

ENZYMOLYSIS
of
CEREAL HEMICELLULOSES

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C O N T E N T S

| | Page. |
|---|-------|
| General Introduction | 1 |
| Section I : Reducing group production in β -glucan enzymolysis | 19 |
| Section II : Qualitative study of oligosaccharides produced in the enzymolysis of β -glucan | 59 |
| General Discussion | 80 |
| Bibliography | 94 |
| Acknowledgements | 100. |

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GENERAL INTRODUCTION

It was Schulze (1891) who first employed the term "hemicellulose" to denote a group of substances entering into the composition of plant cell-walls. These materials were prepared by extraction of the source material with 4% soda, followed by the addition of dilute mineral acids. On hydrolysis with mineral acids, hemicelluloses gave primarily pentoses but also hexoses and uronic acids. He considered these substances as immediate precursors of cellulose, a belief to be proved wrong afterwards. Tollens and Stone (1888) have been accredited to be the first to prepare hemicellulose from brewer's spent grains. Schulze (1892) detected arabinose and xylose in the hydrolysates of the preparations from wheat and rye. In the same year, Schulze and Tollens (1892) prepared a xylan after washing spent grains with dilute ammonia. Preece (1931) working with spent grains isolated three principal products namely xylan, urono-xylan and urono-araban; these were similar to the hemicelluloses obtained from beechwoods by O'Dwyer (1923) who had also established the presence of uronic acids in hemicellulose.

The technique of hemicellulose fractionation was much advanced by the work of Norris and Preece (1930).

These workers employed wheat bran as the source material. Pectic substances were removed by ammonium oxalate followed by treatment with alcoholic soda to minimise the lignin content of the products. The product obtained by the acidification of the subsequent alkaline extract was a polysaccharide fraction A; addition of acetone to the acid solution at concentrations of approximately 30% and 60% by volume gave two fractions B and C. Each of these fractions A, B and C was redissolved in alkali solution separately. Fractions A₁ and B₁ were obtained as copper complexes by precipitation with Fehling's solution; C₁ was not obtained. A₂, B₂ and C₂ were obtained as copper complexes by precipitation with Fehling's solution and acetone. Examination of acid hydrolysates of each fraction revealed that B₂ consists substantially of glucosan material and others namely A₁, B₁, A₂ and C₂ consist of either pentosan or uronopentosan. In view of the objection of Norman (1937) that this procedure is not without effect upon the hemicelluloses themselves especially when high temperatures are employed in presence of alkali for prolonged periods, Preece and Aitken (1953) and Preece and Mackenzie (1953) employed autolysis techniques to obtain evidence of an indirect nature as to the behaviour of the initially soluble hemicellulose in a number of barley varieties. Preece and Hobkirk (1954) distinguished

between two types of cereal grain hemicelluloses extractable by 4% caustic soda (a) Husk type hemicelluloses; these are distributed throughout the grain; they have a low viscosity in solution but a high pentosan content, along with the presence of small proportions of uronic acid residues. These hemicelluloses seem to be quite similar in character to those obtained from cereal straws. This type of hemicelluloses has purely structural function. (b) Endosperm type hemicelluloses are found most abundantly in the inner endosperm; they have high viscosity in solution, have relatively high glucosan content and the uronic acids are substantially absent. From all the available evidence these workers concluded that the endosperm type hemicelluloses act as gum precursors during the earlier stages of germination and autolysis.

The relationship between initially soluble and initially insoluble hemicelluloses was earlier realized by Norman (1929). This conclusion was put on a better footing by the work of Preece et al. (1950) who found that, upon prolonged treatment of barley gum C₂ with barley enzyme, a water-soluble polysaccharide, identical with one obtained from malt, is obtained. The work of Preece and Ashworth (1950) further confirmed the view that the initially insoluble hemicelluloses are chemically similar to barley gums, particularly C₂, and are rendered soluble during malting. In the light of these important researches

it was necessary that Preece (1952) pointed out the artificiality of the distinction between initially soluble hemicelluloses and water-soluble gums, and stressed the far more chemical resemblance between them. He argued that the change from one water insoluble form to a water soluble form is an enzymic one under natural conditions. Hence, it is quite justified to refer to the water-soluble barley gums as hemicelluloses.

Another field of study which contributes substantially towards the knowledge of the cereal hemicelluloses is the work on water-soluble gums. The earliest work on the water-soluble gums or initially soluble hemicelluloses is that of O'Sullivan (1882). Unfortunately, owing to an assumed relationship to starch, these substances were referred as amylangs. The amylangs were obtained by a water extraction of the ground barley at 40°C., preceded by exhaustive extraction with alcohol at the same temperature. The total crude amylangs were further separated into two fractions: α -amylang, soluble at 40°C. but insoluble at room temperature; β -amylang, soluble at room temperature. Both these fractions were regarded by O'Sullivan as being mainly glucosan in composition. It was also reported that the highly viscous α -amylang could not be detected in the finished malt.

Mention may be made of the work of Lindet (1903)

who extracted the ground barley with an aqueous solution of mercuric sulphate. The extract was treated with baryta and sulphuric acid to remove metallic salts. The polysaccharide material was obtained by fractional precipitation with alcohol. A laevorotatory product of specific rotation -146° , and mainly of pentosan nature, was obtained. This could be regarded as corresponding to O'Sullivan's β -amylan.

Piratzky and Wiecha (1938) obtained a substance resembling α -amylan, from the worts of under modified malt. The gum was precipitated from the wort by the addition of Fehling's solution. It was further purified to give a product which yielded glucose on acid hydrolysis. It was considered that this substance is a mixture of gums of varying molecular weights of which the maximum figure recorded was 65,000. These workers also did not detect α -amylan in malt extract.

