, 1950).

In the design of the first type of experiment, no other carbon compounds should be available as sources of carbon; this includes amino acids which would possibly be utilised as source of both carbon and nitrogen.

Of the many possible errors, the most important is associated with autoclaving. The initial pH should be determined after sterilisation to guard against the possibility. Compounds formed from sugars during autoclaving are known to inhibit growth of both bacteria and fungi (Barner and Cantino, 1952; Lilly and Barnett, 1953) although other workers have described the production of growth stimulants
(Cheldelin and King, 1953; Lein and Appleby, 1951). Break-down is more extensive when the sugar is in contact with phosphate (Englis and Hanahan, 1945) or with amino acids (McKeen, 1956). Keto-hexoses, e.g. fructose and sorbose, break down to 5-hydroxymethyl furfural more rapidly than do the aldohexoses (Newth, 1951), and furfural formation from autoclaved xylose is sufficient to inhibit bacterial growth (Lockwood and Nelson, 1946). Oligosaccharides may be partially hydrolysed during autoclaving, especially at low pH (Ball, 1953; Bretzloff, 1954), and probably many claims of slight growth on oligosaccharides reflect merely the partial hydrolysis of a non-utilisable sugar to a utilisable monosaccharide. To prevent such errors, carbohydrates are normally autoclaved separately, and added aseptically to the cold standard medium.

**Hexoses:** Of the hexoses, D-glucose, fructose and mannose are biologically the most important, and are utilised for growth by virtually all cultivable fungi. In some cases growth on fructose and mannose is much poorer, as is shown by the work of Sistrom and Machlis (1955) on *Allomyces macrogyrus*. However, on prolonged incubation, the final growth was comparable to that of glucose. It was also shown that
addition of a small amount of glucose to a medium in which the main carbon source was mannose or fructose permitted normal growth without delay. These observations are consistent with the hypothesis that this fungus, and by implication perhaps others, utilises fructose and mannose only under conditions which permit or encourage the formation of an "adaptive" or induced enzyme, although other observations remain to be reconciled with this hypothesis. Therefore, the utilisation of a particular sugar is dependent on an enzyme system which is constitutive in some organisms and inducible in others; strains of a given species may fall into different groups in this regard.

Pentoses: D-xylose is the most generally utilisable of the pentoses, and has been reported to be superior to glucose for some organisms (Beckman et al., 1953). Some fungi and actinomycetes grow poorly or not at all with xylose, and species within the same genus may differ markedly (Steinberg, 1939; Tamiya, 1932). Xylose is often a good source of carbon for fungi, and other pentoses appear in general to be poor sources of carbon or not utilisable at all.

Oligosaccharides: Other sugars important in the nutrition of fungi include five oligosaccharides
- maltose, cellobiose, trehalose, sucrose and lactose - and one trisaccharide, raffinose. With regard to utilisation of these oligosaccharides, two general problems are relevant at this point: (1) the enzymic basis of disaccharide utilisation, and (2) adaptive growth with oligosaccharides. Most of the fungi grow on maltose, though there are a few exceptions. Cellobiose, like maltose, is composed of two glucose residues, but the linkage is of the $\beta$-configuration. Utilisation of cellobiose is like that of maltose, and this reflects the general occurrence of $\beta$-glucosidase in fungi. Sucrose is generally a good source of carbon for fungi but is not nearly so universally available as maltose. Lactose, i.e. $\beta$-galactosidoglucose, is used by few fungi. Raffinose attacked at one linkage yields galactose and sucrose, but attacked at the other yields fructose and melibiose. From the study of Lilly and Barnett (1953), it would appear that the majority of the fungi utilise raffinose but non-utilisation is not uncommon.

**Polysaccharides:** Polysaccharides are polymers of sugars and the number of units may be as small as 10; the natural polysaccharides, however, have as a rule very high molecular weights and may contain several thousand saccharide units. There are two types of polysaccharides - reserve
polysaccharides, e.g. starch, glycogen and inulin, and structural polysaccharides, e.g. cellulose, pectic materials, and chitin.

Starch is often an excellent source of carbon for fungi, even for fastidious forms. That some like *Penicillium digitatum* do not grow with starch finds its explanation in an inability to form amylase (Holden, 1950). Most investigations have been made with soluble starch, or dextrins which are modified starch, and different results are obtained with raw starch. Utilisation of glycogen is similar to that of starch.

Cellulose, a structural polysaccharide, is the largest reservoir of biologically utilisable carbon in nature, and probably fungi play a major part in its decomposition. The literature on the utilisation of cellulose by fungi is very large, but unfortunately inadequate criteria make it difficult to evaluate this work. It was shown that visual estimation of growth on filter paper was not a good index of growth or cellulolytic capacity (Marsh and Bollenbacher, 1949), and growth on reprecipitated cellulose was equally unreliable (Norman, 1931).

Utilisation of mixed carbon sources may be explained in part by the induction of new enzymes. The effect of a small amount of glucose in permitting rapid growth on fructose or
mannose, sugars which by themselves support growth only after a long lag, may be explained by postulating that the glucose allows sufficient growth so that an enzyme essential to fructose or mannose utilisation can be synthesised. This enzyme is produced only in the presence of the "inducing" sugar: fructose or mannose. Extensive data on the utilisation of mixed carbon sources have been reported by Lilly and Barnett (1953). The results of Steinberg (1939) on the utilisation of mixed carbon sources by Aspergillus niger are not satisfactorily explained by the hypothesis of enzyme induction. Here a mixture of two unutilisable compounds supports good growth. It is clear from these results that the interaction of different carbon sources requires further study.

The industrial application of fungi is increasing very rapidly and their ability to attack a large variety of substrates is being increasingly exploited. Nordstrom and Hutlin (1948) prepared enzymes, previously not recorded, by placing thirty species of moulds in contact with dextran, a glucose polymer containing $\alpha-1,6$-linkages. Penicillium lilacinum, P. funiculosum, and Verticillium coccorum were able to hydrolyse the polymer after exposure for one week. Amylase and sucrase were not produced
in media containing dextran, while on starch only amylase was elaborated. A similar procedure was followed by Gottlieb, Day and Pelczar (1949, 1950) in obtaining the cultures of *Polyporus abietinus* and *Poria subacida*, able to utilise lignin. Originally grown in a mixture of lignin and glucose, successive transfers to media increasingly dilute with respect to glucose, eventually resulted in cultures dissimilating lignin. Foster et al. (1946) found that *P. chrysogenum* grown previously on glucose required 50 hours to ferment lactose, while lactose-grown cells accomplished the fermentation in 26 hours. Nielsen and Nilsson (1950) have demonstrated the practicability of xylose as a substrate for fat formation in *Rhodotorula gracilis*. Initially, xylose was fermented at 25 per cent of the rate of glucose, but continuous transfer to xylose increased the rate to 70 per cent. The economic coefficient (gram cells formed per gram sugar utilised) eventually attained the same value for both sugars. According to Wasserman and Gould (1947), a strain of *Neurospora crassa* fermented glucose more rapidly than fructose, but failed to produce sucrase in the presence of glucose. Sugars yielding glucose also inhibit sucrase production, while fructose and derivatives enhance production.
Goodman (1950) has followed the changes in amylase production in fungi responsible for the deterioration of stored grain. Mycetial preparations of *Aspergillus flavus*, *A. niger*, and *Penicillium notatum* did not possess amylase activity if grown on glucose, but on starch the enzyme content was high at the end of five days. Subsequent loss of activity may be attributable to dilution of the enzyme by inert cell-wall material. Exocellular production appeared to be limited to a period between five and eleven days. Similar results were obtained with lipase.

The growth characters and enzymic activities of bacteria grown in different environments are well recognised, and have been reviewed by Karström (1938). Karström himself studied the ability of bacteria grown on a given sugar to ferment a variety of sugars. *Escherichia coli* grown on maltose was able to ferment maltose but not lactose, and vice versa. Karström postulated that growth on a given carbon source could cause formation of a special enzyme capable of fermenting that carbon source. These enzymes were named "adaptive enzymes", but now they are often called "inducible enzymes".

Karström found that some carbon sources such as glucose were fermented no matter what medium was used for growth of the bacteria. Enzymes
involved in this sort of fermentation were named "constitutive", to differentiate them from the inducible ones. However, even glucose fermentation is not a completely fixed property in the case of certain bacteria, but varies at least fivefold depending on prior growth conditions (Stephenson and Gale, 1937). Constitutivity appeared to be an idealised extreme response of enzyme formation to nutritional conditions.

The early observed striking changes in enzyme formation and the ease of investigation of this phenomenon led to numerous investigations and to hypotheses designed to provide an explanation for the induction. As early as 1938, a mass induction hypothesis involving enzyme-substrate combination was proposed (Yudkin, 1938). The experiments on mechanisms of enzyme induction were reviewed recently by Pollock (1959) and Halvorson (1960).

Gale (1943) and his associates carried out numerous experiments on effects of environment on enzyme formation. Induction by substrates was described for a dozen enzymes of *E. coli*. The effect of pH on the production of these enzymes was also determined. Certain amino acid decarboxylases were formed only in acid media; their reactions tend to increase the pH, bringing it toward the optimal value for growth.
Conversely, amino acid deaminases were produced only in alkaline media; these enzymes tended to decrease the pH.

Certain other enzymes that destroy toxic products of metabolism are formed in very different amounts at different pH values. For example, alcohol and formic dehydrogenases are produced in the smallest amounts at approximately neutral pH where their specific activities are greatest. The resulting total activity is approximately constant over a wide range (Gale, 1943). Thus approximately 2.5 times as much alcohol dehydrogenase is formed at pH 5 as at pH 8, but the specific activity of the enzyme is only about one-fourth as great at the former pH as at the latter. Gale (1943) suggested that by this means the bacteria maintained an enzyme activity adequate to remove the toxic metabolites. The mechanism, possibly a stimulation of enzyme formation by the toxic metabolites, remains undetermined.

The effects of temperature on enzyme formation have not been extensively studied. The subject was reviewed by Knox (1953). The interesting observation is that Salmonella paratyphi B formed adaptive tetrathionate reductase below 37°C, but not at 44°C. This temperature effect, unlike most, was specific because the
activity of the preformed enzyme and the growth of the bacteria adapted to tetrathionate were not abolished at 44°C. The effect is usually attributed to a temperature-sensitive enzyme-forming system.

In the investigation of enzyme induction, numerous studies have been made on β-galactosidase. Now it is believed that the enzyme and its induction are under the control of specific genes; that it is induced by some compounds with almost no affinity for the enzyme and is not induced by other compounds which have affinity for the enzyme; that the enzyme is synthesised completely from amino acids almost at once after inducer is added, and synthesis stops almost as soon as the inducer is removed. Conditions, defined as "gratuitous", have been devised under which the induction of the enzyme can be studied without influencing the over-all metabolism of the bacteria. This work has been summarised in recent reviews by Cohn (1957) and Monod (1958).

Sheinen and Crocker (1961) tried to explain the nature of the actual or immediate inducer in an investigation of the concurrent induction of α-galactosidase and β-galactosidase in E. coli B. So much is known about induction of β-galactosidase of E. coli that it has become a sort of yardstick against which to compare data
obtained with other inducible enzymes.

Whether all or even most inducible enzymes behave in this way is open to question, but there is no reason to believe that a single general solution is available, applicable to all organisms. Other carbohydrates have been much less studied, and the present desire is to investigate both extracellular (culture filtrate) and intracellular (mycelial) enzymes with special reference to utilisation of sugars and hemicelluloses, because carbon sources play an important role in hemicellulase production. It is comparatively difficult to isolate enzymes from the culture filtrate than from the mycelium unless the concentration in culture filtrate is very high. Yield of mycelium does not always go parallel with the concentration of enzymes in the culture filtrate. Accordingly, activity of the culture filtrate was followed along with the weight of the mycelium.

Further, since present interest is in both hexosanases and pentosanases, a wide variety of sugar sources was investigated, including simple hexoses and pentoses, as well as certain oligo- and polysaccharides. In this way it has been possible to learn something of the peculiarities of the individual carbohydrates, not only for support of growth, but also in relation to enzyme induction.
EXPERIMENTAL

Materials and Methods

Isolation of fungi from air-borne spores. - Czapek-Dox medium, having the following composition, was used in this phase of the work:

- Sodium nitrate: 3.0 g.
- Potassium dihydrogen phosphate: 1.0 g.
- MgSO₄·7H₂O: 0.5 g.
- Potassium chloride: 0.5 g.
- FeSO₄·7H₂O: 0.01 g.
- Water (glass distilled): to 1 litre

The medium, at pH 4.3, was brought to 1.0% (w/v) in respect of glucose, and was solidified with 2.0% agar; initially sterile plates were exposed for 5 min. in the laboratory. After closing, the plates were incubated at 25°C for one week, by which time numerous mould colonies had developed. Spores from each of 20 well-separated colonies were transferred individually to 20 slants of "Sabouraud agar (maltose)" supplied by Oxo, Ltd. All the organisms grew well in this medium and each of them was purified on the Sabouraud medium by using the spore-dilution method. The pure cultures were preserved on slants for further examination.
Preparation of β-glucan from barley. — The method of Preece and MacKenzie (1952) was followed for the preparation of β-glucan. Barley (Ymer) ground with the hand-mill was extracted thrice, with 85% boiling aqueous alcohol, each time for 30 min. and with occasional stirring. The final residue, after air-drying, was extracted thrice, each time with 3 times its own weight of water and for 30 min. with stirring. Muslin filtrations were used to separate the extracts. The combined extracts were centrifuged, passed through a bed of celite and concentrated to small volume. β-glucan was precipitated with ammonium sulphate added to 20% (i.e. 20 g. per 100 ml. of extract), and was purified by repeated resolution and alternate precipitation with ammonium sulphate and acetone. Finally, the redissolved material was dialysed for 2 days against running water (thymol as antiseptic), precipitated with acetone and taken to dryness by repeated washing with acetone; drying was completed in the desiccator.

Preparation of arabinoxylan from rye. — The method will be described in Section III.
Liquid culture for assessing extracellular $\beta$-glucanase

Preparation of inoculum. - The inocula for this survey were prepared from the spores of the cultures grown on Sabouraud agar (maltose) for at least 7 days at 25°C. To inoculate a known number of spores to a culture medium, a uniform suspension of spores was necessary. As the spores could not be dispersed uniformly in water, a Squez (Domestos Ltd., SP 798,069) solution (0.3 ml./l litre) was used. A well sporulated culture of the organism was flooded with 5 ml. of the sterile Squez solution, and the spores were mixed with the solution by gentle tapping. The spore suspension was then transferred to a 100 ml. Erlenmeyer flask containing 20 ml. of sterile Squez solution and mixed well by revolving the flask alternately clockwise and anticlockwise for some time. Counting of the spores in this suspension was done by using the haemocytometer. A known volume of the suspension representing a known number of spores was then transferred to another flask containing 20 ml. of the sterile Squez solution so that the final suspension contained approximately a million spores per ml.

Liquid culture. - Liquid Czapek-Dox medium was used under three sets of conditions: (a) with
added glucose (1.0%), (b) with added β-glucan (0.2%), and (c) with added glucose (1.0%) and β-glucan (0.2%). Carbohydrate solution (triple strength) and basal medium (triple strength) were sterilised separately and combined aseptically when cold. The carbohydrate solution was sterilised separately to prevent any degradation, and the sterilised water was added aseptically to the medium where necessary to give the requisite strength of the medium. In each 100 ml. serum-meyer flask, 30 ml. of medium was inoculated with a 0.5 ml. portion of the standard spore suspension, and the flasks with different cultures were incubated at 25°C for 14 days. After incubation, approximately 6 ml. of each culture filtrate was withdrawn and filtered; filtrates were stored in the refrigerator.

β-glucanase activity. — The method of Preece and Aitken (1953) was used for the determination of extracellular β-glucanase activity. The reaction mixture consisted of 8.0 ml. 0.6875% (w/v) β-glucan solution, 1.0 ml. acetate buffer (pH 5.0) and 2.0 ml. of culture filtrate. All the solutions were brought to 25°C before mixing. Times of flow in the viscometer were noted at intervals over a period of 90 min. From the results, specific viscosities were obtained;
from these, reciprocals of specific viscosities were calculated. The increase in reciprocal specific viscosity caused by 2.0 ml. of culture filtrate per hour gave the required arbitrary measure of $\beta$-glucanase activity. Where the increase in reciprocal specific viscosity exceeded 0.2, the filtrate was diluted to give change below this value, and the activities were then calculated to the original filtrate.

The culture filtrates of most of the mould strains tested in this way – strains of Aspergillus and Penicillium – proved to be without substantial $\beta$-glucanase activity, but two strains of Penicillium chrysogenum (strains HW 3 and HW 18) appeared promising, but only strain HW 3 was taken for further study.

**Growth, Enzyme Activity and Carbohydrate source**

**Influence of different carbohydrates.** – The Penicillium strain HW 3 was grown in Czapek-Dox media as in the previous section, using the different carbohydrates shown in Table I. All carbohydrates were in 1.0% concentration, except $\beta$-glucan and araboxylan which were 0.2%. $\beta$-glucanase activity was determined in each filtrate as before. Additionally, the mycelial mats were filtered off in each case, washed well with
water, blotted between filter papers, and dried in vacuo at 40°C to constant weight. Growth and β-glucanase activities are shown in Table I, where are shown also the arabinofuranosidase activities determined by the viscometric method of Preese and MacDougall (1958).

It was observed from the results in Table I that a wide variety of results could be obtained by using different carbohydrate sources; good growth was not parallel with high total β-glucanase activity, and high β-glucanase activity was not necessarily accompanied by high arabinofuranosidase activity. It was interesting to note that pentoses and pentosan gave good to moderate β-glucanase activity together with high arabinofuranosidase activity, whereas hexoses and β-glucan gave good to moderate β-glucanase activity with little or no arabinofuranosidase activity. Sucrose gave little activity of either type. It appeared that hemicellulase preparations containing β-glucanase but devoid of arabinofuranosidase could be directly obtained, but the converse would not be possible though both could be obtained together.

It was an ultimate aim of this investigation to study both extracellular (filtrate) and intracellular (mycelial) enzymes of this mould, and it was thought that, at this early stage, an enzyme
preparation having both types of activity would be suitable for further study. It was true that much more attention was given to extracellular enzymes, but there was borne in mind the above consideration; accordingly growth conditions were sought which would not only give high extracellular activity but also a satisfactory weight of mycelium. Moreover, it was time-consuming to prepare sufficient hemicelluloses to use in culture media and it was hoped to avoid using them for this purpose. Accordingly, culture media containing xylose were considered most suitable, though, for comparison, experiments were carried out initially with hemicelluloses paralleled by experiments with glucose.

**Carbohydrate concentration and extracellular \( \beta \)-glucanase.** - It was known that growth and \( \beta \)-glucanase activity production by strain HW 3, did not run together and for this, a more detailed examination was made for the interaction of different carbohydrates. The general method was as before. In two series of experiments, glucose and xylose were added in increasing concentrations \((0.0 - 1.0\%, \text{ w/v})\) to a standard growth medium containing \(0.2\% \text{ (w/v)}\) of \( \beta \)-glucan (Table II), while in another two series (Table III) the sugar concentrations were kept at \(1.0\%\),
but with increasing concentrations of $\beta$-glucan. Further, in another two series (Table IV) of experiments, xylose or glucose was used as sole source of carbon at increasing concentrations (0.4 - 2.0%). The results of Tables II-IV are shown graphically in Figs. 1-6.

Growth. - Study of the growth of the organism was not at this stage of much importance, but the results were noted since they might be useful for further detailed investigation. In Fig. 1, the lines relating mycelial dry weight to sugar concentration are almost parallel, whether or not $\beta$-glucan is present, and the slopes are consistent with a 1.0% concentration of either sugar leading to the production of approximately 80 mg. of mycelium. When the lines are extrapolated from 0.4 - 2.0% to zero for sugar alone, they do not pass through the origin; the first 0.4% of sugar gives some 20 mg. of mycelium more than would be expected. The reason for this is not clear.

When $\beta$-glucan is added to 1.0% sugar, the results (Fig. 5) are much less regular, but addition to xylose gives less growth response than addition to glucose. Though results seem to be interesting in terms of metabolic relationship, it would not be useful to discuss them here.
It may be noted here that close reproduction of results for growth experiments is very difficult to achieve; thus, there may be compared the lines (Fig. 1) for glucose with $\beta$-glucan and xylose with $\beta$-glucan, both of which would be expected to start at the same point. Such differences may be due to the difficulty of precise control of the numbers of spores used for inoculation, but probably more to the ages of cultures from which inoculum is taken. Though some of the absolute values are on occasion suspect from such causes, the same cannot be true for the patterns of behaviour which have always been consistent for xylose and glucose. A most important conclusion can be reached without any doubt: that for the growth of this organism, as measured by increase in mycelial dry weight, xylose is at least as efficient as glucose as carbohydrate source. Another pentose, arabinose, gives growth of the same order (Table I), though this has not been investigated in detail.

**Development of extracellular $\beta$-glucanase.** - It is clear that more extracellular activity appears in the filtrate with a given concentration of xylose than with the same concentration of glucose (Fig. 2). Since this activity was
determined in the filtrate, it may be that some of the activity is held at the surface of the mycelium. Since the mycelium weights are not dissimilar for the two sugars, errors due to this cause may be expected to affect both sets of results equally, and the conclusion that xylose is the more effective remains valid.