The purification and fractionation of the water-soluble gums of barley, as well as of other cereals, remained unaccomplished till late, when Preece et al. (1950) published a report describing a method fractionating the barley gums. The barley enzymes were first inactivated by refluxing the ground grain with 85% alcohol and the gums were named according to their solubility relationships in the hemicellulose fractionation scheme of Norris and Preece (loc. cit.). Chemical analysis of B₂ and C₂ gums revealed that the

former consisted mainly of glucan accompanied by 5-10% pentosan. Subsequently similar work was undertaken by Canadian workers at the Winnipeg Grain Research Laboratory. Meredith et. al. (1951) reported the isolation of gums from barley, malt and wort. They did not inactivate the enzymes; consequently the yields of gums were greater than obtained by Preece et. al. (loc. cit.) and because of the action of co-precipitated enzyme these gums were unstable in solution. However, the monosaccharides detected in their hydrolysates were the same (with the addition of mannose, galactose, galact-uronic acid and possibly ribose.) In 1952 (Anderson) the Canadian school realized the importance of enzyme inactivation prior to aqueous extraction. Later Meredith et. al. (1953) employed preliminary refluxing of the ground grain with alcohol in all the methods of isolating water-soluble gums. The dried grain was extracted with 1% aqueous solution of papain; in the case of barley a product was obtained, which they believed to be the undergraded and naturally occurring water-soluble polysaccharide. In other methods, extraction was carried out in presence of aqueous solution of hydrochloric acid (pH.1.0) and 6% (V/V) trichloroacetic acid (pH 1.0), and products of similar composition and lesser degree of polymerisation (as indicated by relatively lower viscosities of their solutions) were obtained. In a later publication Meredith and Anderson (1955) reported

~~It~~ have^{ing} employed a 0.0025% aqueous solution of papain. Djurtoft and Rasmussen (1955) also employed the above procedure but, in addition, they had activated papain by treatment with sodium sulphide and sodium cyanide. The impression that the product obtained by papain treatment represents a greatly degraded product cannot be easily overlooked. The criticism of Preece (1955) against the use of papain has much weight. He pointed out that papain may contain enzymes capable of attacking hemicelluloses and that the apparent effectiveness of papain could be more due to its inhibition of degradation.

Realising the limitations of the papain method, Preece and Mackenzie (1952) evolved a method of preparing water-soluble gums from barley; this method eliminates major factors responsible for the degradation of gums and perhaps the products obtained are the nearest approach to the products present in the kernel of barley. Here also the enzymes were inactivated by refluxing the ground grain with 80% alcohol and the dried grains were 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 graded quantities of ammonium sulphate to it nearly homogenous^e fractions were obtained. The fraction precipitated at 20% ammonium sulphate (W/V) after repeated resolution and reprecipitation was purified

to a laevorotatory glucan which was free from any pentosan material. Aspinall and Telfer (1954) undertook chemical investigations on this glucan, and it was found that the molecules are linear with the glucopyranose units in the chain linked with each other by an approximately equal number of β -1.3 and β -1.4 glucosidic linkages. The observation of Brown and Morris (1890) suggest that, during germination, the cell-walls of barley endosperm are gradually dissolved, this resulting in considerable softening of the tissues. Later Grüss (1902) and Ling (1904) showed that the cell-wall is not entirely dissolved away - a transparent skeleton which was revealed by staining, remained intact. Hopkins and Krause (1937) stated that one or more "hemicellulases or cytases" attack the endosperm cell-wall and a skeleton permeable to starch-splitting enzymes is left behind. These investigations point out the important part played by the enzymic breakdown of the endosperm cell-wall materials viz. hemicelluloses, and water soluble gums. The breakdown of cell-wall materials appears to be an important preliminary change which must facilitate amylolysis and proteolysis and must, therefore, play a significant part during germination.

Brown and Escombe (1898) observed that enzymic breakdown of starch granules never takes place as long as the walls of cells containing them are intact. More recently it has also been shown by Dickson and Shands (1941)

that during malting the cell-walls of the endosperm are progressively dissolved from the germ portion to the distal portion of the kernel. This suggests that the embryo is secreting enzymes, i.e. 'cytases', which diffuse towards the distal region. It was also observed that amylolysis does not occur until the cell-wall is broken, and even then it occurs first in regions adjacent to embryo. Hopkins and Krause (loc.cit.) report similar conclusions. Adrian and Brown (1904) soaked thin sections of barley endosperm in extracts of green malt and oats and observed the dissolution of cell-wall. All these investigations clearly indicate the important part played by the enzymolysis of cell-wall material of the barley endosperm during germination.

The enzymes degrading the cell-wall materials of barley endosperm were named "cyto-hydrolytic" enzymes by Brown and Morris (1890); lately the name has been abbreviated to "cytase". The term "cytase" is rather vague and covers all the enzymes or enzyme systems attacking cell-wall materials. Many workers have been more specific, and have named the enzymes according to the particular substrates attacked, e.g. cellulase, hemicellulase, xylanase, etc. Lüers and Volkämer (1928) studied the action of extracts of green malt on xylan preparations from elder pith and from barley. The enzymolysis of the substrate was followed by the observed increase in reducing groups in the reaction mixture at pH 5.0 and 45°C; the enzymic activity was also estimated

on the basis of reducing group production. The enzyme system was called "xylanase" and was claimed to have been purified by adsorption from phosphate buffer, (pH 5.0) on alumina, and elution with a phosphate buffer pH 8.5. Ziese (1931), by employing a synthetic substrate, ethyl hydroxy-ethyl cellulose, demonstrated the presence of "cellulase" in barley malt.

The characterisation and purification of enzymes degrading water-soluble gums of barley and other cereals was not attempted until later, when Preece and Ashworth (loc.cit.), working with mixed barley gum C₂ (approximately 40% pentosan) laid the foundations of modern nomenclature of enzymes attacking water-soluble gums. These workers distinguished two types of enzyme systems involved in the degradation of cell-wall materials: (a) a cytoclastic system of enzymes which effects a rapid decrease in molecular complexity of the substrate, as observed by decrease in viscosity of the reaction mixture, with little production of reducing groups (responsible for the degrading effect); and (b) a cytolytic system which is characterised by the prolonged production of reducing groups. It was, however, realised that with a mixed substrate no single action could be investigated in greater detail. This work could not go any further than establishing general principles of the enzymolysis. The Canadian workers Bass et al. (1952, 1953) undertook similar work, but they also were obstructed by the non-availability of a homogeneous substrate.