When a fixed weight of $\beta$-glucan is present in the culture medium and glucose concentration increases in the same medium, $\beta$-glucanase production becomes more efficient than with a fixed weight of $\beta$-glucan with increasing xylose concentration (Fig. 2), an effect which cannot be ascribed to greater development of mycelium. Moreover, $\beta$-glucanase production in the $\beta$-glucan-glucose mixture is far greater than with glucose alone; the stimulation in the $\beta$-glucan-xylose mixture is relatively small, and tends to disappear as the xylose concentration increases.

When sugar concentration is constant (1.0%) and $\beta$-glucan is added, the sugars again differ; with increasing concentration of $\beta$-glucan (Table III, Fig. 6) total activity with xylose rises to a maximum at 0.1% $\beta$-glucan and then falls sharply, whereas with glucose the activity has perhaps reached the maximum figure (similar to the xylose maximum) only at 0.2%.

Regularities are also seen if the production
of extracellular $\beta$-glucanase per mg. of mycelium is considered and if this production is plotted against sugar concentration (Fig. 3) or mycelial weight (Fig. 4). In the absence of $\beta$-glucan, the fall in activity per mg. shows an inverse and substantially linear relationship with sugar concentration and growth. In presence of 0.2% $\beta$-glucan, the activity per mg., initially high, falls at a decreasing rate with increase in sugar concentration. The respective lines tend to merge with those for sugar alone (Fig. 3), and this might occur at a concentration near 1.0% for xylose and at a substantially higher concentration for glucose. This behaviour can be explained by suggesting that a 1.0% xylose concentration would be as efficient as 1.0% xylose plus 0.2% $\beta$-glucan concentration, though 1.0% glucose concentration would be much less efficient in $\beta$-glucanase production than 1.0% glucose plus 0.2% $\beta$-glucan concentration. However, under the conditions investigated, maximal efficiency per mg. is attained in presence of $\beta$-glucan and minimal sugar concentrations; substantial total activity can be obtained with xylose alone or with xylose or glucose plus $\beta$-glucan, though glucose alone gives only moderate activity and even this falls as sugar concentration increases.
Thus, the *Penicillium* strain HW 3 can grow in liquid media in presence of a wide variety of sugars like glucose, sucrose and xylose, etc., and confirming the previous finding $\beta$-glucanase is present in all media but in different concentration. The production of $\beta$-glucanase can be stimulated again by a wide variety of carbohydrates like $\beta$-glucan, araboxylan, cellobiose and xylose, etc. The production of araboxylanase along with $\beta$-glucanase is only caused in presence of pentoses and pentosan but $\beta$-glucanase can be produced in absence of araboxylanase.

**Preparation of Hemicellulases from Culture Filtrate and Mycelium**

**Method of culture.** - It is clear that a culture filtrate highly active in respect of $\beta$-glucanase and araboxylanase can be obtained by growth of strain HW 3 on Czapek-Dox medium containing 1.0% of xylose. Possibly use of 1.0% $\beta$-glucan would give similar or better $\beta$-glucanase activity, though here there would be no pentosanase; further, but such a medium would be uneconomic and would be highly viscous, though this latter disadvantage should disappear during growth. Cellobiose gives good $\beta$-glucanase but no pentosanase. As it has already been said that the present desire is to investigate both extra-
cellular and intracellular enzymes, the choice of carbohydrate falls on xylose, since this gives the activities desired in the culture medium and also excellent mycelial growth.

The growth medium used has already been described, addition of 1.0% (w/v) of xylose being made as sole carbohydrate source. For large-scale production of enzymes, 20 large flasks were used and in each flask 150 ml. of medium was placed and inoculated with 1.5 ml. portions of spore suspension (approx. 1,500,000 spores). After 14 days incubation at 25°C, the liquid cultures were in each case filtered on paper at the pump, filtrate and mycelium being thereafter worked up separately.

Preparation of extracellular enzymes. - The culture filtrate was saturated with ammonium sulphate at room temperature and the precipitate was separated by centrifugation. The precipitate was then dissolved in the minimum volume of distilled water and dialysed for two days against running water using thymol as antiseptic. After dialysis, the solution was brought to room temperature and the precipitate formed during dialysis was filtered off and discarded. To the clear filtrate, 4 volumes of cooled acetone was added and the mixture was left at room temperature
for about an hour before centrifuging off the precipitated enzyme. The precipitate was then taken to dryness by repeated washing with acetone at room temperature, completing the drying in a desiccator over calcium chloride.

Preparation of intracellular enzymes. - The mycelium was well washed with cold water and was then macerated with 350 ml. of 0.6% sodium chloride solution in a top-drive macerator at first for 3 min., and it was then cooled in a refrigerator before macerating again for another 3 minutes. The macerate was centrifuged and the crushed mycelium was then separated from the extract which was again macerated with another 350 ml. of sodium chloride solution as before. The clear extracts were mixed and then a dry preparation was obtained involving ammonium sulphate precipitation, resolution and dialysis, and acetone precipitation, being taken to dryness as before.

Both the enzyme preparations were stored in the refrigerator. No further purification of either preparation has so far been attempted.

Activities of the preparations. - Determination of endo- and exo-β-glucanase activities (Preece
and Hoggan, 1956) and of pentosanase activity (Preece and MacDougall, 1958) were made by the standard procedures. The results are shown in Table V, along with the figures for barley enzyme preparations for the purpose of comparison. It should be noted here that the enzyme solutions were made with known weights and diluted in such a way that the observed increases in reciprocal specific viscosity did not exceed about 0.2 per hour and the figures of Table V were calculated proportionately to the original preparations.

It is found that the culture filtrate enzyme preparation is highly active in respect of endo-$\beta$-glucanase and endoxylanase and these activities are some 40 and 700 times as great as those of good barley preparations. Green malt gives highly active preparations of endo-$\beta$-glucanase (Preece and Hoggan, 1956), but still this culture filtrate preparation is 7 times more active than that from the green malt (0.57). It is also found that the culture filtrate preparation is apparently devoid of exo-$\beta$-glucanase activity and this property is sharply different from the barley (see Section II) and mycelium preparations. The mycelium preparation is found to have close resemblance with barley and green malt preparation in respect of both endo-$\beta$-glucanase and exo-$\beta$-glucanase activities. It must be remembered
that the mode of determination of "exo-activity" includes the contribution made by oligo-
saccharases to increase in reducing power, and it is not to be excluded at this stage that the activity in this case may be due to oligo-
saccharidases only. There is no simple method which has yet been devised for the rapid direct assessment of exoxylanase.
Development of extracellular $\beta$-glucanase and araboxylanase activities by a strain of *Penicillium chrysogenum* grown on various carbohydrates (Growth at 25°C; 14 days)

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Dry weight of mycelium (mg.)</th>
<th>$\beta$-Glucanase</th>
<th>Araboxylanase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total activity b</td>
<td>Activity per mg. myc. per 2 ml. CF.</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>114.5</td>
<td>6.54</td>
<td>0.057</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>95.0</td>
<td>4.10</td>
<td>0.044</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>83.5</td>
<td>3.50</td>
<td>0.042</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>69.5</td>
<td>1.77</td>
<td>0.026</td>
</tr>
<tr>
<td>Sucrose</td>
<td>67.0</td>
<td>0.38</td>
<td>0.006</td>
</tr>
<tr>
<td>Raffinose</td>
<td>57.5</td>
<td>1.90</td>
<td>0.003</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>54.5</td>
<td>1.00</td>
<td>0.018</td>
</tr>
<tr>
<td>$\beta$-Glucan</td>
<td>42.5</td>
<td>1.04</td>
<td>0.025</td>
</tr>
<tr>
<td>Lactose</td>
<td>39.0</td>
<td>1.40</td>
<td>0.036</td>
</tr>
<tr>
<td>Araboxylan</td>
<td>33.5</td>
<td>1.34</td>
<td>0.040</td>
</tr>
</tbody>
</table>

a. $\beta$-glucan and araboxylan 0.2% in Czapek-Dox medium; others 1.0%.

b. Increase in reciprocal specific viscosity per 2 ml. culture filtrate per hr. on standard substrate solution.
TABLE II

Carbohydrate source and development of extracellular $\beta$-glucanase activity

(Growth at 25°C; 14 days)

<table>
<thead>
<tr>
<th>Sugar added (g. per 100 ml.)</th>
<th>0.2% $\beta$-glucan in basal medium plus:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Xylose</td>
</tr>
<tr>
<td></td>
<td>Mycelial wt. (mg.)</td>
</tr>
<tr>
<td>0.00</td>
<td>8.0</td>
</tr>
<tr>
<td>0.125</td>
<td>-</td>
</tr>
<tr>
<td>0.20</td>
<td>22.5</td>
</tr>
<tr>
<td>0.25</td>
<td>-</td>
</tr>
<tr>
<td>0.40</td>
<td>35.3</td>
</tr>
<tr>
<td>0.50</td>
<td>-</td>
</tr>
<tr>
<td>0.60</td>
<td>53.5</td>
</tr>
<tr>
<td>0.80</td>
<td>68.5</td>
</tr>
<tr>
<td>1.00</td>
<td>88.5</td>
</tr>
</tbody>
</table>
# TABLE III

Carbohydrate source and development of extracellular β-glucanase activity

*(Growth at 25°C; 14 days)*

<table>
<thead>
<tr>
<th>β-glucan added (g. per 100 ml.)</th>
<th>Mycelial wt. (mg.)</th>
<th>Activity per 2 ml.</th>
<th>Activity per mg. myc. per 2 ml. CF.</th>
<th>Mycelial wt. (mg.)</th>
<th>Activity per 2 ml.</th>
<th>Activity per mg. myc. per 2 ml. CF.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000</td>
<td>58.0</td>
<td>3.60</td>
<td>0.062</td>
<td>58.1</td>
<td>0.75</td>
<td>0.013</td>
</tr>
<tr>
<td>0.025</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>66.0</td>
<td>0.79</td>
<td>0.012</td>
</tr>
<tr>
<td>0.050</td>
<td>63.5</td>
<td>4.80</td>
<td>0.075</td>
<td>72.5</td>
<td>0.72</td>
<td>0.010</td>
</tr>
<tr>
<td>0.075</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>86.5</td>
<td>2.40</td>
<td>0.028</td>
</tr>
<tr>
<td>0.100</td>
<td>65.5</td>
<td>5.80</td>
<td>0.088</td>
<td>89.7</td>
<td>3.10</td>
<td>0.034</td>
</tr>
<tr>
<td>0.150</td>
<td>77.0</td>
<td>4.00</td>
<td>0.052</td>
<td>87.0</td>
<td>5.00</td>
<td>0.057</td>
</tr>
<tr>
<td>0.200</td>
<td>88.5</td>
<td>3.40</td>
<td>0.038</td>
<td>102.4</td>
<td>4.89</td>
<td>0.048</td>
</tr>
</tbody>
</table>
### TABLE IV

Sugar concentration and development of extracellular $\beta$-glucanase (in absence of $\beta$-glucan)

(Growth at 25$^\circ$C; 14 days)

<table>
<thead>
<tr>
<th>Sugar concn. (g. per 100 ml.)</th>
<th>Sugar</th>
<th>Xylose</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mycelial wt. (mg.)</td>
<td>Activity per 2 ml.</td>
<td>Activity per mg. myc. per 2 ml. CF.</td>
</tr>
<tr>
<td>0.4</td>
<td>55.0</td>
<td>2.76</td>
<td>0.051</td>
</tr>
<tr>
<td>0.8</td>
<td>87.5</td>
<td>4.00</td>
<td>0.046</td>
</tr>
<tr>
<td>1.2</td>
<td>123.5</td>
<td>4.40</td>
<td>0.036</td>
</tr>
<tr>
<td>1.6</td>
<td>155.5</td>
<td>3.26</td>
<td>0.021</td>
</tr>
<tr>
<td>2.0</td>
<td>188.5</td>
<td>1.86</td>
<td>0.010</td>
</tr>
</tbody>
</table>
TABLE V

Comparison of activities\textsuperscript{a} of mould and barley hemicellulase preparations

<table>
<thead>
<tr>
<th>Source of enzyme preparation</th>
<th>Endo-$\beta$-glucanase</th>
<th>Exo-$\beta$-glucanase\textsuperscript{b}</th>
<th>Endo-arabinoxylanase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw barley\textsuperscript{b}</td>
<td>0.094</td>
<td>0.203</td>
<td>0.013</td>
</tr>
<tr>
<td>Mould:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>culture filtrate</td>
<td>4.050</td>
<td>0.000</td>
<td>10.000</td>
</tr>
<tr>
<td>mycelium</td>
<td>0.340</td>
<td>0.270</td>
<td>3.600</td>
</tr>
</tbody>
</table>

\textsuperscript{a}. Per mg. of enzyme preparation.

\textsuperscript{b}. Refs. (Preece and Hoggan, 1956; Preece, Garg and Hoggan, 1960); these preparations had received some degree of further purification.

That this activity is largely or entirely due to oligosaccharases is not excluded.
FIG. 1 - Growth of mycelium with increasing concentrations of sugar (mg.) in presence and absence of $\beta$-glucan (0.2%).

- Glucose in presence or absence of $\beta$-glucan (0--0--0--0--0).
- Xylose in presence or absence of $\beta$-glucan (x--x--x).

FIG. 2 - Development of extracellular $\beta$-glucanase activity at various sugar concentrations.

- Glucose with or without $\beta$-glucan (0--0--0--0--0).
- Xylose with or without $\beta$-glucan (x--x--x--x).

(%) in presence and in absence of $\beta$-glucan (0.2%).

FIG. 1 - Growth (mm) of mycelium with increasing concentration of sugar (x).
Influence of sugar conc. on activity per mg. mycelium.

In presence of β-glucan; xylose \( \times x \times x \), glucose \( 0 \times 0 \times 0 \).
In absence of β-glucan; xylose \( x \times x \times x \), glucose \( 0 \times 0 \times 0 \).

In presence of β-glucan; xylose \( x \times x \times x \), glucose \( 0 \times 0 \times 0 \).
In absence of β-glucan; xylose \( x \times x \times x \), glucose \( 0 \times 0 \times 0 \).

**FIG. 4** - Relation between mycelial wt. and activity per mg.

**FIG. 5** - Influence of sugar conc. on activity per mg. mycelium.
FIG. 5 - Increased growth on addition of β-glucan to 1.0% glucose (0) or 1.0% xylose (x).

FIG. 6 - Development of β-glucanase activity in culture medium on addition of β-glucan to glucose (A) or Xylose (B).
DISCUSSION

It is well known that hemicellulases are available from a wide variety of sources. Investigations of $\beta$-D-1,3-glucanase occurrence showed that the enzyme is present in plants, algae and micro-organisms, and fungi are the richest source. Some growth conditions have been studied for the production of this enzyme, but little attempt has been taken to find out relationships of growth with enzyme production. Although in the present work, the ultimate aim was to get better yields of enzyme preparation, it has always been observed how the growth of the organism follows the enzyme production. In the present investigation, a strain of *Penicillium chrysogenum* HW 3 could grow on all the carbon sources tested, but amounts of growth were different for different carbon sources. The organism could utilise sugars of different chemical nature like raffinose, lactose, xylose, etc. The growth of the organism on glucose was better than on raffinose, lactose and galactose, and xylose and arabinose were even better, but it cannot be said that the organism can utilise pentose sugars better than some hexoses and their oligosaccharides, as cellobiose gave the highest amount of growth at the same
concentration as that of other sugars in the medium.

In the case of \(\beta\)-glucanase production again, examples of anomalous behaviour were observed. Sometimes better growth was followed by better enzyme production and sometimes this was not the case. Cellobiose, xylose and arabinose gave high growth with high \(\beta\)-glucanase in the culture filtrate. Sucrose and glucose gave almost equal amounts of growth, but activity in the medium due to the former was very poor. Lactose gave much poorer growth than glucose but activity per mg. of mycelium was higher in the case of lactose than with glucose. The poor growth with comparatively higher activity given by arabinoxylan or \(\beta\)-glucan cannot be compared with other sugar results as the concentration of these hemicelluloses was 0.2% in comparison with 1.0% of the sugars. Arabinoxylanase was only favoured when the organism grew only in presence of pentoses or pentosan. \(\beta\)-glucanase production without much contamination of arabinoxylanase is possible by growing the organism on glucose, cellobiose and \(\beta\)-glucan, but arabinoxylanase without much \(\beta\)-glucanase production is not possible, as high arabinoxylanase production is always associated with high \(\beta\)-glucanase production. As the organism produces \(\beta\)-glucanase in
all media, the enzyme can apparently be called constitutive; however, the production of the enzyme is stimulated by xylose, cellobiose and \( \beta \)-glucan. As the araboxylanase production is only induced by pentoses or pentosan and not by hexoses or hexosan, the enzyme can be named adaptive. The reason for stimulation by both arabinose and xylose is not clear. Simpson (1956) showed that Bacillus subtilis possessed a constitutive pentosanase; but the production of the enzyme was stimulated by arabinose, ribose and maltose, and by a number of complex carbohydrates. B. pumilus, however, possessed an adaptive pentosanase since xylose and those substrates which contained xylose did induce the production of pentosanase, but glucose, galactose and arabinose did not.

More is known about \( \beta \)-glucanase production in relation to growth of the organism. The optimum concentration of xylose for \( \beta \)-glucanase production is approximately 1.0\%, but for glucose the production falls as soon as the sugar concentration exceeds about 1.0\% without reaching the production level given by xylose. With the same concentration of sugar for both xylose or glucose, the same amounts of growth are obtained, but \( \beta \)-glucanase production due to xylose is much higher than that due to glucose. Thus it seems that
xylose has two effects, one being the stimulation of growth of the organism and the other the specific stimulation of \( \beta \)-glucanase production. Glucose seems only to have a stimulatory effect on growth. When gradually increasing amounts of sugar are added to \( \beta \)-glucan, the total extracellular \( \beta \)-glucanase production at first increases, but later decreases. With xylose the increase seems due to both the growth effect and also to the specific stimulatory effect on \( \beta \)-glucanase production, but with glucose the effect seems only due to the additional growth. With both sugars, the \( \beta \)-glucanase production per unit weight of mycelium decreases with the addition of increasing amounts of sugar to \( \beta \)-glucan, and this decrease for each sugar continues to the point reached with the sugar alone. The limiting points are different for each sugar, and this is what would be expected from the two types of effects already described.

Two factors must therefore be distinguished:

(a) stimulation of growth (xylose, glucose) and
(b) direct stimulation of extracellular \( \beta \)-glucanase production (xylose, \( \beta \)-glucan). When sugar is of a particular type, e.g. sucrose, but is sufficient to support abundant growth, development of \( \beta \)-glucanase tends to be inhibited. Glucose is active in factor a but not b, and
accordingly there is little increase in activity at lower concentrations and a sharp decline thereafter. Xylose is active in $a$ and $b$, so that the observed effect becomes dependent on the interaction of both, with activity first increasing and then rapidly decreasing. With other sugars, deficient in both $a$ and $b$, it would be expected that fall in activity would be long delayed. With $a$ deficient and $b$ active, high activity might be expected to persist. The case of glucose plus $\beta$-glucan (up to 0.2%) is of special interest, the former supplying $a$ and the latter $b$, so giving an effect resembling that of xylose alone, though the concentrations so far investigated have not gone far enough to show the expected ultimate decline. With xylose plus $\beta$-glucan, $a$ is supplied by the xylose and $b$ by both; the expected pattern of behaviour is observed. It has already been said that $\beta$-glucanase is a constitutive enzyme and a hypothesis which suggests that the production of constitutive enzyme can be stimulated or inhibited is in good agreement with the present results. The picture of events given here is descriptive rather than explanatory, but it suggests immediately a wide field for further study.
Ammonium sulphate was used to precipitate enzymes both from the culture filtrate and the mycelial extract. The recoveries of $\beta$-glucanase and arabinoxylanase were only approximately 13% and 3% respectively. These low recoveries may be due to several causes, the most important being damage to the enzyme protein molecule during ammonium sulphate precipitation. Very recently Chesters and Bull (1963) reported that ammonium sulphate precipitation caused inactivation of laminarinase; they used acetone instead. There are possibilities that some co-factor left unprecipitated and loss of some metallic ion during dialysis might be responsible for enzyme activity.

However, 149 mg. of culture filtrate and 97 mg. of mycelial enzymes were obtained from 2250 ml. culture filtrate and the mycelium obtained from a typical culture. The activities of these preparations were very high in comparison with those of barley enzyme preparations and these amounts were sufficient for later uses. It was of extraordinary interest that this extracellular $\beta$-glucanase (culture filtrate enzyme) was exclusively an endo-$\beta$-glucanase (see below). It became important to compare its action with that of barley enzymes treated with phenylmercuric salts to free the barley endo-enzyme from exo-
activity. It was found earlier that barley enzyme contains both endo-1,3-ase and endo-1,4-ase activity and, accordingly, mould enzymes were tested for these factors; the results of this test are discussed in a later section.

Concerning the endoxylanase, which appeared to be an adaptive enzyme, little more need be said at this stage. The enzyme preparation was very much more active on araboxylan than any preparation yet obtained from a cereal source. The nature of activity on araboxylan degradation and results has been described later. Similarly, further characterisation of the mycelial enzyme is discussed below.