An attempt was made by Sandegren and Enebo (1952) to overcome the non-availability of a natural homogenous^e substrate by employing artificial substrates, ethyl-hydroxyethyl cellulose and carboxy methyl cellulose. The activities of their cellulase preparations were determined by measuring the rate of viscosity diminution. The use of artificial substrate has been objected to by several workers [Preece and Aitken (loc.cit.); Anderson (1955); van Roey and Hupe' (1955); and Thomas (1956)] who observed that the artificial substrates are much less sensitive to enzyme attack and, therefore, the results obtained from experiments with artificial substrates cannot be really compared with the results of natural substrates; considerable difficulty also arises in relating the results of the enzymolyses of natural and artificial substrates. The limitations of artificial substrates are now much too obvious.

Survey of the literature bears out that the close characterisation of cereal hemicelluloses did not progress until a naturally occurring homogenous^e substrate was prepared by Preece and Mackenzie (loc.cit.). The preparation of a β -glucan uncontaminated with any pentosan material was a land mark in the field, and marked the beginning of a new era. Immediately after the above method of preparation was published, the study of the enzymolysis of β -glucan was undertaken by Preece and his school in Britain and Meredith and collaborators in

Canada. Preece, Aitken and Dick (1954) presented evidence for the presence of (a) an endo- β -glucanase system which could be identified with the cytoclastic system of Preece and Ashworth (loc.cit.), (b) exo- β -glucanase system which in combination with (a) could be identified with the cytolytic system, and (c) a cellobiase system. Bass and Meredith (1955, 1956) and Meredith and Anderson (1955) also continued the study of the enzymolysis of β -glucan and obtained information supporting the work of Preece and his school.

The study of the enzymolysis of any substrate resolves itself into obtaining information about the structure of the substrate, and purifying and fractionating various enzymes or enzyme systems responsible for degradation of the substrate and investigating the actual pattern of degradation. The information about the chemical structure of β -glucan as provided by Aspinall and Telfer (loc.cit.) was far from being complete - the order of distribution of β -1.3- and β -1.4- glucosidic linkages in the molecule was not known. The enzymes used by Preece, Aitken and Dick (loc.cit.) were crude and unfractionated; β -glucanase was not purified and means were not available for studying exo- or endo-activity independently. The work of Preece and Hoggan (1956) went a long way towards clearing up the situation and opening new fields for further research. These workers achieved useful purification of the glucanase

system by extracting the ground barley with citric acid-phosphate buffer (pH 4.5) and precipitating the enzymes by the addition of graded quantities of ammonium sulphate to the dialysed extract. The enzymes thus prepared were of much enhanced activity. Partial inactivation of purified glucanase with phenylmercuric nitrate under specified conditions resulted in a complete removal of exo-activity. There was here a discovery which promised a future no less bright than the preparation of β -glucan. The presence of exo-activity in the glucanase system was as great a hindrance in elucidating a pattern of enzymolysis as was the non-availability of a homogenous^e substrate. On the basis of rigid experimental evidence it was established that there exists a mathematical relation between the reducing power of a sample of β -glucan and the reciprocal specific viscosity of its solution, and that true endo-action is involved when the halving of the viscosity of aqueous solution of β -glucan results in the reducing power being doubled. Under such conditions, the total number of reducing groups produced is not in excess of that calculated from the diminution in viscosity (as an indication of endo-activity), while in an enzyme preparation containing all the three activities viz. endo-, and exo-glucanase, and cellobiase, the total reducing groups produced are in excess of those calculated from viscosity diminution. These excess reducing groups

give a measure of joint action of exo-glucanase and cellobiase activity; the rate of hydrolysis of cellobiose could be taken as a measure of cellobiase activity, but precise estimation of exo-activity remained impossible. Chromatographic and electrophoretic methods of separation were employed to identify the oligosaccharides produced during the enzymolysis of β -glucan, and evidence was obtained for the presence of the oligosaccharides theoretically expected to account for the three activities of barley glucanases.

The present knowledge can be summarised: barley β -glucan is a typically fibrous polysaccharide, made up of straight chains of glucopyranose units which are linked with each other by an approximately equal number of β -1.4- and β -1.3- glucosidic linkages. The aqueous solutions of the polysaccharide are highly viscous. During degradation with barley enzymes, the viscosity of a solution decreases and the reducing power increases. The β -glucanase system of barley, and probably of other cereals too, is made up of endo- β -glucanase, exo- β -glucanase, and one or more β -glucosidases (e.g. cellobiase). Endo- β -glucanase attacks well away from the ends of the molecule producing smaller chains of polysaccharides and oligosaccharides and exo- β -glucanase attacks the ends of molecules producing cellobiose as the only recognizable low molecular material. Cellobiase and probably other oligosaccharases

produce glucose from cellobiose and perhaps from laminaribiose too.

It is well recognised that an enzymic study of naturally-occurring substrates has certain limitations. In the case of the enzymolysis of barley glucan the amounts of oligosaccharides produced are extremely small; therefore, the identification must be done initially by the help of physical methods such as paper chromatography and paper electrophoresis; the possibility of enzymic study is always there. All these factors demand a great restraint and caution in the interpretation of experimental results. In spite of all these handicaps, Preece and Hoggan (loc.cit.) were able to present a picture of enzymic degradation of barley glucan which is fairly elaborate. Besides adding information to the pattern of β -glucan enzymolysis, these researches have pointed out new direction of further investigation and it is quite evident that there is still a great deal which is unknown. Some of the points which need to be clarified by further researches are: (1) the distribution of the two types of linkages - whether they are distributed at random throughout the entire length of the chain or they occur in blocks of 1.3-'s and 1.4-'s; (2) since the decrease in the viscosity of the reaction mixture gives a measure of endo-activity for a short period of 90 minutes only, it is necessary that another criterion of determining endo-activity for longer periods of incubations be evolved;

(3) whether the endo-activity is due to one single enzyme or represents an overall picture of the action of more than one enzyme attacking the chain at points remote from the ends; (4) which of the two types of glucosidic linkages is being preferentially attacked during enzymolysis; and (5) at least an approximate estimate of the relative amounts of β -1.3-ase and β -1.4-ase in exo- and endo-activities.