SUMMARY

1. Extracellular endo-β-glucanase is, for this mould strain, a constitutive enzyme, production of which can be stimulated by certain carbohydrates (xylose, β-glucan), but of which production can be inhibited by high concentrations of sugars favouring mycelial growth (xylose, glucose).

2. With growth medium containing xylose, therefore, the overall effect is dependent on sugar concentration; a similar complex interaction
is seen when the growth carbohydrate is a mixture of glucose and $\beta$-glucan.

3. The extracellular enzyme preparation on a xylose medium is free from exo-$\beta$-glucanase activity; the preparation from the mycelium contains both endo- and exo-$\beta$-glucanase, though the reducing power production ascribed to the last-named may actually be due to oligosaccharases.

4. Extracellular arabinoxylanase activity, involving endoxylanase, is developed on media containing xylose, arabinose or arabinoxylan, but not in any appreciable degree on media containing such other carbohydrates as glucose or its oligosaccharides.

5. Culture filtrate preparations are some 40 and 700 times as active against, respectively, $\beta$-glucan and arabinoxylan as are good preparations from barley; mycelial preparations contain endo- and apparent exo-$\beta$-glucanase activities of the same order as in good barley preparations.
SECTION II
COMPARATIVE ENZYMOLYSIS OF BARLEY $\beta$-GLUCAN

INTRODUCTION

For better understanding of the properties of the enzymes that degrade barley $\beta$-glucan, the enzymolysis of $\beta$-glucan-like substrates will be reviewed here. "Lichenin", a glucan having both $\beta$-1,3- and $\beta$-1,4-linkages, shows striking resemblance in its chemical structure to barley $\beta$-glucan. The polysaccharide was first isolated by Berzelius (1813) from tannin-free residue of Iceland moss. The chemical structure of lichenin was studied by Peat, Whelan and Roberts (1957). Lichenin is an unbranched $\beta$-glucan in which $\beta$-1,4- and $\beta$-1,3-types of glucosidic linkages occur together in the same molecule—the ratio between the two types of linkages varying between 2:1 and 3:1. The presence of a lichenin-like polysaccharide in oats (*Avena sativa*) was first reported by Morris (1942) and was later confirmed by Peat *et al.* (1957). The presence of lichenase activity in takadiastase (from *Aspergillus oryzae*) and in *Aspergillus niger* was reported by Saiki (1906), and also the same type of action was reported by Von Tschermak (1912) in the pancreas of dog. Karrer and Staub (1924) studied the nature of lichenase activity, and believed that enzymes of this type are not
usually present among the digestive enzymes of vertebrates. Pigman (1951) reviewed the occurrence of lichanases, and showed that they are present in various fungi, in barley, rye, wheat, oats, spinach and bean seeds and in vertebrates, higher animals and bacteria. Karrer and Staub (1924), and Pringsheim and Siefert (1923), have shown that an enzyme extracted from digestive juice of snail hydrolysed lichenin almost quantitatively to glucose. Pringsheim and Baur (1928) obtained 60% hydrolysis of freshly prepared solution of lichenin with an enzyme extracted from malt, while Karrer and Staub (1924) had obtained 84% hydrolysis of lichenin by snail enzyme.

Although \( \beta \)-glucan of barley contains both \( \beta \)-1,3- and \( \beta \)-1,4-linkages, it has some measure of relationship with the substances which are built up of \( \beta \)-1,4-linkages alone. Cellulose, cellulose-dextrin and synthetic substrates like carboxymethyl cellulose and ethylhydroxyethyl cellulose have the same \( \beta \)-1,4-linkages as make up about one-half of the linkages in \( \beta \)-glucan, and enzymes acting on these substrates might have some relationship with the enzymes degrading \( \beta \)-glucan. Probably the earliest observations on the cellulase activity of animals were made by Karrer (1930). In recent years, Levinson,
Mandelis and Reese (1951) and Reese and Levinson (1950), from studies on cellulose degradation by fungus enzymes, proposed a mechanism involving three enzymes, (1) $C_1$ responsible for the conversion of the insoluble cellulose into soluble glucan molecules of considerable molecular weight, (2) $C_x$ responsible for the degradation of water-soluble glucan chains, mainly to disaccharide units, and (3) cellobiase responsible for the hydrolysis of cellobiose to glucose.

This mechanism shows a similar degradation pattern to that of barley $\beta$-glucan with barley enzymes (Preece and Aitken, 1953). Whitaker (1953), and Basu and Whitaker (1953) investigated cellulose degradation with highly purified enzymes from the extracts of *Myrothecium verrucaria*, and found that the rate of production of glucose from cellobiose was negligible as compared with that from water-soluble cellulose products. They concluded that glucose was produced by a single enzyme without any production of intermediate cellobiose.

Artificial substrates, carboxymethyl cellulose and ethylhydroxyethyl cellulose, were used by Sandegren and Enebo (1952) to estimate the cellulase activity; but Preece and Aitken (1953) and others have shown from their experiments that results so obtained do not parallel those with
more natural substrate, like \(\beta\)-glucan, because the artificial substrates are less sensitive to enzyme attack.

The structure of a water-insoluble polysaccharide, "Pachyman", from an unidentified species of the fungus \textit{Pachyma hoelen} was investigated by Warsi and Whelan (1957), who suggested that it is a \(\beta\)-1,3-linked glucan; however, much more experimental evidence is required for knowledge of its real structure. As the polysaccharide is not soluble in water, the results of its enzymolysis cannot be strictly compared with those from soluble \(\beta\)-glucan. Another polysaccharide at one time believed to be purely a \(\beta\)-1,3-linked linear glucan is laminarin, a water-soluble polysaccharide found in some brown algae (\textit{Laminaria}) as a reserve carbohydrate (Barry, 1939; Barry \textit{et al.}, 1942). Peat \textit{et al.} (1958), after analysing water-insoluble and water-soluble laminarin by the method of partial acid hydrolysis, confirmed the \(\beta\)-1,3-linkages in laminarin but they also suggested the presence of \(\beta\)-1,6-linkages, with some of the chains terminated by mannitol. From the investigation of Hirst, O'Donnell and Percival (1958), laminarin was found to have a branched structure. Smith and Unram (1959) have reported the presence of D-mannose in laminarin. Very recently Chesters
and Bull (1963) showed that an insoluble laminarin contained D-mannose and \( \beta \)-1,6-linked branching points and also mannitol. From all these results, laminarin cannot be considered as merely a \( \beta \)-1,3-linked glucan and its enzymolysis cannot be strictly compared with that of barley \( \beta \)-glucan. Nevertheless, with its partial relationship to \( \beta \)-glucan, the enzymolysis of laminarin can give very useful results. Dillon and O'Colla (1950) found laminarase in extracts of wheat, oats, barley, potatoes and hyacinth bulbs. They also showed the presence in hydrolysates of glucose, laminaribiose and higher oligosaccharides by paper chromatography. MacWilliam and Harris (1957) isolated two enzyme fractions from malt extract by selective elution from alumina. One broke down laminarin to glucose and laminaribiose, and the other produced higher oligosaccharides only. The two enzymes were considered as exo-\( \beta \)-1,3- and endo-\( \beta \)-1,3-polyglucosidases respectively.

Preece and Aitken (1953) used a modification of the method of Sandegren and Enebo (1952) to determine the rate of \( \beta \)-glucan breakdown with barley enzyme extract, and found that the activity of the extract as determined viscometrically was independent of the initial viscosity of the substrate solution. They also showed that the
enzyme degrading barley $\beta$-glucan was not cello-
biase. Preece and Hoggan (1956) also showed the
rapid drop in viscosity of $\beta$-glucan solution
with the enzyme extracted from barley and
characterised glucose, laminaribiose, cellobiose
and other oligosaccharides in the enzymolysate.
Preece, Aitken and Dick (1954) suggested the name
endo-$\beta$-glucanase for the enzyme attacking the
$\beta$-glucan molecule centrally and producing
smaller molecular polysaccharides and oligo-
saccharides, and exo-$\beta$-glucanase for the enzyme
attacking the molecules terminally producing disaccharides.

$\beta$-glucanase activity may be measured by
following the viscosity fall and reducing power
increase in the conversion mixture for shorter
periods only. Preece and Garg (1961) thought
that, as the endo-action involves attack on $\beta$
-glucan chains at points remote from the ends, the
increase in the reducing power of a dextrin
should also provide a criterion for assessing
enzyme action when low viscosities of conversion
mixtures, either at the initial stage or after 90
minutes, limit the study of the enzyme action by
viscometer over longer periods. They determined
the increase in the reducing power of the reaction
mixture, and the recovery of dextrin and its
complexity, during the enzymolysis of $\beta$-glucan
for periods of 48 hours. It was expected that such a study would confirm or disprove the view that endo-action over longer periods is not as simple as it appears from the decrease in solution viscosity; the decrease in the molecular complexity of the dextrin might provide a more correct picture of the degradation.

Preece and Hoggan (1956) employed differential inactivation of the barley enzyme preparation and showed that, when an enzyme preparation was treated with phenylmercuric nitrate, the action of the residual enzyme did not produce reducing groups in excess of those calculated from the viscosity drop, while with the enzyme preparation not treated with phenylmercuric nitrate, the reducing power increase of the standard reaction mixture was far in excess of that calculated from viscosity drop alone; the excess reducing power was taken as a measure of joint action of exo-\(\beta\)-glucanase and cellobiase, the presence of the latter being shown by the hydrolysis of cellobiose to glucose. On the basis of rigid experimental evidence, they obtained a relation between the reducing power of different \(\beta\)-glucan solutions and their viscosities satisfying the equation

\[ g = 20.38 \text{ U} \pm 0.168 \pm 0.23 \]

where \(g\) is the \(\mu\text{g. glucose equivalent per mg. of}\)
substrate and \( U \) is the reciprocal specific viscosity of 0.5% aqueous solution of the \( \beta \)-glucan at 25°C. When enzyme preparations were treated with phenylmercuric nitrate, the \( \beta \)-glucan break-down measured viscometrically and the reducing power increase in the early stages were in accord with the above modified equation (see experimental).

Aspinall and Telfer (1954) studied the structure of barley \( \beta \)-glucan prepared by Preece and MacKenzie (1952). Hydrolysis of methylated barley gum afforded approximately equal proportions of 2,3,6- and 2,4,6-tri-O-methyl-D-glucose, thus indicating the presence of linear chains of \( \beta \)-D-glucopyranose residues with \( \beta \)-1,4- and \( \beta \)-1,3-linkages in equal proportions. Gilles, Meredith and Smith (1952) concluded from their work that the two types of linkages were alternately distributed in the molecule as they could not obtain any phenylosazones of oligosaccharides after using the periodate-oxidised polysaccharide. Peat, Whelan and Roberts (1957) characterised cellobiose, laminaribiose, cellotriose and two trisaccharides containing mixed linkages but no laminaritriose as products of partial acid hydrolysis of oat \( \beta \)-glucan, and on the basis of these results proposed a structure in which isolated 1,3-linked \( \beta \)-D-glucopyranose residues
were separated by two or three 1,4-linked units. This could not be confirmed by Smith and Sorger-Domenigg (1960). They degraded periodate-oxidised oat β-glucan, by reduction with sodium borohydride, followed by mild acid hydrolysis and isolated 2-0-β-laminaribiosyl-D-erythritol and 2-0-β-laminaritriosyl erythritol. This suggested the presence of blocks of two and three β-1,3-linked glucose residues in part of the molecular structure, in addition to isolated 1,3-linkages.

Using chromatographic and electrophoretic techniques, Hoggan (1957) characterised glucose, laminaribiose, cellobiose, laminaritriose and two trisaccharides of the structure G-β-1,3-G-β-1,4-G-OH and G-β-1,4-G-β-1,3-G-OH in the enzymolyses of barley β-glucan with barley enzymes. The presence of these oligosaccharides contradicted the results of Gilles et al. (1952) but was in accord with the results of Smith and Sorger-Domenigg (1960). Preece, Hoggan and Garg (1960) took a more elaborate way of determining the distribution of the two types of linkages in β-glucan. They degraded β-glucans of barley and oats with treated and untreated barley enzyme preparations, and the oligosaccharides produced were characterised by chromatographic and electrophoretic methods. They also characterised the
oligosaccharides produced after partial acid hydrolysis of $\beta$-glucan limit-dextrin of the enzymolysates. The enzymolysates yielded qualitatively the same oligosaccharides whether substrate was barley or oat $\beta$-glucan at the lower oligosaccharides level. There were differences in the barley and oat products at the tetrasaccharide level. They showed that the oligosaccharides produced from the $\beta$-glucan limit-dextrin after degradation of barley $\beta$-glucan with treated and untreated barley enzymes, were rich in $\beta$-1,4-linkages but also possessed $\beta$-1,3-linkages. They concluded from the results that groups of up to at least three $\beta$-1,3-linkages must be present together for the production of laminaritetraose. Preece and Garg (1961) suggested four consecutive $\beta$-1,3-linkages from the knowledge of degradation pattern of $\beta$-glucan and other similar substrates by different enzymes. They expressed a similar view about the disposition of $\beta$-1,4-linkages in the barley $\beta$-glucan.

Parrish, Perlin and Reese (1960) used "laminarinase" from *Rhizopus* species and "cellulase" from *Streptomyces* species in the degradation of oat $\beta$-glucan and characterised the oligosaccharides by chemical methods. Cellulase converted the polysaccharides to a trisaccharide, 4-O-$\beta$-D-laminaribiosyl-D-glucose,
and two tetrasaccharides, 3-O-β-D-cellobiosyl-D-cellobiose and 4-O-β-D-laminaribiosyl-D-cellobiose. Laminaribiose produced a tri-
saccharide, 3-O-β-D-cellobiosyl-D-glucose, and a tetrasaccharide, 3-O-β-D-cellettrosyl-D-glucose. They concluded that oat β-glucan is
composed of two types of structural sequences: one is a tetrameric unit in which a single β-1,3-linkage alternates with two β-1,4-linkages, and the other a pentameric unit in which a single β-1,3-linkage alternates with three consecutive β-1,4-linkages. They also used cellulase in
the enzymolysis of barley β-glucan and pointed out the close relationship of structures with the oat β-glucan but did not mention the disposition of two linkages in the barley β-glucan as the ratios of the two types of linkages are different in the two polysaccharides.

Moscatelli et al. (1961) described the enzymic properties of a β-glucanase from Bacillus subtilis. They used this enzyme to
catalyse the hydrolysis of diastase-treated barley β-glucan. Three minor products like glucose, laminaribiose and laminaritriose were identified by paper chromatography. Two major products
were fractionated from the hydrolysate by cellulose column chromatography and were characterised by chemical methods. These two oligosaccharides
were $3-O-\beta-D$-celliobiosyl-$D$-glucose and $3-O-\beta-D$-cellotriosyl-$D$-glucose. They could not detect any cellobiose in the products.

The application of enzymic techniques of these types to the analysis of structural polysaccharides is becoming well founded. Although the main structural features of a polysaccharide can usually be determined by chemical methods, these procedures are not simple and they do offer certain limitations. The successful application of enzymes to starch and protein analysis suggests that they may prove equally helpful in the analysis of other natural polymers. Enzymes are highly specific; cellulase hydrolyses only cellulose, xylanase only xylan and so forth. Therefore, it is theoretically possible to remove specifically any component from a mixture by means of the appropriate enzyme; such specific action would be very difficult if not impossible by chemical procedures.

An enzymic study of the structure of naturally occurring polysaccharides has, nevertheless, certain limitations. In the case of degradation of $\beta$-glucan with enzymes, very small amounts of oligosaccharides are available. Therefore, identification of these oligosaccharides is only possible by the help of physical methods, such as paper chromatography.
and electrophoresis. The possibility of enzymic synthesis is always present and, therefore, very great caution and restraint are needed in interpreting the experimental results. In spite of all these handicaps, Preece and co-workers were able to present a picture of enzymic degradation of cereal gums which is fairly elaborate. Besides adding information on the pattern of cereal gums enzymolyses, these researches have pointed out new directions for further investigation, and it is quite evident that there is a great deal which is unknown. Further researches are needed to clarify the distribution of the two types of linkages in $\beta$-glucan and the mode of action of different enzymes from different sources.

In all enzymic experiments, the possibility of resynthesis can hardly be ignored and, therefore, in the present work enzymolysis was carried out under conditions most unlikely to permit any resynthesis. It must also be mentioned that extremely small quantities of oligosaccharides did not permit the application of chemical methods of analysis; paper chromatography and paper electrophoresis were employed to characterise the oligosaccharides. One cannot rely entirely on the results obtained by physical methods, but even chemical methods of analysis -
such as methylation, partial hydrolysis and peridate oxidation, are not absolutely perfect, incomplete methylation, reversion and over-oxidation being their respective drawbacks. The technique of paper electrophoresis has in recent years developed simultaneously with the techniques of column and paper chromatography. Although paper electrophoresis is already extensively used in clinical methods for the separation of proteins, its uses in the carbohydrate field have been realised comparatively recently. An important step in the development of electrophoretic methods for carbohydrates was the use of borate buffer, introduced in 1952 by Consden and Stanier (1952), by Michl (1952) and in the same year by Jaenicke (1952). The majority of early investigations were concerned with monosaccharides, and some useful separations of these were achieved which had hitherto been very difficult by paper chromatography.

From a study of the electrophoretic behaviour of the mono-0-methyl-D-glucose, it seemed likely that the reducing disaccharides of D-glucose with 1,2- or 1,4-linkages would have much smaller mobilities than those containing 1,3- or 1,6-linkages. This was confirmed by Foster (1953). It is apparently the linkage by which the "remainder" of the molecule is attached
to the reducing moiety, rather than the structure of the "remainder", which determines mobility. An interesting application of this observation has been in the examination of the products of enzyme action on \(\beta\)-glucan (Hoggan, 1957). In addition to laminaritriose (containing two \(\beta-1,3\)-linkages), a trisaccharide fraction, apparently pure by chromatography, was found to resolve, upon electrophoresis in borate buffer, into two components, whose chromatographic and electrophoretic mobilities could only be accounted for by the structures, \(\alpha-\beta-1,4-\alpha-\beta-1,3-\alpha-\beta-\text{OH}\) and \(\alpha-\beta-1,3-\alpha-\beta-1,4-\alpha-\text{OH}\).

The true mobilities of different molecules during electrophoresis cannot be determined directly, since there is always an electro-endosmotic flow; a marker, such as 2,3,4,6-tetra-O-methyl-D-glucose, which does not react with borate ions may, however, be used for reference. In this connection it is important to note the observation of Bourne et al. (1956) and of Foster et al. (1956) that during electrophoresis different substances which do not complex with borate migrate at identical rates, under the influence of the electroendosmotic flow, there is little or no selective adsorption of low molecular weight carbohydrates.

Although carbohydrates having different
molecular weight may have identical mobilities when submitted to electrophoresis in borate buffer, there are other buffers in which the molecular weight of an aldose determines its mobility. Thus Barker et al. (1956) have shown that the \( \text{N} \)-benzylglycosylamines of aldoses migrate as glycosylammonium ions during electrophoresis in a formic acid-sodium formate buffer of pH 1.8; the mobilities are inversely proportional to the molecular weights of the ions and also apparently independent of the actual structure of the sugars, and of the constituent linkages in di- and higher oligosaccharides. Observations were made on a hexose series from the mono- to the hexa-saccharide level and, although the Mg. values (represented by the mobilities of the \( \text{N} \)-bensylglycosylammonium ion, relative to that of \( \text{N} \)-benzyl-D-glucosylammonium ion) for the higher oligosaccharides are perhaps too close to be unequivocal, those up to, at least, the tetrasaccharide level are satisfactory.

A similar method for the determination of molecular size of carbohydrates by electrophoresis was reported by Frahn and Mills (1956), who used a buffer of aqueous sodium bisulphite. An aldose apparently reacts slowly with bisulphite to form an anionic complex in equilibrium with the free sugar. During electrophoresis the
complex migrates, but slowly decomposes during migration. Thus two spots may be observed, firstly that of complex and secondly that due to electroendosmosis; the distance between them is a measure of the true ionic mobility of the charged complex. The mobilities relative to glucose, for which Mg. is taken as 1.00, were found to be virtually identical for isomers and to decrease regularly, but not linearly, with increase in the molecular weight of the aldose; in fact \( \frac{1}{Mg} \) is proportional to the degree of polymerisation of the sugar unit.

After the isolation of pure \( \beta \)-glucan from barley by Preece and MacKenzie (1952), a considerable knowledge has been obtained about the structure of \( \beta \)-glucan by both chemical and enzymic methods but so far is known, very little \( \beta \)-glucan enzymolysis has been done by using different enzymes under similar conditions except the work of Parrish et al. (1960). In the present work, enzymolyses of \( \beta \)-glucan by using enzyme preparations from culture filtrate and mycelium of a \textit{Penicillium} species and from barley, were carried out under identical conditions and the subsequent analyses were also performed by using similar techniques. It was thought that this type of work might be fruitful for better understanding of \( \beta \)-glucan enzymolysis and its
structural significance. Thus, workers as is shown above have reported different oligo-
saccharides being produced when enzymes from different sources are used. The aim of the present work, therefore, has been to determine whether these differences are real, and if so to attempt to find what characters in the enzyme preparations are responsible for the differences.
**Experimenhal**

Materials and Methods

Preparation of substrates. - The method of $\beta$-glucan preparation has been described in Section I. A water-soluble laminarin used in the present work is a commercial preparation supplied by Messrs. Light.

The method of Preece and Garg (1961) has been followed for the preparation of cellodextrin. 8 g. of cellulose powder was gradually added to 136 ml. of 70% sulphuric acid (80 ml. sulphuric acid (conc.) + 56 ml. water). The mixture was gently stirred for six hours at room temperature. The cellulose powder went into solution by this time. The solution was then poured into 500 ml. ice cold water. The gelatinous precipitate was removed at the centrifuge. Cellodextrin was precipitated from the centrifugate with an equal volume of cold acetone. The white precipitate was separated and washed several times with 50% acetone to minimise the acidity. The precipitate was then dissolved in the minimum volume of water at room temperature and dialysed for two days against running water. Cellodextrin was finally precipitated with an equal volume of acetone and taken to dryness by repeated washing with acetone. The precipitate was soluble in water after initial gentle heating.
Preparation of enzymes. - The methods for the preparation of mould enzymes from culture filtrate and mycelium have been described in Section I.

The method of Preece and Hoggan (1956) has been followed for the preparation of barley enzyme used in the work of this section. About 300 g. of the ground barley (Ymer) was extracted with 1000 ml. of citrate-phosphate buffer (Britton, 1942) of pH 4.5 for one hour at room temperature with stirring. The extract after centrifugation was left overnight for autolysis; it was then dialysed for two days against running water using thymol as antiseptic. After dialysis, the pH of the extract was adjusted to 4.5 with normal acetic acid. Precipitate at the 30% ammonium sulphate concentration was removed and discarded and then the precipitate at the 50% ammonium sulphate concentration level was collected. This precipitate was dissolved in the minimum quantity of water and dialysed for two days. After dialysis, the solution was passed through celite and the final precipitate of enzyme was obtained by adding four volumes of acetone and taken to dryness in the usual way.

Barley enzyme preparations devoid of exo-activity were prepared by the method of Preece and Hoggan (1956). To the enzyme solution
containing 1 mg. of enzyme per ml., phenylmercuric nitrate was added to a concentration of 5 mg. per ml. and the mixture was allowed to stand for 3 hours at room temperature. After this period, the mixture was centrifuged, dialysed for two days and then the enzymes were precipitated by the addition of four volumes of acetone and the enzymes separated were taken to dryness in the usual way. The enzyme preparation was tested and found free of exo-activity. The barley enzyme treated with phenylmercuric nitrate has been used in the work of this section.

**Determination of endo-\(\beta\)-glucanase activity.**

The method of Preece and Aitken (1953) was used for this purpose. 8 ml. of 0.6875% \(\beta\)-glucan, 2 ml. of enzyme solution (containing 1 mg. per ml. in case of barley enzyme, 0.01 mg. per ml. in case of mould enzymes) and 1 ml. of acetate buffer of pH 5 were mixed (all solutions were at 25°C before mixing) and 10 ml. of the reaction mixture were transferred to an Ostwald viscometer. Times of flow were noted at intervals over a period of 90 min. and the specific viscosities were calculated from the relation \(T_s - T_w/T_w\), where \(T_s\) and \(T_w\) are the times of flow of the reaction mixture and water respectively. The enzyme activity expressed by Hultin's (1946) principle
which was also employed by Sandegreh and Enebo (1952) in the equation,

\[ E = K^1 \cdot C^2 \cdot \frac{du}{dt} \]

where \( E \) is the enzyme activity, \( K^1 \) a constant, \( C \) the substrate concentration, \( U \) the reciprocal specific viscosity and \( t \) the time in hours from the moment of mixing. Since the substrate concentration changes only slightly during reaction time of 90 minutes, \( C^2 \) may be considered constant, and the above equation becomes, \( \frac{E}{K} = \frac{du}{dt} \).

The function \( \frac{E}{K} \), which can be easily determined by plotting \( du \) against \( dt \), was taken as a measure of endo-\( \beta \)-glucanase activity. The slope of the curve \( \frac{du}{dt} \) was linear over 90 minutes. Enzyme activity was expressed as increase in reciprocal specific viscosity of 0.5\% \( \beta \)-glucan solution per hour by per mg. of enzyme at 25\(^\circ\)C.

**Search for presence of exo-\( \beta \)-glucanase.** - The following procedure was used to determine whether exo-\( \beta \)-glucanase activity was present. Reducing power of the 5 ml. aliquot of the standard reaction mixture of \( \beta \)-glucan and enzyme at 25\(^\circ\)C was determined at intervals of one hour for four hours by employing the Somogyi (1945) copper-reduction method. The results were corrected for initial reducing power of the substrate and enzyme blanks. On the basis of findings of
Preece and Hoggan (1956) it was expected that, in a \(\beta\)-glucan conversion with enzymes involving endo-activity alone, the total number of reducing groups produced would be accounted for the drop in viscosity, while in presence of enzymes having exo-activity, some additional reducing groups would be produced. Reducing power increase per hour of 11 ml. standard reaction mixture could be calculated from the equation

\[
\text{gm} = 1.165U + 0.000 \pm 0.012
\]

where \(\text{gm}\) is the glucose equivalent of the reducing power change per hour of the reaction mixture and \(U\) is the reciprocal specific viscosity change per hour. From Somogyi's determination, reducing power increase per hour for 11 ml. aliquot can be calculated and from these two sets of results, excess reducing groups production, if any, can be found out. It was found that the barley enzyme treated with phenylmercuric nitrate and mould culture filtrate enzyme did not produce reducing groups in excess of the calculated values. Hence exo-activity was absent.

**Enzymolyses of substrates.** - Enzymolyses of substrates were carried out in 0.5% solution at 37°C (using thymol as antiseptic). Each reaction mixture was prepared by mixing 80 ml. of 0.6875% substrate solution, 20 ml. of enzyme solution and
10 ml. of acetate buffer of pH 5; before mixing, all solutions were brought to 37°C. The barley enzyme solution contained 1 mg. per ml. and the viscometric endo-activities of mould culture filtrate and mycelial enzymes were made equal to that of the barley preparation by diluting the enzyme solutions. A mixture of barley and mould culture filtrate enzymes was prepared by adding equal volumes of each enzyme solution after final adjustment of endo-activity. It was shown by Preece and Aitken (1953) that the relation between enzyme concentration and its activity was linear, at least at the point where the increase in reciprocal specific viscosity per hour was not greater than ca. 0.2. In the present study, the reciprocal specific viscosity increase (as measured, solutions being diluted as necessary) per hour for all enzyme activities were not greater than ca. 0.2. Four sets of conversions of β-glucan were carried out using (a) mould culture filtrate enzyme preparation, (b) a barley enzyme preparation treated with phenylmercuric nitrate, (c) a mixture of equal activities of the culture filtrate and treated barley preparations (in total equalling the activity of the barley conversion), and (d) mould mycelial enzyme preparation. Other comparative hydrolyses were made of (a) β-glucan, (b) laminarin, and (c) cello-
dextrin, each with barley treated enzyme, culture filtrate enzyme and mycelial enzyme preparations with the same initial endo-activity. Substrate blanks and enzyme blanks were run under exactly similar conditions to allow for the autolysis of the substrate and enzyme in presence of buffer. It was found that the enzymes and substrates were stable.

Recovery of dextrin. - 10 ml. aliquots of conversion mixtures were withdrawn at different time intervals and, each time, were kept in a boiling water bath for 3 minutes to inactivate the enzyme. The solutions were cooled, any precipitate being removed by centrifuging. Dextrin in each aliquot was precipitated from the clear liquid by adding 4 volumes of acetone. The dextrin precipitate after acetone washing was separated by filtering on a sintered crucible (No. 3) and the product was dried at first in a vacuum and then at 100°C for 5 hours before cooling and weighing. From the known weight of the dried sintered crucible and the final weight, weight of the recovered dextrin was found. In this way, weights of the recovered dextrins were determined at different time intervals.

Complexity of dextrin. - Dextrins were precipitated
in 10 ml. aliquots withdrawn at the same time intervals with 4 volumes of 95% ethanol and each precipitate was washed with dilute acetone several times and dissolved in 10 ml. of hot water. The reducing power of the dextrin solution was determined by Somogyi's (1945) method. Knowing the dry weights of dextrins from the previous experiment, mg. glucose equivalent per mg. of dextrin (R) were calculated. The function (1000/R) was used as a rough measure of the complexity of the dextrin. It is realised that reducing power is not a true inverse measure of complexity, especially when $\beta-1,3$- and $\beta-1,4$-linkages are involved, since glucose equivalents of laminaribiose and cellobiose are different. The concept "complexity" is not employed to represent strictly the molecular size or the number of glucose units in the dextrin, but merely expresses, in a broad sense, that the molecular size of dextrin is gradually decreasing during enzymolysis.

Reducing power of glucose and oligosaccharides and total reducing power of the conversion mixture. - The total reducing powers of 10 ml. aliquots withdrawn at the same time intervals, were determined by Somogyi's method. The difference between the total reducing power of an
aliquot and the reducing power obtained in the recoverable dextrin of an aliquot withdrawn at the same time interval gave the reducing power of glucose and oligosaccharides together (Preese and Garg, 1961).

**Celllobiase and laminaribiase activity.** - 8 ml. of 0.3433% sugar solution (celllobiose or laminaribiose) were mixed with 1 ml. of acetate buffer of pH 5 and 2 ml. of enzyme solution, and the mixture was incubated at 37°C in presence of thymol as antiseptic; the solutions were brought to 37°C before mixing. Substrate and enzyme blanks were concurrently run for applying any necessary correction due to hydrolysis of the disaccharide in presence of buffer and autolysis of the enzyme. Glucose equivalents of the reaction mixture were determined on 0.5 ml. aliquots by Somogyi's method. Activities were expressed in terms of mg. of sugar hydrolysed per mg. of enzyme per hour. Calculations for celllobiase activity were done as following (see Thesis; Garg, 1959).

Let \( c \) be the number of mg. of celllobiose originally present for 0.5 ml. of the reaction mixture and \( x \) be the number of mg. of celllobiose remaining after \( t \) hours of reaction, then

\[
(c - x) \cdot \frac{360}{342} \text{ mg. of glucose are produced.}
\]
Let \( T \) be the number of ml. of \( \text{N}_2/200 \text{Na}_2\text{S}_2\text{O}_3 \) solution equivalent to 0.5 ml. of the reaction mixture after correction for enzyme control.

\[
T = \frac{x}{0.208} + (c - x) \frac{360}{342} \cdot \frac{1}{0.150} \text{ ml.} \quad (A)
\]

where 1 ml. of \( \text{N}_2/200 \text{Na}_2\text{S}_2\text{O}_3 \) solution is equivalent to 0.208 mg. pure cellubiose or 0.150 mg. pure glucose.

Equation (A) on simplification would become

\[
T = 4.808x + (c - x) 7.018
\]

or \( x = \frac{7.018c - T}{2.21} \) mg. per 0.5 ml. of reaction mixture and \( c - x \) = no. of mg. cellubiose hydrolysed per 0.5 ml. of the reaction mixture after \( t \) hours.

In case of laminaribiose determinations equation (A) would become

\[
T = \frac{x}{0.329} + (c - x) \frac{360}{342} \cdot \frac{1}{0.150} \quad (B)
\]

where 1 ml. of \( \text{N}_2/200 \text{Na}_2\text{S}_2\text{O}_3 \) solution is equivalent to 0.329 mg. pure laminaribiose, and \( c \) is no. of mg. of laminaribiose present in 0.5 ml. of reaction mixture.

Equation (B) on simplification becomes

\[
T = 3.039x + (c - x) 7.018
\]

or \( x = \frac{7.018c - T}{3.979} \) mg. per 0.5 ml. of the reaction mixture and \( c - x \) = no. of mg. of laminaribiose hydrolysed per 0.5 ml. reaction mixture after \( t \) hours.
Transglycosylation. — It was shown by Preece, Garg and Hoggan (1960) that barley enzymes did not produce any oligosaccharides during enzymolysis when the sugar concentration was 0.2%. In the present work, enzymolysis was carried out at 0.25% $\beta$-glucan concentration for oligosaccharide analysis and this concentration has been tested further for glucose, cellobiose and a mixture of half glucose and half cellobiose with mould culture filtrate, mould mycelium and barley enzymes. The reaction mixture contained 8 ml. of sugar solution, 1 ml. of acetate buffer of pH 5 and 2 ml. of enzyme solution (barley enzyme 1 mg. per ml., culture filtrate 0.25 mg. per ml., and mycelium 0.1 mg. per ml.) and incubated at 37°C for 48 hours. After this period, all the reaction mixtures were placed in a boiling water bath for 3 minutes and then evaporated to dryness over a water bath. All were examined chromatographically and there were no signs of oligosaccharide synthesis by any enzyme preparation. It was interesting to note that cellobiose and laminaribiose were not broken down by culture filtrate enzyme whereas both these sugars were broken down to glucose by barley and mycelium enzymes.

Enzymolysis of $\beta$-glucan. — Enzymolysis of
\( \beta \)-glucan was carried out at 37°C for 48 hours for oligosaccharides analysis at 0.25\% \( \beta \)-glucan concentration with barley (1 mg. per ml.), culture filtrate (0.25 mg. per ml.) and mycelium (0.1 mg. per ml.) enzymes as previously described. After 48 hours of enzymolysis, 110 ml. of each reaction mixture, each in a suitable flask was placed in a boiling water bath for 3 minutes and then cooled. Then the reaction mixture was adjusted to pH 7 by adding dilute sodium hydroxide solution and 4 volumes of 95\% ethanol was added to it, and it was kept in refrigerator overnight. Materials of high molecular weight were removed by filtration. The filtrate was then evaporated to dryness under vacuum at 50-60°C and was kept in the refrigerator for further analysis.

Fractionation of oligosaccharides. - Separation of oligosaccharides by direct chromatography was not possible, as the enzymolysates contained salts of buffer and excess glucose. The concentrated solution was applied to a charcoal-celite column as described by Whelan (1953). A charcoal-celite column was prepared by mixing equal quantities of acid-washed charcoal and celite 545 with water (322 ml. per 100 g. of the mixture). The slurry was poured, 2-3 inches at a time, into a glass column which contained glass wool as
support in the bottom. After allowing the column to settle, it was washed with about 500 ml. of water. The water coming from the column should be neutral. The concentrated solution of mixture of oligosaccharides from an enzymolysate was applied to the column, which was then exhaustively washed with water for removing the buffer salts and glucose; the remaining oligosaccharides were removed by eluting the column with about 2 litres of 30% (v/v) ethanol. The eluate was concentrated under vacuum at 50-60°C. The concentrate was then applied in a streak on 3 mm Whatman filter paper which was irrigated by descending flow of butanol:acetic acid:water (4:1:5) solvent (upper layer) for 6 days at constant temperature (25°C). The positions of different oligosaccharides were located by spraying side test strips and heating at 110°C for 10 min., using aniline hydrogen oxalate spray reagent. Strips of chromatogram corresponding to each spot on the test strips were cut and were eluted by the method of MacLeod (1951). Eluates were concentrated and separately spotted for each oligosaccharide, and the chromatogram was run as before using the upper layer of butanol:acetic acid:water. This type of double run gave oligosaccharides well separated from adjacent ones. $R_f$ values of the spots on each chromatogram were calculated with reference to cellobiose (0.090).
Plate 1. - Chromatogram to show oligosaccharides produced from barley $\beta$-glucan by barley enzyme treated with phenylmercuric nitrate (Bar.), mould culture filtrate enzyme (CF.) and mould mycelial enzyme (Myc.). Mixture of oligosaccharides from each source was freed from glucose by charcoal-celite column chromatography.

Solvent, Butanol : Acetic acid : Water :: 40:10:50

K, Known - laminaribiose (lam.) and cellobiose (cell.).
Electrophoresis. - (A) In sodium bisulphite: The method of Frahn and Mills (1956) was employed to determine the molecular weights of the oligosaccharides. Single chromatographically separated spots were eluted as before and concentrated and mixed separately with equal volumes of 0.4 M solution of sodium bisulphite; each mixture was allowed to stand for half an hour, during which bisulphite formed its complex with the oligosaccharide. These oligosaccharide complexes were spotted on Whatman No. 4 paper repeatedly to get a high concentration of each spot. The electrophoresis was carried out for six hours using 0.4 M solution of sodium bisulphite as electrolyte at 40-50 milliamps and 8 volts/cm., the paper being 11.5 cm. wide. After this period, the paper was dried in the oven at 110°C and sprayed with aniline hydrogen oxalate solution acidified with glacial acetic acid and heated for 10 min. at 110°C. Two spots separated by a faint streak were formed for each oligosaccharide, the position of the back spot representing electro-endosmotic movement of unchanged oligosaccharide, whereas the other spot represented a charged complex moving towards the anode. The distance between the spots being taken as measure of true mobility, $Mg.$ values for oligosaccharides were calculated with reference to cellobiose (0.70).
Plate II. - Electrophoresis of oligosaccharides from barley β-glucan by mould culture filtrate enzyme. The oligosaccharides were complexed with bisulphite and electrophoretogram was run in bisulphite solution as electrolyte at 8 volts/cm. and 40-50 milliamps. for 6 hr.

C, cellobiose.
(B) **In borate buffer.** - According to Foster (1953) oligosaccharides having $\beta$-1,3-linkage at the reducing end would form borate complex and would be mobile electrophoretically in borate buffer, whereas oligosaccharides having $\beta$-1,4-linkage at the reducing end would not form any complex with borate and electrophoretically would be immobile. It was observed experimentally that cellobiose does not move while laminaribiose moves towards anode. This criterion of mobility in borate buffer can also be applied in determining the type of linkages at the reducing end of an oligosaccharide. The oligosaccharides were eluted in the usual way and concentrated eluates were complexed by mixing with borate buffer and spotted on to Whatman No. 4 paper. Electrophoresis was carried out for 3 hours using 0.2 M sodium borate solution (pH 10) as electrolyte at 20 volts/cm. and 12-15 milliamps with a paper 11.5 cm. wide. After the period, the paper was dried and spots were revealed by spraying the paper with aniline hydrogen oxalate solution acidified with glacial acetic acid and heating at $110^\circ$C for 10 min.

**Assessment of results.** - In general, the methods used for characterising the oligosaccharides were those used by Freece, Garg and Hoggan (1960), but
Plate III. - Electrophoresis of oligosaccharides produced from barley $\beta$-glucan by mould culture filtrate enzyme. The oligosaccharides were complexed with borate and electrophoretogram was run in borate solution as electrolyte at 20 volts/cm. and 12-15 milliamps. for 3 hr.

G, glucose; C, cellobiose.
Mould (culture filtrate)

To Anode
- owing to the inevitable minor variations in
  technique as between workers - it was deemed
  advisable to prepare new standardisation diagrams
  in respect of the chromatographic and electrophoretic runs. Celllobiose was always used as
  initial reference compound, and preliminary runs
  proved it to have chromatographically a value for
  $\log \left(1 - R_F\right)/R_F$ of 1.00 and electrophoretically
  an $M_g.$ value in sulphite of 0.69. Compound No. 2
  (Table \(X\)) obtained by $\beta$-glucan enzymolysis ran
  exactly with celllobiose chromatographically and
  electrophoretically in sulphite; moreover, it
  was electrophoretically immobile in borate, and
  is therefore evidently celllobiose. Compound
  No. 1, obtained in all three enzymolyses, is
  evidently laminaribiose, since this is known to
  be a major product when $\beta$-glucan is hydrolysed
  by barley enzymes, and all three present products
  give the electrophoretic mobility in sulphite of
  disaccharides, are highly mobile in borate, and
  show the appropriate chromatographic behaviour.
  Similarly, laminaritriose and $\beta$-celllobiosyl-$3$-
  glucose are known to be produced - again as major
  products - when barley enzyme hydrolyse $\beta$-glucan,
  and compounds Nos. 3 and 4 must correspond to
  these. Accordingly, four reference compounds
  are available, permitting construction of the
  diagram in Figure 6 to show the values of
log \( \frac{1 - R_p}{R_p} \) to be expected according to the disposition of \( \beta-1,3- \) and \( \beta-1,4- \) linkages in the oligosaccharides. Again, it is a simple matter to construct the electrophoretic diagram of Figure 7, relating \( \frac{1}{M_g} \) to oligosaccharide complexity. The calculated values for the two functions are shown in Table \( \text{V} \), along with the mean observed values for the experimentally-obtained oligosaccharides. The two sets of values now given are closely similar to, though not identical with, those given earlier.

**Oligosaccharides obtained.** - In all three enzymolyses glucose was obtained with, in addition (Tables IV, V, VI): laminaribiose (structure 1), cellobiose (2), laminaritriose (3), \( \beta- \) cellobiosyl-3-glucose (4), and one or more tetrasaccharides with two \( \beta-1,4- \) linkages and one \( \beta-1,3- \) linkage (8), except that laminaritriose was not detected when the mycelial enzyme was used. As was to be expected, the barley enzyme gave additionally the mixed trisaccharide relatively immobile in borate, \( \text{viz.} \) \( \beta- \) laminaribiosyl-4-glucose; this trisaccharide was also obtained with the mycelial, but not with the filtrat enzyme. The earlier work had shown the barley enzyme to give one or more tetrasaccharides corresponding to structure No. 7, but these were
not obtained with barley on this occasion, though one such - mobile in borate - was obtained in small amount with the culture filtrate enzyme. It should also be noted that the three enzymes gave different results for materials corresponding to linkage-distribution No. 8. The slight differences seen here as compared with previous results for barley are interesting, but are not thought to have any great significance, since such differences might merely be due to different balances of oligosaccharase factors in different enzyme preparations from different barleys, and perhaps also in duration of enzymolyses. Full assessment of what oligosaccharides may or may not be produced is only possible when endo-$\beta$-glucanases free from oligosaccharases are used. It may be noted that material corresponding to distribution No. 8 was, in the earlier work, obtained with enzymes acting on oat $\beta$-glucan and also by acid hydrolysis of glucan dextrin. It may also be suggested here that there is no guarantee that preparations of $\beta$-glucan from different samples of barley - and especially in different seasons - will be identical in the balance of linkages which they contain.