Therefore, already, having a technique available for complete inactivation of exo-activity, opportunity was taken in the present work to compare the degradation of barley glucan, and other β -glucans too, with enzyme preparations treated with phenylmercuric nitrate free from exo-activity and untreated enzyme preparations which are supposed to contain endo-activity as well. Quantitative estimation of higher oligosaccharides (upto tetra- and penta-saccharide level) produced during enzymic degradation was undertaken.

In order to determine the character of an enzyme preparation in respect to a certain linkage, other glucans containing -1.4- or 1.3-linkage only were employed. Cellodextrin and laminarin were regarded as water-soluble substrates representing β -1.4 and β -1.3 linked glucans respectively. Cellodextrin is a degraded product of cellulose powder and can safely be assumed to represent a β -1.4-linked glucan, but laminarin is reported to be having a structure much more complicated than a mere

β -1.3 - linked straight chain of glucopyranose residues. On the basis of the present knowledge of the chemical structure of laminarin, it can still be expected to provide enough regions which will behave like a β -1.3-linked glucan during enzymolysis. A water-soluble glucan from oats was also studied; this glucan was prepared in exactly the same way as the barley glucan. A presumably similar product from oats, though prepared by a different method was studied by Peat, Whelan and Roberts (1957) under the name of "oat lichenin", and was reported to contain β -1.3- and β -1.4- linkages in 30:70 ratio. It is ^{not} necessary that the two preparations from oats viz. "oat glucan" and "oat lichenin" have the same ratio of two types of linkages. However it should be quite reasonable to expect that the glucan from oats and barley are not exactly alike. Hence in the present approach the enzymolysis of the glucan from oats was also studied, so that any differences in the structure might manifest themselves in the pattern of enzymolysis.

In all the enzymic experiments, the possibility of resynthesis can hardly be ignored and therefore in the present work enzymolyses were 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 identify the

oligosaccharides. One cannot rely entirely on the results obtained by physical methods, but even the chemical methods of analysis such as methylation, partial acid hydrolysis and periodate oxidation are not absolutely perfect - incomplete methylation, reversion and over-oxidation being their respective drawbacks. Hence, although the information available from the present work, in regard to the structure of the glucan and oligosaccharides may not be considered finally conclusive in all respects, it is claimed that it does advance knowledge both of substrate and enzymes in certain directions, and does indeed form a basis for later structural investigations and for the studies which are now necessary of enzyme fractionation.

SECTION I

REDUCING GROUP PRODUCTION IN β -GLUCAN ENZYMOLYSIS.

INTRODUCTION

High molecular β -glucan may have a structural function in the cell-walls of raw barley and other cereals. Its rapid degradation and ultimate almost complete elimination during malting is an interesting phenomenon whose explanation promises to throw considerable light on the physiology of germination. Preece and Mackenzie (1952) have put forward considerable evidence for the degradation of β -glucan in barley during early stages of malting process. Preece and Hoggan (1956) reported that under the action of barley enzyme, an aqueous solution of β -glucan undergoes a rapid fall in viscosity, the reducing power of the substrate increases, and glucose, cellobiose, laminaribiose and other oligosaccharides are produced in the enzymolysate. Earlier, Preece and Aitken (1953), employing a modification of the method of Sandegren and Enebo (1952), made diminution in viscosity the basis for assessing cytoclastic action in respect of β -glucan. With the realisation that the glucan is related to lichenin rather than to cellulose (resemblance in the chemical structure of the two substrates is discussed in Section II), and assuming that rapid diminution in viscosity accompanied by relatively small liberation of reducing groups, was caused by attack on the molecule at

points remote from the ends, Preece, Aitken and Dick (1954) preferred the name "endo- β -glucanase" or "endo- β -glucosanase" for cytoclastic action of barley enzymes. Differential inactivation of barley enzymes was successfully attempted by Preece and Hoggan (loc.cit.), by treatment of the crude enzyme preparation with phenylmercuric nitrate; these workers were able to obtain enzyme preparations whose action on β -glucan did not produce reducing groups in excess of those calculated from the viscosity drop, while enzyme preparations not treated with phenylmercuric nitrate attacked the glucan in such a way that the total number of reducing groups produced in a reaction mixture was far in excess to that calculated from viscosity drop alone; these excess reducing groups were taken as a measure of joint action of exo- β -glucanase and cellobiase - the presence of the latter being shown by hydrolysis of cellobiose to glucose. On the basis of rigid experimental evidence it was found that the relation between the reducing power of a

β -glucan sample and its viscosity satisfy the equation:

$$g = 20.38 U + 0.168 \pm 0.23,$$

where g is μg glucose equivalent per mg. of substrate and U is the reciprocal specific viscosity of 0.5% aqueous solution of the glucan at 25°C. The above relation was always made use of in calculating the number of reducing groups produced on the basis of viscosity drop. It appeared that in experiments with enzyme preparations treated with phenylmercuric nitrate, the attack on the

glucan chain was, in the earlier stages, accompanied by an interior split in the chain or, in other words, reducing power was doubled when the viscosity was halved. It was, therefore, concluded that when an endo- β -glucanase action is involved the glucan chains are attacked at points remote from the ends.

The production of large quantities of cellobiose and glucose during enzymolysis of β -glucan with enzyme preparations not treated with phenylmercuric nitrate could not be accounted for by endo-action alone. It was, therefore, assumed that the disaccharide was being liberated from chain ends by an exo- β -glucanase acting in a manner analogous to the attack on amylose by

β -amylase. Reducing groups produced in excess to those calculated from the drop in viscosity of the conversion mixture gave a measure of joint action of exo- β -glucanase and cellobiase; cellobiase activity was determined separately by the rate of hydrolysis of cellobiose to glucose.

Bass and Meredith (1955) also studied the action of enzymes from green-malt on β -glucan. It was revealed by chromatographic analysis that glucose was produced right from the early stages; after some thirty hours a trace of cellobiose appeared and later a second disaccharide, possibly laminaribiose, also appeared. Meanwhile, a high molecular oligosaccharide had appeared near the starting line on the chromatogram; it was later followed by oligosaccharides of greater chromatographic mobility.

It was concluded that the combined action of endo- and exo- β -glucanases provided an explanation consistent with production of various oligosaccharides.