It must be the case that the present method of preliminary characterisation of oligosaccharides loses precision as the molecular size
of the oligosaccharides increases. This is especially true for the chromatographic method, where crowding at the top of the paper precludes any really accurate assessment of $R_f$ values. For this reason, only compounds up to the tetrasaccharide level have been included in Tables IV-VI. Nevertheless, it must be recorded that two apparent hexasaccharides ($M_g. 0.32$ and $0.31$; expected value from Fig. 7 is $0.31$) were obtained with the culture filtrate enzyme; these were chromatographically distinct, but both showed mobility in borate. Similarly, a hexasaccharide ($M_g. 0.31$) was obtained with the barley enzyme, and this also showed borate mobility. It would appear that here we are dealing with compounds with mixed linkages, but the present evidence is insufficient to allow further characterisation.

Finally, it is necessary to indicate the relative abundance of the various oligosaccharides produced in the three conversions, so far as this can be done by visual assessment from the density of colour of chromatographic spots; this evidence is given in Table V.
<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Reduction: mg./100 mg. glucan</th>
<th>Reaction time (hr.):</th>
<th>0</th>
<th>6</th>
<th>12</th>
<th>24</th>
<th>36</th>
<th>54</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate</td>
<td>Dextrin</td>
<td>0.24 0.47 0.79 1.11 1.36 1.68</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oligosacch. + glucose</td>
<td>0.00 0.18 0.31 0.55 0.74 1.18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Recovery (%)</td>
<td>100 96.8 95.2 94.2 92.4 91.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reduction (μg./mg.)</td>
<td>2.4 4.9 8.3 14.8 19.7 18.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Barley</td>
<td>Dextrin</td>
<td>0.24 0.63 1.01 1.45 2.02 2.36</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oligosacch. + glucose</td>
<td>0.00 0.64 1.45 4.68 7.15 9.84</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Recovery (%)</td>
<td>100 94.6 92.6 89.2 84.8 80.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reduction (μg./mg.)</td>
<td>2.4 6.7 10.9 16.3 23.8 29.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixture of above</td>
<td>Dextrin</td>
<td>0.24 0.54 0.97 1.42 1.92 1.97</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oligosacch. + glucose</td>
<td>0.00 0.58 1.24 3.22 3.89 5.82</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Recovery (%)</td>
<td>100 95.6 94.6 93.0 89.4 81.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reduction (μg./mg.)</td>
<td>2.4 5.7 10.3 15.3 21.4 24.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mycelium</td>
<td>Dextrin</td>
<td>0.24 0.54 0.98 1.30 1.60 1.94</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oligosacch. + glucose</td>
<td>0.00 0.66 2.40 4.35 8.26 8.98</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Recovery (%)</td>
<td>100 98.3 96.8 95.2 91.8 89.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reduction (μg./mg.)</td>
<td>4.5 6.1 10.2 13.6 17.4 21.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N.B. In all cases, total crude endo-activity (by viscometry) was the same. In the case of the mixture of filtrate and barley enzymes, equal activities of the two were taken, to give the same endo-activity as with the individual enzymes. The barley enzyme was treated to suppress exo-activity.

*= 48 hr.
### TABLE II
Total Reduction on Various Substrates with Different Enzymes

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme source</th>
<th>Reaction time (hr.):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>β-Glucan</td>
<td>Barley</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>Filtrate</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>Mycelium</td>
<td>0.47</td>
</tr>
<tr>
<td>Laminarin</td>
<td>Barley</td>
<td>1.53</td>
</tr>
<tr>
<td></td>
<td>Filtrate</td>
<td>1.53</td>
</tr>
<tr>
<td></td>
<td>Mycelium</td>
<td>1.53</td>
</tr>
<tr>
<td>Cellodextrin</td>
<td>Barley</td>
<td>9.16</td>
</tr>
<tr>
<td></td>
<td>Mycelium</td>
<td>9.16</td>
</tr>
</tbody>
</table>

### TABLE III
Laminaribiose and Cellobiose Activities of the Enzyme Preparations

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>mg. hydrolysed/hr./mg. of enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Laminaribose</td>
</tr>
<tr>
<td>Barley (treated)</td>
<td>0.111</td>
</tr>
<tr>
<td>Culture filtrate</td>
<td>0.000</td>
</tr>
<tr>
<td>Mycelium</td>
<td>1.010</td>
</tr>
</tbody>
</table>
TABLE IV
Oligosaccharides produced from $\beta$-Glucan by Various Enzyme Preparations
(m, present in hydrolysate and mobile in borate; i, present in hydrolysate and immobile in borate; - not detected in hydrolysate)

<table>
<thead>
<tr>
<th>Group*</th>
<th>Chromatography $\log \frac{1 - R_f}{R_F}$</th>
<th>Electrophoresis $Mg.$</th>
<th>Presence in hydrolysate and borate mobility</th>
<th>$\beta$-linkage distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calo.*</td>
<td>Obs.</td>
<td>Calo.*</td>
<td>Obs.</td>
</tr>
<tr>
<td>1**</td>
<td>0.90</td>
<td>0.90</td>
<td>0.69</td>
<td>0.69</td>
</tr>
<tr>
<td>2</td>
<td>1.00</td>
<td>1.00</td>
<td>0.69</td>
<td>0.69</td>
</tr>
<tr>
<td>3</td>
<td>1.12</td>
<td>1.20</td>
<td>0.54</td>
<td>0.55</td>
</tr>
<tr>
<td>4</td>
<td>1.22</td>
<td>1.24</td>
<td>0.54</td>
<td>0.53</td>
</tr>
<tr>
<td>5</td>
<td>1.32</td>
<td>-</td>
<td>0.54</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>1.33</td>
<td>-</td>
<td>0.44</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>1.43</td>
<td>1.42</td>
<td>0.44</td>
<td>0.42</td>
</tr>
<tr>
<td>8</td>
<td>1.53</td>
<td>1.54</td>
<td>0.44</td>
<td>0.40</td>
</tr>
<tr>
<td>9</td>
<td>1.64</td>
<td>-</td>
<td>0.44</td>
<td>-</td>
</tr>
</tbody>
</table>

* See standardisation graph, Fig. 6.

** From standardisation graph, Fig. 7.

*** These numbers have the same significance as in Preece, Garg and Hoggan, 1960. Tables II, III and IV.
**TABLE V**

Oligosaccharide Production from \(\beta\)-Glucan

(+++ abundant, ++ moderate, + trace, - not detected)

<table>
<thead>
<tr>
<th>Group</th>
<th>Characterisation</th>
<th>Structure (\beta)-linkages</th>
<th>Enzyme source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Culture filtrate</td>
</tr>
<tr>
<td>1</td>
<td>Laminaribiose</td>
<td>(G.3.G^)</td>
<td>+++</td>
</tr>
<tr>
<td>2</td>
<td>Cellobiose</td>
<td>(G.4.G^)</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Laminaritriose</td>
<td>(G.3.G.3.G^)</td>
<td>+</td>
</tr>
<tr>
<td>4a</td>
<td>Other tri-saccharides</td>
<td>(G.4.G.3.G^)</td>
<td>+++</td>
</tr>
<tr>
<td>b</td>
<td></td>
<td>(G.3.G.4.G^)</td>
<td>-</td>
</tr>
<tr>
<td>7a</td>
<td>Tetrasaccharides</td>
<td>(G.4.G.3.G.3.G^})</td>
<td>+</td>
</tr>
<tr>
<td>c</td>
<td></td>
<td>(G.3.G.3.G.4.G^)</td>
<td>-</td>
</tr>
<tr>
<td>c</td>
<td></td>
<td>(G.3.G.4.G.4.G^})</td>
<td>-</td>
</tr>
</tbody>
</table>

I See Table \(V\).

II \(G\) represents a glucosyl residue, with \(G^\) the reducing end of the molecule;

3. represents a \(\beta-1,3\)- and 4. a \(\beta-1,4\)-glucosidic linkage.
<table>
<thead>
<tr>
<th>Group</th>
<th>Characterisation (all straight-chain compounds)</th>
<th>Acid hydrolysis</th>
<th>Enzymic hydrolysis</th>
<th>Parrish et al.</th>
<th>Present results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Laminaribiose</td>
<td>+ + + + + + +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Celllobiose</td>
<td>+ + + + + + +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Laminaritriose</td>
<td>+ + + + + + +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4a</td>
<td>$\beta$-cellobiosyl-3-glucose</td>
<td>+ + + + + + +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>$\beta$-laminaribiosyl-4-glucose</td>
<td>+ + + + + + +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Cellotriose</td>
<td>+ + + + + + +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Laminaritetraose</td>
<td>+ + + + + + +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7a</td>
<td>$\beta$-glucosyl-4-laminaritriose</td>
<td>+ + + + + + +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>$\beta$-laminaribiosyl-4-laminaribiose</td>
<td>+ + + + + + +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c</td>
<td>$\beta$-laminaritriosyl-4-glucose</td>
<td>+ + + + + + +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8a</td>
<td>$\beta$-cellotriosyl-3-glucose</td>
<td>+ + + + + + +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>$\beta$-cellobiosyl-3-celllobiose</td>
<td>+ + + + + + +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c</td>
<td>$\beta$-glucosyl-3-cellotriose</td>
<td>+ + + + + + +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Cellotetraose</td>
<td>+ + + + + + +</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FIG. 1 - Reduction (mg. per 100 mg. original β-glucan) in oligosaccharides and glucose.

FIG. 2 - Reduction (mg. per 100 mg. original β-glucan) in dextrin.

FIG. 3 - Loss of dextrin (% original β-glucan) during enzymolysis.

FIG. 4 - Reducing power (µg. per mg.) of residual dextrin.
FIG. 5 - Comparative hydrolyses with barley endo-β-glucanase, culture filtrate and mycelial enzyme.
**FIG. 5**

Graphs showing the production of glucose per 100 mg initial substrate over time of enzymolysis (HR) for different substrates and sources:
- **β-Glucan**
  - Barley
  - Mycelium
  - Filtrate
- **Laminarin**
  - Barley
  - Mycelium
  - Filtrate
- **Cellodextrin**
  - Barley
  - Mycelium
  - Filtrate
FIG. 6 - Chromatographic reference diagram for gluco-oligosaccharides containing β-1,3- and β-1,4-linkages. Reference points were provided by compounds 1, 2, 3 and 4.

G, glucose; GC, cello-oligosaccharides; GL, laminari-oligosaccharides.
NO. OF GLUCOSE RESIDUES AS REVEALED ELECTROPHORETICALLY.

FIG. 5
FIG. 7 - Electrophoresis reference diagram for gluco-oligosaccharides (in sulphite). Reference points were provided by compounds 1, 2, 3 and 4.
Preece and Hoggan (1956) established an equation correlating reducing power and reciprocal specific viscosity of $\beta$-glucan samples, and also increments of reducing power and changes in reciprocal specific viscosity of $\beta$-glucan under the influence of endo-$\beta$-glucanase. The endo-$\beta$-glucanase activity was discussed on the assumption that the decrease of viscosity to half its original of a substrate solution by endo-action would involve doubling the initial reducing power of that solution. It was shown that when barley enzyme preparation was treated with phenylmercuric nitrate, its action on $\beta$-glucan, satisfied the equation. They also showed that this relationship between reducing group production in endo-action and viscosity change did not persist when the reducing groups present exceeded about 1.0%, when calculated as glucose equivalent percentage of substrate. With this limitation, the equation was used in testing the present mould enzyme preparations, and it was found that the culture filtrate enzyme possessed endo-activity only and the mycelial enzyme apparently possessed both endo- and exo-activities. Whether this exo-activity was real, or whether it was due entirely to other factors
such as oligosaccharases is yet to be determined.

Preece and Garg (1961) employed a different method to compare quantitatively the joint endo- and exo-activities of enzyme preparations with the activity of endo-$\beta$-glucanase alone, and this quantitative approach was also meant to give information on the relative dispositions of 1,3- and 1,4-linkages in the $\beta$-glucan molecule. In each degradation, they measured per cent recovery of high molecular material at different time intervals, reducing power increase in the recoverable high molecular material and also reducing power increase due to glucose and oligosaccharides production. The same idea and measurements were applied to give knowledge of comparative enzymolyses of barley $\beta$-glucan with barley, mould culture filtrate and mycelial enzymes. Other substrates were used to study linkage specificity of the enzymes.

Extracellular (culture filtrate) mould enzyme. — Though the initial activities (viscometric) for the three enzymes, barley, culture filtrate, and mixture of the two were the same in $\beta$-glucan degradation, different rate of degradation is observed from the progress curves of glucose-oligosaccharide reduction production with prolonged time (Table I). Measurable amounts of
glucose-oligosaccharide reduction are evident after 6 hrs. and further production of glucose-oligosaccharide reduction is almost linear with time. The barley enzyme is about 8 times as active as the culture filtrate enzyme and the mixture about 5 times. Similarly, the mixture gives result between those for barley and filtrate enzymes for reduction in the residual dextrin; taking the reciprocals of times required to reach a given degree of reduction (1.5%) due to dextrin (Fig. 2), the three activities come out in approx. ratio 2:1.5:1, which is different from the above ratio 8:5:1, but the mixture again gives approximately the mean value. However, to take reduction due to dextrin as a measure of activity is a very crude approximation, since it is obvious that rapid loss of dextrin is accompanied by transferring reducing groups to the alcohol-soluble (glucose-oligosaccharide) fraction.

When loss of dextrin (Fig. 3) is considered to give more useful results, it is found that for equal loss of dextrin (at 8% loss), the reciprocal times for three enzymes come to the ratio 2.8:2.2:1. It seems, therefore, that after the earliest stages with enzymes by definition equally efficient viscometrically - the barley enzyme is approximately 3 times as efficient as that from the culture filtrate in
eliminating dextrin. This ratio is not at variance with the ratio of 2:1.5:1 for reducing groups in residual dextrin but rather accords, because if the dextrin is more efficiently hydrolysed, there will be less to recover by alcohol precipitation and the apparent activity will be less.

The ratio for glucose-oligosaccharide reducing group production by barley and culture filtrate enzymes is 8:1, and this difference may in part be due to different reducing potentials of 1,3- and 1,4-linked oligosaccharides, but the difference between the ratios 8:1 for glucose-oligosaccharide and 3:1 for loss of dextrin is too great to be accounted for in this way. This difference, in fact, gives indirect evidence that the barley enzyme contains a greater proportion of oligosaccharases relative to endo-β-glucanase than is found in culture filtrate enzyme. This is confirmed directly by Table III.

The complexity of residual dextrin from barley enzyme action was found smaller by reducing power measurement than the complexity of residual dextrin produced from culture filtrate enzyme action. This is expected from the results discussed earlier; the barley enzyme gives a higher proportion of glucose-oligosaccharides with greater loss of dextrin than the
mould enzyme. The mixture again gives intermediate results (Fig. 4). It would seem, from all these results (Table I, Figs. 1-4), that the effect of mixing the two enzymes is merely to dilute an important factor in the barley enzyme preparation, and the dilution effect seems rather accurately to give the arithmetic mean.

Initially, the barley and culture filtrate enzymes were adjusted to have the same chain-splitting capacity (measured viscometrically), but differences appeared with prolonged action of enzymes. Preece and Garg (1961) have shown that the barley enzyme system is rich in endo-$\beta$-1,3-ase but it has also endo-$\beta$-1,4-ase activity, and the residual dextrin is rich in $\beta$-1,4-linkages. Hence it seems logical to attach great importance to the splitting of $\beta$-1,3-linkages in the degradation of $\beta$-glucan. However, as the results of Table II and Figure 5 show, the barley enzyme is — as expected — far more effective in prolonged degradation of $\beta$-glucan than is the extracellular mould enzyme. Barley enzyme is efficient in degrading laminarin and cellodextrin whereas mould culture filtrate enzyme degrades laminarin slowly and is without any action on cellodextrin. Thus, the mould culture filtrate enzyme has $\beta$-1,3-ase activity, and it is devoid of $\beta$-1,4-ase activity — at least against
molecules of the chain-length of this cello-
dextrin. It seems reasonable to suggest an
overall deficiency in endo-β-1,4-ase activity
for the mould culture filtrate enzyme.

The activity of barley enzyme on laminarin
is far more than the activity of mould culture
filtrate enzyme. This was found by measuring
total reducing power increase against time in
laminarin degradation using both enzymes having
equal endo-β-glucanase (viscometric) activity.
As both enzymes had equal initial activity, and
the barley enzyme had some β-1,4-ase activity in
addition to β-1,3-ase activity, the overall
activity of barley enzyme should be slower than
the mould culture filtrate enzyme. The reverse
activity is, in fact, found; the answer to this
must be in concomitant oligosaccharase activities,
in which the barley enzyme is known to be rich.
The results of Table III show that the mould
culture filtrate enzyme is devoid of laminaribiase
and cellobiase activity. On these bases, there-
fore, the differences between the two enzymes
seem to be reasonably accounted for: barley
enzyme provides endo-β-1,3-ase, endo-β-1,4-ase
and oligosaccharases, whilst the mould culture
filtrate enzyme gives endo-β-1,3-ase alone.

This view gives unexpected importance to the
role of β-1,4-ase in the degradation of β-
glucan. It is true that $\beta$-1,3-ase alone can bring about an important degree of hydrolysis, giving a residual dextrin with a reducing power of approximately 20 $\mu$g. per mg.; this is thereafter slowly "saccharified" (with diminution in dextrin amount but little change in its character), presumably in a manner analogous to that in which $\alpha$-amylase changes to a slow saccharifying action after its initial rapid dextrinisation action; however, the "limit dextrins" here would appear to be of longer molecular size than those from starch. On the other hand, in presence of the $\beta$-1,4-ase provided by barley (but not by the mould), the initial dextrinisation proceeds more quickly and completely, giving a smaller limit dextrin with a higher reducing power (approx. 30 $\mu$g. per mg.). It is unfortunate that useful estimates of the relative molecular sizes of these two dextrins are not available; it would be rash to attempt such estimates from these reduction figures. However, whatever their relative molecular sizes may be, it is indisputable that loss of dextrin is quicker with barley enzyme, which means - in effect - that whatever the relative rates of dextrinisation, the rate of saccharification is greater when both $\beta$-1,3-ase and $\beta$-1,4-ase activities are present. Moreover, the
differences are further magnified by oligo-
saccharases provided by the barley enzyme. It
is clear that, on this hypothesis of degradation,
the "average" effect observed with a mixture of
the two enzymes is that which would be expected.

It is important to mention here that the
overall activity of the culture filtrate enzyme
preparation in respect of total (viscometric)
endo-β-glucanase activity is far greater than
that of a preparation from barley, the culture
filtrate enzyme preparation needing some 40-fold
dilution to allow comparison in total (visco-
metric) activity with barley enzyme preparation.

**Intracellular (mycelial) mould enzyme.** – From
previous information and from Table III, the
activity ratios of barley and mycelial enzyme
preparations are 1:3 for endo-β-glucanase, 1:9
for laminariobiase, and 1:24 for cellobiase.
Dilution of the mould enzyme to give 1:1 for
endo-β-glucanase, therefore, will give 1:3 for
laminariobiase and 1:8 for cellobiase. It
accordingly follows that discussion of the
glucanase activity of this mould preparation will
be rendered more complex by the presence of the
oligosaccharases, and it is not yet known whether
these biases (or their concomitants) are without
action on higher oligosaccharides.
It will be noted from Figures 1 and 2 that, while the reducing groups in glucose and oligosaccharides with the mycelial enzyme are closely similar in amount to those with the barley enzyme, reduction due to dextrin with the mycelial enzyme is similar to that with culture filtrate enzyme. Clearly, the latter observation is more important than the former in assessing the progress of hydrolysis, and it leads to the assumption that culture filtrate and mycelial enzymes are of similar type. Again, dextrin loss and dextrin reduction per mg. with the mycelial enzyme are much closer to the corresponding results for the culture filtrate enzyme than they are to the barley factor. It must be assumed that the mycelium provides endo-$\beta$-1,3-ase, as does the culture filtrate.

The standard method for exo-$\beta$-glucanase determination gives a result for the mycelial enzyme of the same magnitude as with barley enzyme (Section I). For equal endo-$\beta$-glucanase activity, mycelial enzyme needs 3-fold dilution which brings down the exo-activity to less than one-half of that for barley. It has been shown (Preece and Garg, 1961) that barley yields an exo-$\beta$-1,4-ase. At first sight and taking into consideration results of cellodextrin hydrolysis (Table II), it seems that mycelial enzyme also
contains this factor, but on further consideration this may not be the fact. It has been postulated (Preece and Garg, 1961) that barley enzyme gives laminaribiase and a general glucosidase, but this idea does not seem to help here, where laminaribiase activity is less than that shown against cellobiose. The explanation of the differences observed might well lie in the possession by the oligo-β-1,4-ase of ability to attack chains of moderate length (as in cellobextrin) and a restriction of activity of oligo-β-1,3-ase to chains of short length. This would account for the observed attack on the cellobextrin, but would not account for the relatively inefficient attack of the mycelial enzyme (though it is more efficient than the culture filtrate enzyme) as compared with the barley enzyme, unless the mould mycelial and barley 1,3-ases differ in the length of chain attackable. There are several problems here that would repay further study: in particular, what is the product of action of the mycelial enzyme on cellobextrin - is it glucose (cf. glucamylase) or cellobiase (cf. β-amylase)? And what differences exist between the oligo-β-1,3-ases of mycelial and barley enzymes?

In short, the mycelial enzyme contains endo-β-1,3-ase, oligo-β-1,3-ase and oligo-β-1,4-ase
activities, though the limits of action of the last two remain to be determined. It is preferred to leave open the question whether exo-β-glucanase is also present.

Oligosaccharides from barley β-glucan. - Three types of degradation, with barley, mould culture filtrate and mycelial enzymes, were used to obtain the oligosaccharides. All oligosaccharides will be discussed together, but it will not be a strict comparison as the enzymes used were not pure. The barley enzyme used was treated with phenylmercuric nitrate to suppress exo-activity but still the preparation, as is known from the earlier and present work, contains endo-β-glucanase accompanied by a variety of oligosaccharases. The mould culture filtrate enzyme used contains endo-β-1,3-ase unaccompanied by endo-β-glucanase and typical oligosaccharases, whereas the mould mycelial enzyme used contains, like barley enzyme, endo-β-glucanase and a variety of oligosaccharases. The present evidence, therefore, relates not to the range of oligosaccharides which can be produced by the various endo-enzymes, but to the oligosaccharides which - under the experimental conditions chosen - escape further degradation.

Before discussing the oligosaccharides of
the present work, it would be better to review the oligosaccharides already known to be produced by various degradations. Thus, Ono and Dazai (1959) obtained $\alpha-L-\beta-D$-glucopyranosyl-(1→4)-$\alpha-L-\beta-D$-glucopyranosyl-(1→3)-D-glucose ($\beta$-celllobiosyl-3-glucose compound 4a of Table V) from a barley mash fermented by the amylo process.

Parrish, Perlin and Reese (1960) with an enzyme ("laminarinase") from Rhizopus arrhizus obtained from oat $\beta$-glucan this compound and also the tetrasaccharide, $\beta$-cellotriosyl-3-glucose (8a of Table V), whilst with "cellulase" from Streptomyces sp. they obtained $\beta$-laminaribiosyl-4-glucose (4b), and two tetrasaccharides, $\beta$-cellobiosyl-3-celllobiose (8b) and $\beta$-glucosyl-3-cellotriose (8c). Peat, Whelan and Roberts (1957), using partial acid hydrolysis of oat $\beta$-glucan, characterised laminaribiose (1), celllobiose (2), two trisaccharides with mixed linkages (4a, 4b), and cellotriose (5, Tables IV-VI). Smith and Sorger-Domenigg (1960), also using acid hydrolysis, showed oat $\beta$-glucan to contain blocks of 2 and 3 contiguous 1,3-linked glucose residues as well as isolated $\beta$-1,3-linkages.

Preece, Garg and Hoggan (1960) obtained the same oligosaccharides from both barley and oat $\beta$-glucans, and suggested that the only difference between these two glucans lay in the possession
of a greater proportion of $\beta-1,3$-linkages by barley $\beta$-glucan. With endo-$\beta$-glucanase from barley they obtained from both glucans, compounds corresponding to structures 1, 2, 3, 4a, 4b, 6, 7a, 7b and 7c of Tables IV-VI. More recently, Moscatelli, Ham and Ricks (1961) with an enzyme preparation from *Bacillus subtilis*, and using barley $\beta$-glucan as substrate, obtained 1, 3, 4a and 8a. This information is summarised in Table VI.

It will be observed that, of the 14 theoretically-possible straight-chain oligosaccharides from di- to tetra-saccharide level, all have been characterised, though some have been obtained only by acid hydrolysis and others only by enzymic degradation. Of those obtained by enzymic degradation, Nos. 1, 2, 3, 4a, 4b, 8a, 8b and 8c leave no room for doubt. The oligosaccharides corresponding to the structures 4a and 8a, are major products of the laminarinase enzyme of *Rhizopus arrhizus* (Parrish et al., 1960) and also of the enzyme of *Bacillus subtilis* (Moscatelli et al., 1961), and it is extremely interesting to note that these are also major products (noting that the product of the structure 8a has high borate mobility) of the *Penicillium* culture filtrate enzyme, which therefore resembles these two enzymes. The striking
difference is that both *Rhizopus arrhizus* enzyme and *Bacillus subtilis* enzyme do not produce any cellobiose in \( \beta \)-glucan degradation whereas the *Penicillium* culture filtrate enzyme produces cellobiose; though it is small in amount, its production is definite. The *Penicillium* culture filtrate enzyme has no cellobiase enzyme but there was no mention of this enzyme in two other enzyme preparations. The barley endo-\( \beta \)-glucanase produces oligosaccharides characteristic of both laminarinase of *Rhizopus arrhizus* and cellulase of *Streptomyces* sp. action; it might therefore be held that the barley enzyme, consistent with previous belief, contains both types of enzyme and the same is true for the mycelial enzyme. However, it must be remembered, what has already been said, about the influence of oligosaccharases, which are abundantly present in both the barley and mycelial enzymes and which could upset the results. However, taking only what appears to be well founded, it is reasonable to conclude that (a) the culture filtrate enzyme is of laminarinase type, whilst the barley endo-enzyme is a combination of both laminarinase and cellulase (Parrish et al., 1960); and (b) the principal oligosaccharides obtained enzymically from barley \( \beta \)-glucan are laminaribiose, cellobiose, laminaritriose, two trisaccharides with
one $\beta$-1,3- and one $\beta$-1,4-linkage, and three tetrasaccharides with one $\beta$-1,3- and two $\beta$-1,4-linkages.

This view of the nature of the culture filtrate enzyme is entirely in accord with that reached as a result of the purely quantitative study reported above, namely that — in contrast to a barley preparation combining $\beta$-1,3- and $\beta$-1,4-activities — the culture filtrate enzyme shows substantially only $\beta$-1,3-activity. Moreover, while the barley enzyme is rich in oligosaccharases, the filtrate enzyme is not.

One important point still remains to be discussed. All enzymolyses of $\beta$-glucan have produced glucose, though the amounts vary with the enzyme source. In the case of culture filtrate enzyme, it is in relatively small amount. It follows that glucose can be a direct product of endo-$\beta$-glucanase action, as was claimed by Whitaker (1953) for glucose production from cellulose by a purified enzyme from Myrothecium verrucaria. In the case of the barley enzyme, therefore, the glucose has a dual origin — direct from endo-$\beta$-glucanase action and indirect by oligosaccharase action on the oligosaccharide products of endo-$\beta$-glucanase action.
SUMMARY

1. The extracellular endo-β-glucanase preparation from the mould Penicillium chrysogenum HW3 has β-1,3-ase activity but no β-1,4-ase activity; it is devoid of laminaribiase and cellobiase activities.

2. Its action when prolonged can produce glucose directly from β-glucan.

3. Major oligosaccharide products of its action on β-glucan are laminaribiose, β-cellobiosyl-3-glucose and a tetrasaccharide which is apparently β-celiotriosyl-3-glucose.

4. The mycelial endo-β-glucanase is accompanied by considerable oligosaccharase activity, and the major products of its action on β-glucan are cellobiose and β-cellobiosyl-3-glucose; additionally, some β-laminaribiosyl-4-glucose is produced.

5. Whether a true exo-β-glucanase is present in the mycelial preparation cannot be decided on the present evidence, but the results can be explained in terms of endo-β-1,3-ase and oligosaccharase activities.

6. It is noted that barley endo-β-glucanase has both β-1,3-ase and β-1,4-ase activities, and in preparations from barley is accompanied by exo-β-glucanase and oligosaccharases.
Differences between the activities of the barley and mould preparations are clearly accounted for on this basis.
SECTION III
ENZYMOLYSIS OF ARABOXYLAN

INTRODUCTION

To introduce this section, it will be convenient to recapitulate and extend something of what has already been said of the pentosans in the general Introduction. Tollens and Stone in 1883 isolated a group of polysaccharides from brewer’s spent grains. On hydrolysis, xylose and arabinose were produced. Schulze (1891) isolated similar substance from plant cell-walls by extraction with 4% sodium hydroxide solution. Since he thought that these substances might be intermediates in cellulose formation, he termed them hemicellulose. Later work showed that although these substances contained primarily pentoses with some hexoses and uronic acids. Using the same method, Schulze (1892) isolated similar hemicelluloses from straws of wheat and rye, and from wheat and rye flour. Again, xylose and arabinose were detected in the hydrolysates. Wróblewski (1897) isolated from diastase a soluble polysaccharide which yielded L-arabinose on hydrolysis; he termed it "araban" but more recently Ford and Peat (1941) extracted a polysaccharide from a wheat $\beta$-amylase preparation with dilute alcohol, and found xylose and galactose in addition to arabinose residues in
the hydrolysate. A soluble araban of wheat flour was also described by Geoffrey (1937) who found only arabinose in the hydrolysate of a laevorotatory polysaccharide prepared from an aqueous extract of wheat-flour. All these polysaccharides were readily soluble in water and appeared to be composed principally of anhydro-pentose sugar residues. The difference in composition between isolates of different workers may be explained by the variety of procedures used in isolation, and is also due to difficulties in characterising the mixture of sugars, especially for early investigators. Later O'Dwyer (1923) detected xylose, arabose, mannose and galactose in the hydrolysates of hemicellulose preparations from American white oak. The detection of uronic acid residues in some beech-wood hemicelluloses by O'Dwyer (1926) and in spent grain by Preece (1931) revealed the complex nature of these hemicellulosic polysaccharides.

Preece and Hobkirk (1954) showed that there are two types of hemicelluloses present in cereal grains; the husk-type hemicelluloses, also present in smaller amounts throughout the grain, containing mainly pentosan (together with uronic acid) and giving a low specific viscosity in water solution; and endospermic hemicelluloses which are free from uronic acid, showing high
specific viscosity in aqueous solution. They also showed that endospermic hemicelluloses might act as precursors of soluble hemicelluloses during autolysis or germination but their solubilisation involved a decrease in the xylan:araban ratio of pentosan-rich fractions. A similar view was also proposed by Preece, Aitken and Dick (1953) on the basis of an autolysis experiment with different varieties of barley.

Lindet (1903) used mercuric sulphate to inactivate the enzyme before extraction of barley at various stages of germination. Metallic salts were removed from the extract with baryta and sulphuric acid. Using alcoholic fractionation he obtained a non-cupric-reducing gum, identical with O'Sullivan's β-amylans which yielded a mixture of pentoses on hydrolysis. Brown (1906) investigated the water-soluble polysaccharides of barley and malt, though the products—owing to the method of extraction employed—do not necessarily represent the water-soluble form in the original barley. Malt extract was used to liquefy the homogenate prepared by treating ground barley with boiling water. A crude amylase was precipitated with alcohol and the hydrolysate was found to be composed of glucose and pentoses. These results suggested that amylans and starch were not related.
Preece and Hobkirk (1953) commenced a more detailed examination of fractionation of gums from five cereals. Among other fractions, they obtained a reasonable amount of pure arabinoxylan; such arabinoxylan was substantially absent from maize, oats and barley, but was present in smaller amounts in wheat.

Soluble pentosans of wheat-flour were investigated by Perlin (1951) and these pentosans on graded hydrolysis preferentially lost L-arabinose units, leading to formation of an insoluble residue composed principally of anhydro-D-xylose units. Methylation analysis of the pentosans yielded: 2, 3,5-trimethyl-L-arabinose (3 moles), 2,3-dimethyl-D-xylose (3 moles), 2-methyl-D-xylose (1 mole), and D-xylose (1 mole). Periodate oxidation coupled with graded hydrolysis indicated that the pentosans from wheat-flour were straight chain compounds of anhydro-D-xylose residues formed by $\beta$-1,4-linkages, to which were appended single units of anhydro-L-arabofuranose through 1,2- and 1,3-linkages, the latter linkages being predominant. In some respects this structure closely resembled that of pentosans isolated from other sources; for example the xylans of certain woods and straws had been shown to consist principally of D-xylopyranose residues linked by 1,4-$\beta$-glyco-
sidic bonds, and associated with very little amount of arabinose units (Hampton et al., 1926; Haworth et al., 1934) or free of arabinose (Freeman et al., 1932). These xylans were only sparingly soluble in water but more soluble in dilute sodium hydroxide. Pentosans isolated from wheat-straw were found to be soluble only in boiling water or dilute sodium carbonate solution at room temperature; they were predominantly xylans with 5-10% uronic anhydride, but contained in addition 12-19% arabinose. Pentosans from wheat-flour contained 35% or more arabinose and were soluble in cold water. From these results Perlin concluded that a series of xylans existed in which the solubility increased with increasing arabinose content. Gradual removal by hydrolysis of arabinose units from soluble pentosans yielded compounds of decreasing solubility but increasing xylose content. The cleavage proceeded to the point where a substantially pure xylan remained which was sparingly soluble in alkali and chemically closely similar to the known xylans of wood and straw. This theory was discussed by Preece and Hobkirk (1953). Using ammonium sulphate fractionation they isolated a hexosan-free fraction of pentosan from rye, and also isolated pentosan-rich fractions from barley, wheat, oat and maize by 40% saturation of
ammonium sulphate. The pentosans from wheat showed increasing solubility in water with increasing araban content, supporting Perlin's hypothesis. However, from barley and rye, the most soluble product contained least araban.

The contradictory results were explained thus: the type of molecule isolated by Perlin might be built up on principal xylan chains of different lengths, in which case the short chain molecules would have inherently greater solubility in water though associated with fewer or shorter araban side-chains. Fractions of greater solubility isolated by Preece and Hobkirk might then be built up on molecules of similar xylan chain length, but with more, or longer, araban side-chains.

Xylan isolated from various sources usually yielded some arabinose on hydrolysis. Originally it was not certain whether the arabinose represented a component of the xylan molecule or a component of an araban admixed with a xylan containing xylose units. Adams (1952) showed that the data from methylation and hydrolysis of a xylan from wheat straw were consistent with a structure in which single arabinose units were attached along a chain of \( \beta-1,4 \)-linked xylo-pyranoside units. However, proof of this structure required isolation from the hydrolysed
xylan of the xylose and arabinose containing oligosaccharides. Dilute acids were not a suitable hydrolysing agent for this purpose since they split arabofuranoside residues more readily than xylopyranose residues. A purified cellulolytic enzyme preparation from *Myrothecium verrucaria* (Whitaker, 1953) was found to hydrolyse linear chains of $\beta$-1,4-linked xylopyranose units. By hydrolysing wheat-straw xylan (Adam, 1952; Bishop and Whitaker, 1955) with this enzyme, mixed arabinose-xylose oligosaccharides were obtained. No evidence of oligosaccharide synthesis was found when the enzyme was incubated under the same conditions with mixtures of arabinose and xylose. The disaccharide produced during enzymolysis of wheat-straw xylan was only of xylose but all the other oligosaccharides were mixed.

Preece and MacDougall (1958) used barley enzymes to degrade araboxylan; the process involved four enzymes or enzyme systems: (1) arabinosidase, liberating free arabinose from the araboxylan and from oligosaccharides; (2) endo-xylanase, degrading xylan chains, whether or not these carried arabinose side chains; (3) exo-xylanase, producing xylobiose from araboxylan and from oligosaccharides after some degree of arabinose removal and perhaps,
also from xylotriose; and (4) xylobiase. The oligosaccharides characterised were xylobiose, xylotriose, xylotetraose and the higher oligosaccharides containing varying proportions of arabinose residues in side-chains.

Aspinall et al. (1960) used a number of enzyme preparations for araboxylan degradation and the enzymes could break down araboxylan to the constituent sugars. Only xylobiose and its polymer homologues could be detected as products of incomplete hydrolysis. Conchie and Levy (1957) showed that the glycosidic activities of certain enzymes could be inhibited in presence of aldonolactones of the corresponding configuration. Aspinall et al. (1960) degraded rye-flour and cocksfoot grass araboxylan in the presence of 0.1 M-L-arabonolactone and a series of oligosaccharides were detected chromatographically containing both xylose and arabinose residues. A trisaccharide, 0-L-arabinofuranosyl-1,3-O-β-D-xylopyranosyl-β-1,4-D-xylose (cf. Bishop, 1956), was characterised amongst them. The trisaccharide was not an artefact of enzymic synthesis. The carbohydrate concentration in digests was low, which would favour hydrolysis rather than enzymic transfer of arabinofuranosyl residues to a xylobiose acceptor. A control experiment also showed that, under the conditions
employed during enzymic hydrolysis of the polysaccharides, free arabinose was not transferred to xylobiose or xylotriose.

The arabinoxylan prepared from rye-flour by Preece and Hobkirk (1954) was used by Aspinall and Sturgeon (1957) for a structural investigation. Xylose (60%), arabinose (29%) and glucose (5%) were obtained after hydrolysis. Glucose was considered to be an impurity and not part of the xylan molecule. Methylation, followed by hydrolysis, produced 2,3,5-tri-O-methyl-L-arabinose (30%), 2,3-di-O-methyl-D-xylose (36%), 2-O-methyl-D-xylose (31%) and D-xylose (2.5%).

With additional evidence from periodate studies, Aspinall and Sturgeon (1957) concluded that rye arabinoxylan was a linear molecule containing chains of 1,4-linked \( \beta \)-D-xylopyranose residues with approximately every second xylose residue carrying a terminal L-arabinofuranose residue linked through position 3.

The selective oxidation of primary alcoholic groups in the simple hexopyranosides by gaseous oxygen in presence of a platinum catalyst provides the simplest method for the formation of glycosiduronic acid (Mehltzetter, 1953). This procedure has been used by Aspinall and Cairncross (1960) in the oxidation of a proportion of the primary alcoholic groups in rye-flour
araboxylan, which are found only in the terminal L-arabinofuranose residues. They isolated the aldobiouronic acid of 3-D-xylose-L-arabinofuranoside and confirmed the presence of terminal L-arabinofuranose residues in the polysaccharide, attached as single-unit side chains to the position 3 of D-xylopyranose residues in the 1,4-linked basal xylan chain.

Much was not known about the disposition of the L-arabinofuranose residues as side-chains in the backbone of β-1,4-linked D-xylose residues. Ewald and Perlin (1959) applied a modified Barry's method to the degradation of periodate-oxidised wheat-flour arabinoxylan with phenylhydrazine. They also degraded rye-flour arabinoxylan by Smith's procedure to get periodate-resistant fragments, in the form of glycerol-glycosides. In both series of experiments, they isolated the degradation products which contained one, two and three xylose units. From this it was concluded that, in the arabinoxylan, side-chains were attached to isolated D-xylose residues and to adjacent D-xylose residues, less frequently to three, but not to four or more contiguous D-xylose residues along the chain. More evidence is required to generalise the conclusion.

Like β-glucan, though considerable knowledge has been obtained about the structure
and the pentosanase systems, more work on this line should reveal the nature of both substrates and pentosanase systems. It was shown by Preece and MacDougall (1958) that cereal enzymes had very weak degradating capacity. In the present work, a highly active enzyme preparation from a *Penicillium* species has been used. As in the work on the enzymolyses of β-glucan, it has thus been possible to compare degradation of samples of the same substrate by enzymes from different sources. Thus, the varying potentialities of the different enzyme complexes can be brought in relation to one another.
EXPERIMENTAL

Materials and Methods

Preparation of pancreatic $\alpha$-amylase. - The method of Meyer et al. (1947) has been followed for the preparation of this enzyme. Crude pancreatic was extracted with 0.5 N sodium acetate solution for 12 hours at about $5^\circ$C with occasional stirring. Insoluble material was removed by centrifugation. A small portion of enzyme extract was boiled, cooled and added to the remainder of the extract which was then dialysed for 2 days against running water. This procedure has been reported (Meyer et al. 1947) to delay enzyme inactivation during dialysis. Again, insoluble material was removed by centrifugation and the enzyme was precipitated by adding 3 volumes of acetone and taken to dryness in the usual way. The enzyme preparation was preserved in the refrigerator.

Preparation of araboxylan from rye. - The method of Preece and MacDougall (1958) was followed. 1 Kg. of rye ground by hand-mill was enzyme-inactivated as in the case of $\beta$-glucan preparation. Araboxylan extraction was also carried out like $\beta$-glucan extraction but at room temperature. Extracts were mixed, centrifuged, passed
through a Celite bed and concentrated to a small volume. From this, precipitate at 20% ammonium sulphate concentration was obtained and removed by centrifugation and discarded. Precipitate at 50% ammonium sulphate concentration level was collected, dissolved in the minimum volume of water and dialysed for 2 days against running water using thymol as antiseptic. After dialysis, the solution was treated with pancreatic α-amylase preparation at 37°C for 24 hours. The treated solution was again dialysed for 2 days as before. Araboxylan was precipitated, after removing the precipitate formed during dialysis, with an equal volume of acetone and the product was taken to dryness in the usual way.