The degradation of β -glucan, by the action of enzyme preparations from sources other than barley, malt or seeds, is also observed. Bass et. al. (1952) isolated an enzyme system from a commercial bacterial α -amylase preparation by chromatography on activated alumina. These workers claimed that the purified enzyme behaved similarly to enzymes isolated from green-malt, reducing viscosity and increasing the reducing power of aqueous solutions of barley gums. Blackwood (1953) has made a survey of bacterial cultures in the genus Bacillus for the production of enzymes capable of degrading barley gums. Some 114 cultures were examined, all showed some "cytase activity"; Bacillus subtilis gave the most active enzymes. The action of cell-free culture filtrates and acetone precipitated enzymes of Myrothecium verrucaria was studied by Aitken et. al. (1956), reductionetric and chromatographic methods were employed. It was observed that the viscosity of β -glucan-culture filtrate mixtures rapidly diminished while the reducing power showed a corresponding rise; the presence of glucose and cellobiose in the reaction mixture was ascertained by paper chromatography. The conclusion that the degradation of the glucan to disaccharide was brought about by a system of exo- and endo-glucosidases was inescapable.

The presence of the β -glucanase system in enzyme preparations from other cereals such as oats and maize was observed by Preece and Hoggan (loc.cit.); different cereals were found to have a different balance of exo- and endo- β -glucanases and cellobiase. The pattern of degradation of β -glucan seems to be nearly the same under the action of barley enzymes or enzymes from other sources such as oats, rye, maize, bacteria and fungi.

Chemical investigations of Aspinall and Telfer (1954) showed that both β -1.3- and β -1.4-glucosidic linkages are present in the linear chains of the barley glucan. It is expected that the pattern of enzymic degradation of the barley glucan would show some resemblance to the enzymolysis of other glucans having both types of linkages. "Lichenin" from Iceland moss shows striking resemblance in its chemical structure with the barley glucan. This polysaccharide was first isolated by Berzelius (1813) from tannin-free residue of Iceland moss. The chemical structure of "lichenin" is discussed elsewhere (Section II); the latest information comes from the work of Peat et.al. (1957) who have confirmed that the lichenin is an unbranched β -glucan in which 1.4- and 1.3- types of glucosidic linkages occur together in the same molecule - the ratio between the two types of linkages being between 2:1 and 3:1. The presence of lichenin in oats (Avena sativa) was first reported by Morris (1942) and was later confirmed by Peat et al.(loc.cit.)

Saiki (1906) reported that the enzymes of taka-diastase (Aspergillus oryzae) and of Aspergillus niger attack lichenin. The presence of similar enzyme action in dog pancreas was reported by von Tschermak (1912). The nature of the lichenases was studied by Karrer and his school. Karrer and Staub (1924a) believe that lichenases are not usually present among the digestive enzymes of vertebrates, although found in the stomachs of cattle and swine. The literature concerning the occurrence of lichenases has been reviewed by Pigman (1951). These enzymes are distributed in various fungi, and present in barley, wheat, rye, oats, spinach, bean seeds and invertebrates, higher animals and bacteria (cytophaga). The work of Karrer and Staub (1924) and Pringsheim and Siefert (1923) has shown that snail enzyme hydrolyses lichenin almost quantitatively to D-glucose. Pringsheim and Baur (1928) obtained 60% hydrolysis of freshly prepared solutions of lichenin with malt enzyme, while Karrer and Staub (1924) had obtained in 24 hours 84% hydrolysis of 0.3 g. of lichenin by snail enzyme. It is interesting to note that Pringsheim and Kusenack (1924) and Pringsheim and Leibowitz, (1923) studied the enzymolysis of lichenin with aged malt solution and, on the basis of measurements of reducing power and optical rotation, claimed that lichenin was quantitatively converted to cellobiose. It was also inferred that the malt solutions had lost the cellobiase

activity during ageing but retained lichenase activity. The findings of Karrer and Lier (1952) are contradictory to the above. These workers were able to separate snail lichenase and cellobiase by fractional adsorption on aluminium hydroxide, and the action of essentially cellobiase free snail enzyme (lichenase) on lichenin did not result in the conversion of lichenin to cellobiose; on the contrary, more complex products were found to be present in the enzymolysate and cellobiose could not be identified. Karrer et al. (1923; 1924) found that the degradation of lichenin by snail and plant enzyme, at least during the early stages, follows the first order equation and then follows the Schutze rule, which states that the amount of material degraded is proportional to the square root of the time. The rate of hydrolysis for the same period is increased by a factor of 1.45 when the enzyme concentration is doubled. But according to Pringsheim and Baur (loc.cit.) the enzymolysis of lichenin with malt enzyme follows a first order equation whose reaction constant is proportional to enzyme concentration.

Because of the presence of β -1.3- and β -1.4 - linkages, the chemical structure of the barley glucan has some measure of relationship with other glucans, where glucopyranose residues are linked with each other either by β -1.3- or β -1.4- glucosidic linkages. Cellulose, cellulose-dextrins, and synthetic substrates such as

carboxymethyl cellulose or ethylhydroxyethyl cellulose are representative of β -1.4-linked glucans. Naturally, it is expected that there would exist certain relationship in the enzymic degradation of β -glucan and cellulose, cellodextrin or such synthetic substrates as carboxymethyl cellulose and ethylhydroxyethyl cellulose. Probably, the earliest observations on the degradation of cellulose by animals were made by Karrer (1930). Greenfield and Lane (1953) studied enzymes from borers of the genus Toredo, and presented evidence that more than one enzyme was involved in the degradation of cellulose. In recent years, considerable interest has been taken in the mechanism of cellulose degradation by fungal enzymes. Levinson et al. (1951) and Reese et al. (1950) have proposed a mechanism covering the complete conversion of cellulose to glucose. It was concluded that at least three different enzymes were involved: (1) C_1 responsible for the breakdown of the insoluble complex cellulose into single, presumably, linear, water-soluble glucan molecules of high molecular weight; (2) C_x responsible for the degradation of water-soluble glucan chains into mainly disaccharide units; and (3) cellobiase responsible for the hydrolysis of cellobiose into glucose. This mechanism shows remarkable resemblance to the degradation of the barley glucan with barley enzymes wherein exo- and endo-glucanase and cellobiase are involved. Whitaker (1953) and Basu and Whitaker (1953) investigated the degradation

of cellulose with highly purified and protein stimulated enzymes from the extracts of Myrothecium verrucaria, and found that the rate of production of glucose from cellobiose was negligible as compared to that from water-soluble cellulose product. It was concluded that glucose was produced without the intermediate formation of cellobiose and that a single enzyme was responsible for the total degradation.