Preparation of enzymes. - The methods of preparing mould culture filtrate and mycelial enzymes have already been described in Section I. The barley enzyme preparation used in the work of this section was different from that used in the previous section.

The method of Preece et al. (1954) was used for this new preparation. Barley (Ymer) ground with the hand-mill was extracted with 0.6% sodium chloride solution (100 ml. per 15 g.) at room temperature for 1 hour with occasional stirring. The extract was centrifuged, passed through a bed
of Celite and allowed to stand overnight to permit of autolysis; it was then dialysed for 2 days against running water using thymol as antisepic. After dialysis, the extract was centrifuged to remove any precipitate formed during dialysis and the enzyme was precipitated by adding 4 volumes of acetone and was taken to dryness in the usual way.

**Determination of endo-arabinoxylanase activity.** - The endo-arabinoxylanase activities of different enzyme preparations were measured following the method of Preece and MacDougall (1958) which was a modification of the method of Preece and Aitken (1953). The reaction mixture contained 8 ml. of 0.6875% arabinoxylan solution, 2 ml. of enzyme solution and 1 ml. of acetate buffer of pH 5; all solutions were brought to 25°C before mixing. 10 ml. of the reaction mixture were transferred to an Ostwald viscometer and specific viscosities were found at 25°C at different time intervals. Activity of each enzyme preparation was expressed as increase of reciprocal specific viscosity per mg. of enzyme per hour.

**Quantitative liberation of arabinose and xylose.** - Enzymes from barley, mould culture filtrate and mycelium were tested. For comparison, the
increase in reducing power per hour of a standard reaction mixture was determined for each enzyme solution. The reaction mixture used, which was buffered to pH 5, contained 5 mg. of araboxylan and 0.5 mg. in the case of barley enzyme, 0.02 mg. in the case of mould culture filtrate enzyme and 0.05 mg. in the case of mould mycelial enzyme per ml. of the reaction mixture. Three parallel conversions were incubated at 37°C, and a 5 ml. aliquot was withdrawn from each at 1 hour, 2 hours and 3 hours. Each time, withdrawn aliquots were placed in the boiling water-bath for 3 min. to ensure enzyme inactivation and the reducing power of each was determined by Somogyi's (1945) copper-reduction method. After plotting reducing power against time, the initial per hour reducing power increase was obtained for each enzyme solution. Mould enzyme solutions were diluted to give reducing power increase per hour equal to that of barley enzyme solution.

Three conversion mixtures were prepared as before using the enzyme solutions with equal initial per hour reducing power increase and incubated at 37°C. Aliquots of 5 ml. were withdrawn from each conversion mixture at 5, 18, 29 and 43 hr.; at the end of the appropriate time, each aliquot was placed in the boiling water-bath for 3 minutes and, after cooling, 1 ml. of ribose
solution (equivalent to 500 µg. of ribose) was added, followed by 12 ml. of 95% ethanol. After shaking and then allowing to stand in the refrigerator for at least 6 hr., the precipitate was centrifuged off and the liquid filtered through Kieselguhr and taken to dryness. The residue was dissolved in a small volume of 30% ethanol and streaked on Whatman 3 MM paper (7½ in. x 3½ in.) and developed in ascending chromatogram for 8 hours using n-propanol-water (78:22, cf. Held et al., 1955); this preliminary run was necessary to remove materials which tended to decrease the efficiency of descending chromatography. Two side strips were cut and sprayed with aniline hydrogen oxalate and heated for 5 min. at 110°C. By placing the strips along the sides of unsprayed paper, paper at the monosaccharide level was cut out and the sugars were eluted by the method of MacLeod (1951). After concentration of the eluate, the residue was dissolved in a small volume of 30% ethanol and streaked on Whatman No. 1 paper with control spotting on both sides of the streak. This time the chromatogram was run in descending flow using the upper layer of n-butanol-ethanol-water (45:5:50) for 3 days at 25°C; separation of arabinose, xylose and ribose thus took place. After obtaining the positions of these sugars in
FIG. 1 - Quantitative liberation of arabinose and xylose from rye araboxylan during its incubation with enzyme preparations from barley, mould culture filtrate and mould mycelium.

x represents xylose
A represents arabinose
FIG. 1

PENTOSE (µG.) PER 5 ML.

TIME (HR.)

BARLEY (O)
MYCELIUM (△)
FILTRATE (X)
MYCELIUM (A)
FILTRATE (A)
BARLEY (X)
control strips, corresponding portions of unsprayed paper were eluted for each sugar as before, taken to dryness and transferred to pyrex test-tube (15 cm. x 1.5 cm.) by washing with 2 ml. of distilled water. Determinations were carried out according to the method of Fernell and King (1953). The amounts of arabinose and xylose corresponding to 500 μg. ribose, and hence to the original 5 ml. aliquot, could therefore be calculated. The linear relationship between sugar concentration and colorimeter reading (EEL colorimeter, No. 608 filter) was the same for all three pentoses.

Test of enzymic synthesis. - Mixtures of arabinose and xylose, each at 0.125 g. per 100 ml., were incubated separately with barley, mould culture filtrate and mould mycelial enzymes in reaction mixtures consisting of 8 ml. sugar solution, 2 ml. enzyme solution and 1 ml. acetate buffer of pH 5. The enzyme solutions contained 1 mg. per ml. of barley enzyme, 0.05 mg. per ml. of mould enzymes. After 48 hours of incubation at 37°C, each reaction mixture was dried over the water-bath and examined chromatographically. No sign of oligosaccharide synthesis was observed.

Enzymolysis of araboxylan. - Enzymolysis of
arabinoxylan was carried out for oligosaccharide analysis only with mould culture filtrate and mycelial enzyme preparations. The reaction mixture was prepared by mixing 80 ml. of 0.3438% arabinoxylan solution, 20 ml. of enzyme solution (each was 0.05 mg. per ml.) and 10 ml. of acetate-buffer of pH 5 and was incubated at 37°C for 48 hours. After this period, the pH of the reaction mixture was adjusted to 7 by adding dilute sodium hydroxide solution and then the temperature of the solution was raised to boiling to ensure enzyme inactivation. To the cooled solution, 4 volumes of 95% ethanol were added to precipitate high molecular materials, the mixture being left in the refrigerator overnight. The precipitate was filtered off and the filtrate was concentrated in vacuum at 50-60°C and was stored in the refrigerator for further analysis.

Separation of oligosaccharides. - The concentrate so obtained after enzymolysis was dissolved in a small quantity of 30% ethanol and streaked on chromatograms of Whatman 3 MM paper which were then irrigated by the descending method using n-butanol-ethanol-water (45:5:50, upper layer) for 13 days at 25°C. Two side strips were cut, sprayed with aniline hydrogen oxalate and heated at 110°C for 5 min. Oligosaccharides separated
Plate I. - Chromatogram to show oligosaccharides produced from rye araboxylan by mould culture filtrate (CF.) and mould mycelial (Myc.) enzymes.

Solvent, Butanol : Ethanol : Water :: 45:5:50

K, Known - cellobiose (cell.).
Plate II. - Chromatogram to show oligosaccharides with different $R_f$ values produced from rye arabinoxylan by mould culture filtrate enzyme.

Solvent, Butanol : Ethanol : Water :: 45:5:50

K, Known - cellobiose (cell.) and raffinose (raff.).
above glucose level were eluted as before and evaporated. These oligosaccharides were further purified by streaking on Whatman 3 MM paper and running chromatogram as before using control spots of each oligosaccharide. Separated oligosaccharides were also individually spotted on Whatman No. 1 paper and the chromatogram was run as before using cellobiose as control spot. \( R_F \) values for each oligosaccharide were measured with reference to cellobiose (0.032). It was found that both culture filtrate and mycelial enzymes gave identical \( R_F \) values for the two series of oligosaccharides and the intensities (visual estimation) of oligosaccharides of the same \( R_F \) value were the same. It was also observed that arabinose and xylose liberation during araboxylan enzymolysis were of similar nature for both enzymes. From these, it appeared that oligosaccharides produced from araboxylan by culture filtrate and mycelial enzymes were the same. All further analyses were carried out only with the oligosaccharides separated from araboxylan degradation by mould culture filtrate enzyme.

**Acid hydrolysis of oligosaccharides.** - Two types of acid hydrolysis, complete and partial, were carried out for oligosaccharide analysis. For
complete hydrolysis, each oligosaccharide was eluted from an unsprayed portion of chromatogram and concentrated. The concentrate was then dissolved in about 0.5 ml. of 0.5 N HCl acid and hydrolysed in a sealed tube by placing in the oven at 105°C for 4 hours. The hydrolysate was taken to dryness at 40°C on a hotplate, with sodium hydroxide as desiccant. The products were separated chromatographically on Whatman No. 1 paper using the upper layer of n-butanol-ethanol-water (45:5:50). Determination of the constituent sugars of each oligosaccharide was carried out by the method of Pernell and King (1953).

It was known that arabofuranose residues in the araboxylan molecule are easily removed during mild acid hydrolysis. For this, oligosaccharide eluted from unsprayed chromatogram was dissolved in 0.5 ml. portion of 1 N acetic acid and hydrolysed in a sealed tube by placing in the oven at 105°C for 4 hours. The products were taken to dryness as before and were used for further examination. After spotting on Whatman No. 1 paper and irrigating the chromatogram with the upper layer of n-butanol-ethanol-water (45:5:50), the products were resolved on the paper. Rf values of different products were measured with reference to cellobiose (0.032).
Plate III. - Chromatogram to show oligosaccharides produced from each separated oligosaccharide from rye arabxylan degradation by mould culture filtrate enzyme after mild acid (1.0 N acetic acid) hydrolysis.

Solvent, Butanol : Ethanol : Water :: 49:5:50

K, Known - celllobiose (cell.) and raffinose (raff.).
The products were also used for electrophoretic examination.

**Electrophoresis.** In presence of 2 N formic acid:

For the determination of the molecular weights of pento-oligosaccharides, electrophoresis was first carried out in presence of formic acid-sodium formate (pH 1.8), the electrolyte used by Barker et al. (1956). The determination was possible, but when the same method was applied to determine the molecular weights of a mixture of oligosaccharides formed after mild hydrolysis of an oligosaccharide, it was found that separation was not well marked owing to tailing caused by gradual decomposition of the amine complex of each oligosaccharide. Concentrations of the products were so low that the separation by paper chromatography followed by electrophoresis was not possible. Isbell and Frush (1951) reported that sugar amines are easily hydrolysed at below or above pH 5, but virtually there is no hydrolysis below pH 1.5. The pH of approximately 2 N formic acid solution is 1.3 and this was used as electrolyte to separate electrophoretically the benzylamine complexes of oligosaccharides. Xylose and oligosaccharides, which from their chromatographic positions and acid hydrolysates of oligosaccharides were probably xylobiose,
xylotriose and higher homologues, were dissolved in a solution containing 1 c.c. of benzylamine in 9 c.c. methanol and 5 c.c. N formic acid. After spotting on Whatman No. 1 paper (11.5 cm. wide and 56 cm. long) and immediately before electrophoresis, the paper was heated at 95°C for 5 min. Electrophoresis was carried out at 9 volts/cm. and 8-12 milliamps for 6 hr. The spots were revealed by dipping the dried paper in an acetone solution of silver nitrate, and thereafter spraying with an alcoholic solution of sodium hydroxide. After proper development of the spots, the paper was dipped in a strong solution of ammonia followed by washing in running water for 1 hr. and drying in the oven. The distances separating the complexed from the uncomplexed sugars were measured and $M_X$ values were calculated.
Plate IV. - Electrophoresis of oligosaccharides from rye araboxylan by mould culture filtrate enzyme.
The oligosaccharides were complexed with benzylamine and electrophoretogram was run in 2N formic acid as electrolyte at 9 volts/cm. and 8-12 milliamps. for 6 hr.

X, xylose.
Plate V. - Electrophoresis of oligosaccharides produced from rye arabinoxylan by mould culture filtrate enzyme after mild acid (1.0 N acetic acid) hydrolysis. The oligosaccharides were complexed with benzylamine and electrophoretogram was run in 2N formic acid as electrolyte at 9 volts/cm. and 8-12 milliamps. for 6 hr.

X, xylose.
# TABLE I

Quantitative Liberation of Arabinose and Xylose from Araboxylan by Barley Enzymes (untreated) and Mould Enzymes

<table>
<thead>
<tr>
<th>Enzyme source*</th>
<th>wt. of pentose (μg.) in 5 ml. aliquot</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 hr.</td>
</tr>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Barley (untreated)</td>
<td>147</td>
</tr>
<tr>
<td>Mould (culture filtrate)</td>
<td>42</td>
</tr>
<tr>
<td>Mould (mycelial)</td>
<td>81</td>
</tr>
</tbody>
</table>

* Enzyme concentrations adjusted to give same liberation of total reducing power per hr.

# TABLE II

Oligosaccharide Production from Araboxylan by Mould Enzymes

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Culture filtrate</th>
<th>Mycelial</th>
<th>Mean R&lt;sub&gt;f&lt;/sub&gt;</th>
<th>log 1-R&lt;sub&gt;f&lt;/sub&gt;/R&lt;sub&gt;f&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intensity*</td>
<td>R&lt;sub&gt;f&lt;/sub&gt;</td>
<td>Intensity*</td>
<td>R&lt;sub&gt;f&lt;/sub&gt;</td>
</tr>
<tr>
<td>1</td>
<td>++</td>
<td>0.0462</td>
<td>++</td>
<td>0.0468</td>
</tr>
<tr>
<td>2</td>
<td>+++</td>
<td>0.0164</td>
<td>+++</td>
<td>0.0158</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>0.0098</td>
<td>+</td>
<td>0.0115</td>
</tr>
<tr>
<td>4</td>
<td>+++</td>
<td>0.0057</td>
<td>+++</td>
<td>0.0056</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>0.0037</td>
<td>+</td>
<td>0.0036</td>
</tr>
<tr>
<td>6</td>
<td>+++</td>
<td>0.0021</td>
<td>+++</td>
<td>0.0020</td>
</tr>
</tbody>
</table>

* Visual.
**TABLE III**

Oligosaccharide Production after Partial acid hydrolysis

(1.0 N acetic acid) of each Separate Oligosaccharide

<table>
<thead>
<tr>
<th>Origin of spot (Table II)</th>
<th>Hydrolysed spot no.</th>
<th>Intensity*</th>
<th>$R_F$</th>
<th>$\log \frac{1-R_F}{R_F}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1a</td>
<td>+++</td>
<td>0.048</td>
<td>1.297</td>
</tr>
<tr>
<td>2</td>
<td>2a</td>
<td>++</td>
<td>0.048</td>
<td>1.297</td>
</tr>
<tr>
<td></td>
<td>2b</td>
<td>+++</td>
<td>0.015</td>
<td>1.817</td>
</tr>
<tr>
<td>3</td>
<td>3a</td>
<td>+</td>
<td>0.015</td>
<td>1.817</td>
</tr>
<tr>
<td>4</td>
<td>4a</td>
<td>++</td>
<td>0.048</td>
<td>1.297</td>
</tr>
<tr>
<td></td>
<td>4b</td>
<td>++</td>
<td>0.015</td>
<td>1.817</td>
</tr>
<tr>
<td></td>
<td>4c</td>
<td>+++</td>
<td>0.004</td>
<td>2.396</td>
</tr>
<tr>
<td></td>
<td>Xylose</td>
<td>-</td>
<td>0.140</td>
<td>0.788</td>
</tr>
<tr>
<td></td>
<td>Arabinose</td>
<td>-</td>
<td>0.120</td>
<td>0.865</td>
</tr>
</tbody>
</table>

* Visual.
<table>
<thead>
<tr>
<th>Origin of spot</th>
<th>Spot No.</th>
<th>( M_x )</th>
<th>Mean ( M_x )</th>
<th>( 1/M_x )</th>
<th>Complexity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Table II)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>0.67</td>
<td>(0.67)</td>
<td>1.493</td>
<td>Tri-</td>
</tr>
<tr>
<td>1</td>
<td>1a</td>
<td>0.82, 0.80</td>
<td>0.81</td>
<td>1.235</td>
<td>Di-</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>0.54, 0.57, 0.51, 0.57</td>
<td>0.55</td>
<td>1.818</td>
<td>Tetra-</td>
</tr>
<tr>
<td>2</td>
<td>2a</td>
<td>0.78, 0.76, 0.80, 0.81</td>
<td>0.79</td>
<td>1.266</td>
<td>Di-</td>
</tr>
<tr>
<td></td>
<td>2b</td>
<td>0.64, 0.63, 0.60, 0.59</td>
<td>0.62</td>
<td>1.613</td>
<td>Tri-</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>Not detectable</td>
<td>-</td>
<td>-</td>
<td>?</td>
</tr>
<tr>
<td>3</td>
<td>3a</td>
<td>&quot;</td>
<td>-</td>
<td>-</td>
<td>?</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>0.44, 0.46, 0.45, 0.45</td>
<td>0.45</td>
<td>2.222</td>
<td>Penta-</td>
</tr>
<tr>
<td>4</td>
<td>4a</td>
<td>0.76, 0.76, 0.82, 0.78</td>
<td>0.78</td>
<td>1.282</td>
<td>Di-</td>
</tr>
<tr>
<td></td>
<td>4b</td>
<td>Not detectable</td>
<td>-</td>
<td>-</td>
<td>?</td>
</tr>
<tr>
<td>4</td>
<td>4c</td>
<td>0.52, 0.54, 0.55, 0.50</td>
<td>0.53</td>
<td>1.887</td>
<td>Tetra-</td>
</tr>
</tbody>
</table>

\( M_x \) = Mobility of pento-oligosaccharide relative to that of xylose.
TABLE V

Possible structure of the Oligosaccharides

<table>
<thead>
<tr>
<th>Origin of spot (Table IV)</th>
<th>Complexity</th>
<th>Structure*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tri-</td>
<td>X-X</td>
</tr>
<tr>
<td>1a</td>
<td>Di-</td>
<td>X-X</td>
</tr>
<tr>
<td>2</td>
<td>Tetra-</td>
<td>X-X or X-X-X or X-X-X</td>
</tr>
<tr>
<td>2a</td>
<td>Di-</td>
<td>X-X</td>
</tr>
<tr>
<td>2b</td>
<td>Tri-</td>
<td>X-X-X</td>
</tr>
<tr>
<td>3</td>
<td>?</td>
<td>-</td>
</tr>
<tr>
<td>3a</td>
<td>?</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Penta-</td>
<td>X-X or X-X-X or X-X-X-X or X-X-X-X or X-X-X-X</td>
</tr>
<tr>
<td>4a</td>
<td>Di-</td>
<td>X-X</td>
</tr>
<tr>
<td>4b</td>
<td>Tri-</td>
<td>X-X-X</td>
</tr>
<tr>
<td>4c</td>
<td>Tetra-</td>
<td>X-X-X-X</td>
</tr>
</tbody>
</table>

* X = xylose; A = arabinose.

†, Chromatographic; ††, Electrophoretic.
FIG. 2 - The linear relationship obtained between $\log \frac{1 - R_F}{R_F}$ and complexity in the homologous series of xylo-oligosaccharides which were obtained after mild acid (1.0 N acetic acid) hydrolysis of original oligosaccharides given by rye araboxylan enzymolysis.

FIG. 3 - The linear relationship obtained between $\frac{1}{M_X}$ values and complexity (no. of saccharide units) of the benzylamine complexes of the pento-oligosaccharides.
FIG. 2

NO. OF XYLOSE RESIDUES AS REVEALED ELECTROPHORETICALLY.

FIG. 3
DISCUSSION

In the degradation of water-soluble araboxylan by cereal enzymes, Preece and MacDougall (1956) suggested that there are four enzymes responsible for this purpose and that these are (a) arabinosidase, (b) exoxylanase, (c) xylobiase, and (d) endoxylanase. The presence of arabinosidase causes rapid production of free arabinose from the araboxylan and the rate of release is linear up to liberation of some 10% of the total available arabinose. The enzymes from barley, oats and rye have similar arabinosidase activity. The exoxylanase action is confirmed from the production of xylobiose from the ends of xylan chains which occurs before the production of intermediate oligosaccharides, but the action is dependent on the availability of appropriate xylan ends. The absence of arabinosylxylose and the presence of arabinosylxylobiose in the araboxylan enzymolysate suggest that the enzyme cannot attack the araboxylan molecule immediately in front of a xylose residue carrying an arabinosyl side chain. The presence of xylobiase is confirmed from the rapid degradation of xylobiose, though the enzyme can also attack xylotriose slowly. Endoxylanase is responsible for the rapid fall in viscosity of
araboyxlan solutions, indicating the random attack on the araboyxlan molecule but its action could probably be influenced by the positioning of the arabinosyl side chains. The appearance of chromatographically detectable oligosaccharides by endo-action occurs only in prolonged conversions; but if exo-action takes place there is early production of xylobiose.

In the present investigation, rye araboyxlan was degraded with barley, mould culture filtrate and mould mycelial enzyme preparations to give a comparative degradation pattern. In Table 1, it is seen that the barley enzyme gives a rapid production of arabinose from the start and xylose is available at 18 hr. The ratio xylose/arabinose gradually increases with time. In chromatographic analysis, it is seen that xylose concentration increases with the increase of oligosaccharides. These results are similar to earlier findings of Preece and MacDougall (1958) who have shown the rapid production of arabinose from araboyxlan due to arabinosidase activity and the production of xylose is related to the xylo-biase action on xylobiose which is produced by the exoxylanase action. The mould enzymes both from culture filtrate and mycelial preparations give an early production of arabinose and xylose, but the production of xylose is always higher.
than arabinose in the case of the two mould enzymes, which is the opposite to what is found with the barley enzyme. The ratio xylose/arabinose in the case of the culture filtrate enzyme, though it is not very regular, is different from the ratio for mycelial enzyme. The reason for this difference is difficult to explain without having the comparative xylose/arabinose ratios of the limit-dextrins produced by the two enzymes. From preliminary chromatographic analysis, it is known that the oligosaccharides from mycelial enzymic degradation give the same intensity of spots with the same $R_P$ values as those obtained with culture filtrate enzyme (Table 2).

As the production of arabinose and xylose due to culture filtrate and mycelial enzymes increases similarly with time, the modes of action for the two enzymes will be discussed together. According to Preece and MacDougall (1958), the barley arabinosidase removes arabinose residues from the side chains of arabinoxylan and oligosaccharides produced from it, and its action is independent of the action of other enzymic factors in the barley enzyme. In comparison with the barley enzyme, the mould enzymes are deficient in arabinosidase activity, because mould enzymes always produced less arabinose
than did barley enzyme. The mould enzymes cause rapid fall in viscosity of araboxylan solutions without much increase in reducing power at the initial stage, indicating the presence of endoxylanase action.

The steady increase of arabinose using mould enzymes for degradation indicates that the arabinosidase activity of mould enzymes, like that of barley enzyme, is also independent of other enzymic factors. The production of xylase gives a steady increase with time, but its production may be due to more than one enzymic action. In the araboxylan degradation with mould enzymes, no xylobiose was detected, which indicates the absence of any exoxylanase in mould enzymes. Xylose is probably formed by the action of endo-enzyme followed by oligosaccharase action. This enzyme may be similar to the enzymes, xylanase and xylobiase from bacteria, isolated by Howard et al. (1960). Xylanase could produce xylose from xylan and higher oligosaccharides, and its action was retarded with decrease in degree of polymerisation. Xylobiase could produce xylose from lower to higher oligosaccharides, but not from xylan. In the case of the mould enzyme degradation of araboxylan, it seems that xylose is only produced from that part of the molecule from which arabinose has already been removed by
arabinosidase action, leaving residual molecules with arabinose side chains. When these residues with arabinosyl side chains become low molecular after gradual endo-degradation, they appear as oligosaccharides. This type of degradation would give rise to oligosaccharides with higher arabinose content than with the barley enzyme, and in the present results all the oligosaccharides have high arabinose contents. It therefore appears that, with the mould enzymes, degradation is due to a blend of endoxylanase, arabinosidase and xylo-oligosaccharase activity, with the arabinosidase proportionately deficient in amount as compared with the barley enzyme mixture.

In the rye arabinoxylan degradation with mould culture filtrate enzyme, small amounts of arabinose and xylose were detected. No disaccharide, either xylobiose or arabinosylxylose, was detected. Preece and MacDougall (1958) isolated xylobiose from arabinoxylan degradation products with barley enzyme and the appearance of this oligosaccharide is common, especially when the enzymes contain high arabinosidase activity, but Bishop and Whitaker (1955) also, detected xylobiose in the degradation products of wheat-straw xylan (which contain very little arabinose) with an enzyme from *Myrothecium verrucaria* where
the arabinosidase activity was very low. The disaccharide arabinosylxylose is not yet reported from enzymic degradation of arabinoxylan.

A trisaccharide was, however, detected electrophoretically. It is known that when arabinoxylan or oligosaccharides from it, is subjected to mild acid hydrolysis, the arabinosyl side-chains are removed. When such hydrolysis was applied to this trisaccharide, it yielded along with arabinose a disaccharide which was again by chromatography and electrophoresis characterised as xylobiose. The original trisaccharide is, then, arabinosylxylobiose. The occurrence of this oligosaccharide is common in arabinoxylan enzymolyses (Aspinall et al., 1960; Bishop and Whitaker, 1955), especially when the enzyme contains low arabinosidase.

When the tetrasaccharide fraction was subjected to mild acid hydrolysis, it gave two oligosaccharides, which were characterised as xylobiose and xylotriose. No xylotetraose was detected. From this it appears that the original fraction is composed of two types of oligosaccharides, one containing two arabinose residues and two xylose residues, and another containing one arabinose residue and three xylose residues. Bishop and Whitaker (1955) isolated a tetrasaccharide containing one arabinose unit
attached to three xylose residues.

Before the pentasaccharide fraction (below), there was another fraction, the concentration of which was so low that electrophoresis could not be carried out.

The pentasaccharide fraction was subjected to mild acid hydrolysis, and three oligosaccharides appeared in the chromatogram as xylobiose, xylotriose and xylotetraose. In the electrophoresetogram, only xylobiose and xylotetraose were detected. No xylopentaose was detected in this fraction. Xylobiose in the hydrolysate of this fraction cannot arise by the breakdown of a xylose to xylose linkage; if the production of xylobiose can be confirmed, it would mean that there was a side chain of two units instead of the expected one, e.g. \( \frac{X}{A} - \frac{X}{A} \) or \( \frac{X}{A} - \frac{X}{A} \). There is a further possibility of a structure such as \( \frac{X}{A} - \frac{X}{A} \).

This type of complex side chain has recently been reported in the case of cereal xylans (Aspinall and Farrier, 1957; Whistler and Lauterbach, 1958). Apart from this, the fraction seems to be a mixture of two types of oligosaccharides, one containing two arabinose units and three xylose units, and another containing one arabinose unit and four xylose units. If
structures of these types are possible, it becomes necessary to examine in much more detail the components of the tri- and tetrasaccharides obtainable by mild acid hydrolysis.

The degradation of arabinoxylan by mould culture filtrate enzyme thus gave oligosaccharides with mixed sugar units. In the present work, simple chromatographic and electrophoretic methods were used to separate and to characterise these oligosaccharides. It is seen that mixtures of oligosaccharides with the same molecular weights are present in the same chromatographic fraction, thus offering considerable difficulties in characterising these oligosaccharides firmly. Hence, more elaborate methods are necessary to define the structures of these oligosaccharides. It is obvious that knowledge of the structures of the oligosaccharides would give a better idea about the structure of the original arabinoxylan and its degradation pattern by mould enzyme. Further it would seem that, by removing some of the enzymic factors either by chemical or physical means, and by applying the modified enzyme to the degradation, more knowledge of arabinoxylan degradation might be gained.
SUMMARY

1. The amount of arabinose production from araboxylan degradation by barley enzyme is always greater than that of xylose. The relationship between arabinose production and time is linear from the start, indicating the presence of a strong arabinosidase in the barley enzyme and an action independent of other enzymic factors in the barley enzyme. The results confirm the earlier results obtained by Preece and MacDougall (1958).

2. The mould enzymes are deficient in arabinosidase activity. Xylose production by mould enzyme in araboxylan degradation is always greater than arabinose production. The rapid viscosity drop of an araboxylan solution in the presence of this enzyme without much increase of reducing power indicates the presence of strong endo-activity in the mould enzyme. There is no indication of the presence of exo-activity but probably xylo-oligosaccharase is present.

3. The araboxylan degradation by mould culture filtrate enzyme gives rise to oligosaccharides with mixed sugar units.

4. The presence of xylobiose in the partial acid hydrolysis products of the pento-oligo-
saccharide fraction indicates a probable complex nature of side chains in the araboxylan molecule.
Before the isolation of pure $\beta$-glucan by Preece and MacKenzie (1952) from barley, it was not possible to study quantitatively the degradation pattern of barley gum and there was insufficient comparative knowledge about the hemicellulase systems of different cereals. Preece and Hoggan (1956) could go a long way by using $\beta$-glucan as substrate and were able to measure endo-$\beta$-glucanase, exo-$\beta$-glucanase, and cellulobiase activity of barley, wheat, rye, oats and maize and also that of barley at successive stages during malting. They prepared more active enzymes and were able to suppress exo-activity of enzyme preparations by chemical methods. They showed the relation between reducing power and viscosity from rigid experimental data making it possible to test an enzyme preparation for its relative contents of endo- and exo-activity. Following these developments, Preece and Garg (1961) attempted to obtain more elaborate knowledge of $\beta$-glucan degradation by cereal enzymes. They postulated from their results that in barley $\beta$-glucan, $\beta$-1,3- and $\beta$-1,4-linkages are distributed at random and certain regions are rich in $\beta$-1,3- or $\beta$-1,4-linkage and these runs may be up to four of any
of such linkages. They showed from the degradation pattern of various substrates that the barley enzymes contain endo-1,3-ase, endo-1,4-ase, exo-1,4-ase, laminaribiase, and a general $\beta$-glucosidase.

Preece and MacDougall (1958), using the principle of ammonium sulphate fractionation and improved technique, showed that appreciable amounts of araboxylan could be obtained with only $3-4\%$ of $\beta$-glucan contamination. By using this araboxylan as substrate, they detected four enzymes or enzyme systems responsible for the degradation: (a) arabinosidase, liberating free arabinose from araboxylan and oligosaccharides; (b) endoxylanase, degrading xylan chains, whether or not these carry arabinose side chains; (c) exoxylanase, producing xylobiose from araboxylan and from oligosaccharides after removal of some degree of arabinose; (d) xylobiase, liberating free xylose from xylobiose and perhaps also from xylotriose. They also showed that raw cereals like barley, maize, oats, rye and wheat contain all four enzymes, but in different proportions.

Knowing all these results of cereal gum enzymolyses by cereal enzymes, it was desired to compare the mode of hemicellulose degradation by enzymes from moulds, which are well-recognised
sources of hemicellulases, with that achieved by cereal enzymes. After preliminary investigation, a Penicillium strain was selected for this purpose. Later, from cultural and microscopic studies, the growth was tentatively found to be a strain of Penicillium chrysogenum.

It was seen that the organism could produce both β-glucanase and araboxylanase. Extracellular β-glucanase production without much araboxylanase contamination is possible when there is growth in presence of glucose or its oligosaccharides; araboxylanase cannot be obtained free from β-glucanase, an appreciable amount of extracellular araboxylanase together with β-glucanase is produced only in presence of pentose or pentosan. From these results it can be said that β-glucanase is a constitutive enzyme though its production can be stimulated by xylose or β-glucan whereas araboxylanase is an adaptive or inducible enzyme, the production of which is only stimulated by pentose or pentosan.

In further studies on β-glucanase production with different concentrations of a sugar, or with interaction of a sugar and a hemicellulose, it was seen that high production of enzyme does not necessarily go parallel with high mycelium development. Two factors are
recognised from these studies - (a) stimulation of growth and (b) direct stimulation of extracellular $\beta$–glucanase production. Glucose has effect (a), $\beta$–glucan has effect (b), whereas xylose has both effects (a) and (b). These observations are confirmed from comparative studies using these sugars. Xylose is as effective as glucose in promoting development of mycelium, but, in the case of xylose, $\beta$–glucanase production is higher than with glucose. Although growth on $\beta$–glucan is much poorer than on either of the sugars, $\beta$–glucanase production per mg. mycelium is higher with $\beta$–glucan than with these sugars.

Xylose is found to be most suitable sugar source for the development of extracellular enzyme containing both $\beta$–glucanase and araboxtylanase activity. The recovery of total activity from culture filtrate to enzyme preparation is very poor. It is not yet known whether ammonium sulphate precipitation has any adverse effect on these enzymes. Yet the enzyme preparation from the culture filtrate was some 40 times and 700 times as active against, respectively, $\beta$–glucanase and araboxtylanase, as a good enzyme preparation from barley. The enzyme preparation from the mycelium also contains both $\beta$–glucanase and araboxtylanase.
activity.

Preece and Hoggan (1956) showed that the degradation of \( \beta \)-glucan by barley enzyme is caused by the action of enzyme systems containing endo-\( \beta \)-glucanase, exo-\( \beta \)-glucanase and cello-biase. The action of endo-\( \beta \)-glucanase in the earliest stages produces new reducing groups proportional to the concomitant increase in reciprocal specific viscosity. The action of exo-\( \beta \)-glucanase in presence of endo-\( \beta \)-glucanase produces additional reducing groups from the start, over and above what can be predicted from the viscosity change. They were able to suppress this exo-activity by treatment of an enzyme preparation with phenylmercuric nitrate without destroying other enzymic factors. The presence of cellobiase and laminaribiase activities was proved by their action against cellobiose and laminaribiase respectively. They used these two types of enzyme preparations from barley and oats to give the degradation patterns of \( \beta \)-glucans from oats and barley along with other substrates.

In the present work, the action of mould enzymes on \( \beta \)-glucan together with other substrates was compared with that of barley enzyme treated with phenylmercuric nitrate. Preece and Garg (1961) showed that the barley enzyme
contains endo-1,3-ase, endo-1,4-ase, exo-1,4-ase, laminariibiase and a general β-glucosidase. In comparative studies, they also showed that the presence of exo-activity (in joint-action) in the barley enzyme accelerates the degradation of β-glucan, producing increased reducing power due to glucose and oligosaccharides and decreased recovery of alcohol-precipitable dextrin; it also gives a higher reducing power in the dextrin. The degradation of β-glucan by these two types of action reaches a steady state in average dextrin composition (though not yield) corresponding to a reducing power of approximately 32 μg. per mg. for endo-action and 57 μg. per mg. for the dextrin produced by joint-action. The degradation of β-glucan by mould enzymes reaches a similar steady state giving average dextrin composition corresponding to a reducing power of approximately 20 μg. per mg.; in the present work, the barley enzyme gives average dextrin composition corresponding to a reducing power of approximately 30 μg. per mg. This shows that the barley enzyme containing both endo-1,3-ase and endo-1,4-ase can give more extensive degradation to the β-glucan molecule than that given by mould enzymes containing endo-1,3-ase alone. The presence of oligosaccharases does not seem to have any special influence on the extent of
degradation. The mould mycelial enzyme with oligosaccharases and mould culture filtrate enzyme without any oligosaccharase gives degradation to the same extent as the extracellular enzyme. It seems that oligosaccharases which are common to the barley and the mycelial enzyme, only give increased glucose and oligosaccharides production. The presence of exo-activity in the barley enzyme (Preece and Garg, 1961) gave more extensive degradation than that of endo-action alone, and this shows that, in the mycelial enzyme, the presence of exo-enzyme is very doubtful and differences shown earlier in the estimates of relative amounts of endo- and exo-activity seem to be due to oligosaccharase activity.

The oligosaccharides produced by mould culture filtrate enzyme included the oligosaccharides reported by Parrish, Perlin and Reese (1960) after degradation of oat $\beta$-glucan by "laminarinase" from Rhizopus arrhizus and also those reported by Moscatelli, Ham and Ricks (1961) after degradation of barley $\beta$-glucan by an enzyme preparation from Bacillus subtilis. The mould mycelial and barley enzymes produced oligosaccharides reported by Parrish, Perlin and Reese (1960) when they used "cellulase" from a Streptomyces sp. in oat $\beta$-glucan degradation and also
included oligosaccharides reported by Preece, Garg and Hoggan (1960) after degradation of barley and oat β-glucan by barley and oat enzymes. Parrish et al., from their results, proposed a structure of β-glucan having two types of structural sequences: one is a tetrameric unit in which a single β-1,3-linkage alternates with two β-1,4-linkages, and the other, a pentameric unit in which a single β-1,3-linkage alternates with three consecutive β-1,4-linkages, which is not in agreement with the present results. The detection of laminari-triose and the action of culture filtrate enzyme containing endo-1,3-ase giving oligosaccharides only with β-1,3-linkage at the reducing end strongly support the structure proposed by Preece and Garg (1961), in which a more random arrangement of short and variable runs of β-1,3-linkages alternates with similar short and variable runs of β-1,4. The results of Smith and Sorger-Domenigg (1960) also supported the structure of β-glucan containing blocks of two and three contiguous β-1,3-linked glucose residues in addition to isolated units.

The present results indicate that the mould culture filtrate enzyme contains endo-β-1,3-ase only, the mycelial enzyme contains at least endo-β-1,3-ase, oligo-β-1,3-ase and oligo-β-1,4-
ase, and the barley enzyme after treating with
phenylmercuric nitrate contains endo-\(\beta\)-1,3-ase,
endo-\(\beta\)-1,4-ase, oligo-\(\beta\)-1,3-ase and oligo-\(\beta\)-
1,4-ase. Luchsinger (1962) reported, after
ammonium sulphate fractionation of an extract of
barley green malt, three fractions containing
endo-\(\beta\)-glucanase activity with different optimum
pH values for enzyme action and with different
heat half-inactivation times. They did not give
any specific biochemical difference in action.

In a comparative study, Preece and
MacDougall (1958) showed the relative abundance
of different enzymes in the cereals. All the
cereals they tested are rich in arabinosidase
activity. Oats are rich in endoxylanase
activity, and this activity is also high in
maize, though the yield of enzyme is smaller in
comparison with other cereals. The exoxylase
is greatest in barley; oats and rye are very
near to barley in this respect, while wheat is
very deficient. The xylobiase activity is very
strong in oats, whereas maize shows little of
this activity. The pattern of degradation of
arabinoxylan by these cereal enzyme preparations
depends on the relative proportions of different
enzymes present in a preparation. It was shown
that exo-action accelerates production of higher
oligosaccharides by progressively further
shortening the chains produced by endo-action but the deficiency of exo-action is compensated by the presence of strong endo-action.

These different types of enzyme are also present in different proportion in extracts from algae, fungi and bacteria. Very few quantita-tive investigations have been made with these enzymes on the relative liberation of arabinose and xylose in arabinoxylan degradation, but in qualitative investigations it has been shown that some enzymic degradations produce oligo-saccharides without arabinose side-chains while others produce oligosaccharides containing arabinose side-chains. The enzymes from the *Penicillium chrysogenum* strain of the present work are deficient in arabinosidase activity in comparison with the barley enzyme preparation, and the oligosaccharides produced by culture filtrate enzyme contained both arabinose and xylose. The mould culture filtrate preparation seems to be similar to the enzymes of *Myrothecium verrucaria* (Bishop and Whitaker, 1955) in respect of oligosaccharide production, with one exception. The latter enzyme produced xylobiose from wheat-straw xylan, whereas the present enzyme did not produce any xylobiose from rye arabinoxylan.

Though arabinose contents of these two substrates are very different, the reason for xylobiose
production might be due to exo-action which seems to be absent in the mould culture filtrate enzyme preparation. Bailey et al. (1962) showed that the enzyme preparation from rumen ciliates was different from the enzymes of Butyrovibrio and Bacteroides amylogens (Howard et al., 1960) in their optimum pH of enzyme action and mode of degradation of wheat-flour xylan. In the chromatographic analysis, they observed initial release of arabinose without much accompanying xylose or xylobiose, which was similar to the results of enzymic action of rumen bacteria (Howard, 1957). They detected only xylobiose after initial release of arabinose, with no other oligosaccharides in the hydrolysate at any stage during the incubation. They thought that the xylan chain was hydrolysed by the successive removal of xylobiose units from one end of the chain and not by random cleavage. In the case of enzymes from Butyrovibrio and Bacteroides amylogens, Howard et al. (1960) detected xylo-oligosaccharides of a higher degree of polymerization only during the last stage of the hydrolysis. The degradation of wheat-flour xylan described by Howard et al. (1957, 1960) and Bailey et al. (1962) can be explained by the four enzyme systems postulated by Preece and MacDougall (1958) in the cereals.
In the rye arabinoxylan degradation by mould culture filtrate enzyme, oligosaccharides identified are (a) a trisaccharide containing two xylose and one arabinose unit, (b) two tetrasaccharides, one containing two xylose units and two arabinose units, and another containing three xylose units and one arabinose unit, and (c) three pentasaccharides, one containing four xylose units and one arabinose unit, another containing three xylose units and two arabinose units, and another containing two xylose units in the main chain and three sugar units in the side-chain; these side-chain units may be three arabinose or two arabinose and one xylose unit as shown in Table V of Section III. This type of complex side-chain is known to be present in cereal hemicelluloses (Whistler and Lauterbach, 1958; Montgomery et al., 1957), and Aspinall and Ferrier (1957) isolated 2-0-β-D-xylopyranosyl-L-arabinofuranose from barley husk xylan which are believed to be directly attached to the main xylan chain.

In the present work, it has been observed that various enzymic factors are present in both cereal and microbial enzymes and often, though not always, more than one factor is present in an enzyme preparation. Various workers are attempting to separate these enzymic factors by
salt fractionation, adsorption and electrophoretic methods. It is also hoped that more comparative enzymolyses with enzymes from different sources and also with different substrates and using more elaborate methods in characterisation of oligosaccharides, will give more useful results.
BIBLIOGRAPHY


74. Lindet, L. (1903). Comp. rend., 137, 73.


149. Von Tschermak (1912). Biochem. Z., 45, 452.


ACKNOWLEDGEMENTS

The author wishes to express his deepest gratitude to Professor I.A. Preece, D.Sc., F.R.S.E., for his advice, encouragement and readiness to enter into discussion at all times during his supervision of this work. The supervision and helpful suggestions throughout of Professor E.L. Hirst, C.B.E., D.Sc., F.R.S., and (in the early stages) of Dr A.H. Rose, are also gratefully acknowledged. Thanks must also be extended to Dr Iain Campbell, Mr A.S. Millar, A.H.W.C., and Mr C.S. Johnston, B.Sc., for their co-operation.