Sandegren and Enebo (loc.cit.) and Enebo et al. (1953) employed artificial substrates, carboxymethyl cellulose, and ethylhydroxyethyl cellulose, for estimating the "cellulase" activities of enzyme preparations; but it has been shown by Preece and Aitken (loc.cit.), Anderson (1955), van Roey and Hupe (1955) and Thomas (1956) that in experiments of such a nature the results obtained do not parallel those with the natural substrate, β -glucan, because the artificial substrates are much less sensitive to enzyme attack.

It is rather unfortunate that a purely β -1.3-linked glucan has not been prepared yet and no comparison can be drawn between the enzymic degradation of the barley glucan and a 1.3-linked β -glucan. However, mention must be made of the investigations by Warsi and Whelan (1957) on the structure of "Pachyman" water-insoluble polysaccharide from fungus Pachyma hoelen Rumph. The experimental results of these workers suggest that the polysaccharide is a β -1.3-linked glucan; it needs

much more experimental evidence to establish that Pachyman is a linear β -glucan where glucopyranose residues are interlinked by 1.3-types of glucosidic linkages.

Besides this, the polysaccharide is not soluble in water, and therefore its enzymic degradation cannot be strictly compared with β -glucan from barley. Another polysaccharide which was at one time believed to be β -1.3-linked linear glucan is laminarin, a water-soluble polysaccharide found in some brown algae as a reserve carbohydrate. Barry (1938) was first to isolate it, in pure form, from the dried fronds of Laminaria digitata. Barry (1939) found that laminarin consists entirely of D-glucose residues and has $(\alpha)_D - 10^\circ$ in water. The presence of β -1.3-linkages in the molecule was shown by methylation studies and periodic acid oxidation (Barry et al., 1942). Peat et al. (1958) analysed the linkages in the insoluble and soluble laminarin by the method of partial acid hydrolysis, and confirmed that the principal linkage was of β -1.3-type; they also indicated the presence of other linkages too. The same workers (1958a) further suggested that the laminarin molecule may consist of unbranched chains of β -1.3-linked glucose units occasionally interrupted by β -1.6-linkages, and that some but not all of the chains are terminated by mannitol. Hirst, O'Donnell and Percival (1958) subjected laminarin to Barry degradation; oxidation and reduction were applied in succession through a cycle of three oxidations and two reductions. The

residual non-dialysable polysaccharide was isolated at each stage, weighed and its nitrogen content determined. The values obtained supported the idea that the laminarin molecule has branched structure. Recently Smith and Unrau (1959) have reported the presence of D-mannose in laminarin. On the basis of the above information laminarin can now hardly be considered as being merely a β -1.3 linked glucan and its enzymolysis cannot be strictly taken as a pattern of the enzymolysis of a linear glucan containing β -1.3-type of linkages only.

However, the predominance of β -1.3-linkages in laminarin can still be made use of in determining the enzymic characters with reference to these linkages. Dillon and O'Colla (1950) found a laminarase in extracts of wheat, oats, barley, potato, and hyacinth bulbs. Paper chromatography of the enzymolysate, in the early stage, showed the presence of glucose, laminaribiose, and several higher oligosaccharides. The crude enzyme preparation also showed lichenase activity. It is not possible to say whether it is a specific enzyme or if it is just the β -1.3-polyglucosidase activity of the lichenase system. MacWilliam and Harris (1957), while fractionating malt enzymes by selective elution from alumina, obtained evidence for the separation of two enzymes: (a) producing glucose and laminaribiose from laminarin; and (b) producing higher oligosaccharides. These two enzymes were considered exo- β -1.3- and endo- β -1.3 -polyglucosidases respectively. Since the

activity of these fractions on the barley glucan or lichenin was not tested, the enzymes cannot be regarded as exo- and endo-lamarases.

Decrease in the viscosity of the barley glucan solution in presence of enzymes over a period of 90 minutes, with predictable increase in reducing groups, has been, so far, the only evidence of endo- β -glucanase activity. Since endo-action involves attack on 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 viscometry over longer periods. Therefore, opportunity was taken, in the present work, to determine the increase in the reducing power of the reaction mixture, the recovery of dextrin, and its complexity, during the enzymolysis of β -glucan under endo-action alone for periods of 48 hours. It was expected that such a study would confirm or disprove the view that the endo-action over long periods is not as simple as it appears from the decrease in solution viscosity; also the decrease in the molecular complexity of the dextrin might provide a more correct picture of the degradation. The pattern of degradation of oat glucan should also reflect the presence of different proportions of β -1.3- and β -1.4-glycosidic linkages in it; Peat et al. (1957) have reported that the 1.3- and 1.4-types of linkages are present in the

ratio of 70:30 in "oat lichenin" - a β -linked glucan prepared from oats by the method of Morris (loc.cit.). The production of reducing groups during the enzymolysis of laminarin and cello-dextrin was also studied with a view to determining enzyme characters in respect to β -1.3- and β -1.4-linkages independantly.

Previous workers have taken the reducing groups, produced in excess to those calculated from the decrease in viscosity, as evidence for exo-activity; the conversions were studied over shorter periods. It is quite obvious, that the values obtained for the activities of exo- β -glucanase, and endo- β -glucanase enzymes in these experiments may not hold good in longer enzyme actions. The rate of disappearance of β -glucan, or inversely the recovery of dextrin, under the joint action of exo- and endo- enzymes should be more rapid; consequently, greater amounts of glucose and oligosaccharides should be present in such a conversion. Hence, arose the necessity of studying joint-action (endo- + exo-) also over longer periods.

The aim of this section is, therefore, to present a more detailed picture of the pattern of β -glucan degradation over periods longer than those previously studied.

EXPERIMENTAL

Preparation of Substrates.

β -glucan from barley and oats was prepared by the method of Preece and Mackenzie (loc.cit.); in order to avoid extensive degradation of the glucan during the grinding of the grains, an ordinary coffee-mill was employed instead of a hammer-mill (approximately 3,000 revolutions per minute). Water-soluble laminarin was obtained by courtesy of Dr. Black. Cellodextrin was prepared by the following procedure:

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. At this stage the whole of the cellulose powder went into solution. The solution was poured into 500 ml. of cold water; insoluble cellulose formed a white gelatinous precipitate while the cellulose which was rendered soluble during treatment remained in the liquor. Insoluble cellulose was removed from the mixture at the centrifuge and water-soluble cellodextrin was recovered from the liquor by adding an equal volume of acetone. The white precipitate of cellodextrin was dissolved in the minimum amount of water, the solution was neutralised with barium carbonate and dialysed for 2 - 3 days in presence of thymol as antiseptic. The solution, after dialysis was concentrated to 200 ml. and centrifuged. Cellodextrin was finally precipitated

from the clear liquid by the addition of equal volume of acetone, and the precipitate was taken to dryness in the usual way.

Preparation of enzymes.

The method of Preece and Hoggan (loc.cit.) was employed for the preparation of enzymes. 300 g. of the ground cereal (barley or oats) was extracted with 100 ml. of the citrate-phosphate buffer of pH 4.5 for one hour at room temperature. The buffer was prepared as described by Britton (1942). The extract was left overnight for autolysis, dialysed in presence of antiseptic for 2 - 3 days and the pH of the dialysed extract was again adjusted to 4.5 with normal acetic acid. Material of low enzyme activity was precipitated by the addition of 30 g. of ammonium sulphate per 100 ml. of the extract. This precipitate was removed and another 20 g. of ammonium sulphate was added to the original extract to precipitate the enzymes. The precipitate was dissolved in the minimum quantity of water and enzyme solution was dialysed for 2 days, after which period it was centrifuged; the enzymes were finally precipitated from it by the addition of four volumes of acetone and dried in the usual way.

Preece and Hoggan (loc.cit.) had observed that the exo- β -glucanase activity was completely destroyed by treatment of an enzyme solution with phenylmercuric nitrate; 1 ml. enzyme solution was treated with 5 mg. of phenylmercuric nitrate for 3 hours at room temperature. This observation was made use of in preparing enzymes

completely devoid of exo-activity.

A known amount of phenylmercuria nitrate in aqueous suspension was added to 100 ml. of enzyme solution so that 5 mg. of the salt was present per 1 ml. of the reaction mixture which was allowed to stand at room temperature for 3 hours. The reaction mixture was then centrifuged, dialysed for 2 days, and the enzymes were precipitated from it by the addition of four volumes of acetone; the enzymes were dried in the usual way. Thymol was used as antiseptic throughout the whole procedure of preparing enzymes. The action of these enzymes has been referred to as "endo-action" in the present work, though, it should be kept in mind that cellobiase was always present.

Determination of endo- β -glucanase activity.

8 ml. of 0.6875% β -glucan solution were mixed with 2 ml. of enzyme solution (containing 1 mg. enzyme per 1 ml.) and 1 ml. of acetate buffer pH 5.0; the buffer was prepared as described by Palmer (1951) and all the solutions were brought to 25°C. before mixing. 10 ml. of the reaction mixtures were transferred to an Oswald viscometer, and the viscosity was determined at 25°C. after regular intervals over a period of 90 minutes.

The rate of decrease in the viscosity of the reaction mixture was made the basis for determining the enzyme activity (Preece and Aitken, loc.cit.). The specific viscosity (η_{sp}) of the reaction mixture is represented by $T_s - T_w/T_w$, T_s and T_w being the time of

outflow from the viscometer of the reaction mixture and water respectively; reciprocal specific viscosity $1/\eta_{sp}$ is represented by the symbol U. The enzyme activity is expressed by Hultin's (1946) principle which was also employed by Sandegren and Enebo (loc.cit.) in the equation

$$E = K^1 \cdot C^2 \cdot \frac{dU}{dT}, \text{ where } E \text{ is the enzyme activity,}$$

k^1 a constant, C^2 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 the 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- β -glucanase activity. The slope of the curve dU/dT was linear over 90 minutes. Enzyme activity was expressed as increase in reciprocal specific viscosity of 0.5% glucan solution per mg. of enzyme per hour at 25°C.

Presence of exo-activity.

The presence of exo-activity in enzyme preparations treated with phenylmercuric nitrate was always tested. It was found that the exo-activity was completely eliminated from such preparations. The following procedure was adopted:

Production of reducing groups in 0.5% conversion mixtures of β -glucan at 25°C. was determined on 5 ml. aliquots of the reaction mixtures by employing the

Somogyi (1945) copper-reduction method. The results were corrected for the initial reducing power of the substrate and reduction due to enzyme by concurrently-run substrate and enzyme blanks (enzyme blanks did not in fact show any increment in their reducing power meaning thereby that the enzymes were uncontaminated with labile carbohydrate material). The production of reducing groups was measured over a period of 3 hours. On the basis of the findings of Preece and Hoggan (loc.cit.) it was expected that in a β -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. Therefore, the presence of exo-activity in phenylmercuric nitrate treated enzymes was tested by comparing the observed number of total reducing groups (as mg. glucose equivalent) produced during enzymolysis with the reducing groups calculated on the basis of decrease in viscosity. Reducing power of 11 ml. aliquots of reaction mixture was calculated from the following equation:

$$\text{gm.} = 1.165 U + 0.000 \pm 0.012$$
, where gm. is the glucose equivalent of the reaction mixture, and U is the reciprocal specific viscosity. It was found that the observed reducing power never exceeded the calculated values.

Enzymolysis of substrates.

Enzymolysis of each substrate was carried in

0.5% solution at 37°C. (using thymol as antiseptic) for 48 hours. An 110 ml. aliquot of the conversion mixtures were prepared by mixing 80 ml. of 0.6875% substrate solution with 20 ml. of enzyme solution and 10 ml. of Palmer's (loc.cit.) acetate buffer, pH 5.0; before mixing, the solutions were brought to 37°C. For the experiments recorded in Tables V - XII, endo-activity was the same in each conversion; using enzyme preparations of low activity the enzyme concentration in solution was correspondingly increased. The justification for doing so, came from the work of Preece and Aitken (loc.cit.) who had already shown that the relation between enzyme concentration and its activity was linear, at least to the point where the value of dU/dT was greater than ca. 0.125. Substrate blanks and enzyme blanks were run under exactly similar conditions to allow for the autolysis of the substrate and enzyme in presence of the buffer. It was found, in fact, that enzyme and substrate were both stable.

Recovery of dextrin.

10 ml. of conversion mixture was withdrawn from the main incubation and was kept in a boiling water bath for 3 minutes for inactivating the enzymes. The solution was then cooled and denatured enzyme was removed by centrifuging. Dextrin was precipitated from the clear liquid by adding four volumes of acetone. The dextrin precipitate was separated, dried in increasing concentrations of acetone, and filtered in No.3. sintered

glass crucibles, previously dried in vacuum and then at 100°C. five hours before cooling and weighing. In the same way dextrans were recovered from the endo-action and joint-action conversion mixtures of 0.5% β -glucan solution after 24 hours incubation at 37°C. These recovered dextrans have been referred as "endo-action limit dextrin" and "joint-action limit dextrin"; enzymolysis and acid hydrolysis of these limit dextrans were also studied.

Complexity of dextrin.

Dextrin was precipitated as described above, the precipitate was washed with acetone and dissolved in 10 ml. of hot water. The reducing power of the dextrin solution was determined on 5 ml. aliquots by Somogyi's method (loc.cit.). Knowing the dry weight of dextrans from previous experiment μ g. glucose equivalent per mg. of dextrin (R) was calculated. Function $1000/R$ expressed the complexity of dextrin. It is realized that reducing power is not a true inverse measure of complexity, especially when both β -1.3, and β -1.4- linkages are involved; glucose equivalents of laminaribiose and cellobiose are different. The concept "complexity" is not employed to express 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.

Total reducing groups in the conversion mixtures,
and in the liquor as glucose and oligosaccharides.

Reducing power (mg. glucose equivalents) of the reaction mixtures was determined by the Somogyi method (loc.cit.); correction for substrate and enzyme blank was applied. Glucose equivalents for oligosaccharides and glucose produced during enzymolysis were calculated by subtracting the values for glucose equivalents of dextrin from the total. In experiments where the amounts of glucose and oligosaccharides are reported separately, the liquor obtained after precipitating the dextrin from reaction mixture was concentrated and was submitted to descending paper chromatography in butanol-acetic acid-water (4 : 1 : 5) solvent; spots of glucose and oligosaccharides on the paper were cut separately, the ratio of the two in the liquor was determined by eluting sugars from paper with water and measuring the reducing power of the eluates by Somogyi method (loc.cit.).

Cellobiase and Laminaribiase activity.

8 ml. of 0.3437% sugar solution (cellobiose or laminaribiase) were mixed with 1 ml. of acetate buffer (pH 5.0) prepared according to Palmer (loc.cit.) 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 (loc.cit.). Activities were expressed in terms of the mg. of sugar hydrolysed per mg. of enzyme per hour. Calculations for cellobiase activity were done as following:

Let C be the number of mg. celliobiose originally present for 0.5 ml. of the reaction mixture and X be the number of mg. cellobiose 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 $N/200 \text{ Na}_2\text{S}_2\text{O}_3$ solution equivalent to 0.5 ml. of the reaction mixture after correction for enzyme control.

$$T = \frac{X}{0.198} + (C - X) \cdot \frac{360}{342} \cdot \frac{1}{0.141} \text{ ml. - (A)}$$

where 1 ml. of $N/200 \text{ Na}_2\text{S}_2\text{O}_3$ solution is equivalent to 0.198 mg. pure cellobiase or 0.141 mg. pure glucose.

Equation (A) on simplification would become

$$T = 5.06X + (C - X) \cdot 7.46$$

$$\text{or} = 5.06X + 7.46C - 7.46X$$

$$X = \frac{7.46 - T}{2.40} \text{ mg. per 0.5 ml. of reaction}$$

mixture and $(C - X) =$ no. of mg. cellobiose hydrolysed per 0.5 ml. of reaction mixture after t hours.

In case of laminaribiose determinations equation (A) would become,

$$T = \frac{X}{0.360} + (C - X) \frac{360}{342} \cdot \frac{1}{0.141} \text{ ml. - (B)}$$

where 1 ml. of N/200 $\text{Na}_2\text{S}_2\text{O}_3$ solution is equivalent to 0.360 mg. pure laminaribiose, and C is the no. of mg. of laminaribiose present in 0.5 ml. of reaction mixture.

Equation (B) on simplification becomes

$$T = 2.07X + (C - X) \cdot 7.46$$

$$\text{or} = 0.27X + 7.46C - 7.46X$$

$$X = \frac{7.46 - T}{5.39} \text{ mg. per 0.5 ml. of reaction}$$

mixture and $(C - X) =$ no. of mg. of laminaribiose

hydrolysed per 0.5 ml. reaction mixture after t hours.

TABLE I [⊛]

Endo-action alone of oat enzymes

| | Reaction time (hr.) | | | | | |
|--|---------------------|------|------|------|------|------|
| | 0 | 6 | 18 | 24 | 30 | 42 |
| Reduction : mg. per 100 mg. Original glucan:- Dextrin | 0.47 | 0.57 | 0.86 | 0.98 | 1.33 | 1.52 |
| Oligosaccharides | - | 0.00 | 0.19 | 0.22 | 0.33 | 0.25 |
| Glucose | - | 0.00 | 0.19 | 0.21 | 0.22 | 0.23 |
| Dextrin characters:- Recovery (mg. per 100) | 1.00 | 99.2 | 98.9 | 98.9 | 98.6 | 98.3 |
| Reduction (R ₁ / Ng. per Mg) | 4.70 | 5.75 | 8.70 | 9.91 | 13.5 | 15.5 |
| Complexity (1000/R ₂) | 213 | 174 | 115 | 101 | 74 | 64 |

⊛ All reducing values for the results of this and other tables are as glucose equivalents.

⊣ Represents 20 ml. aliquot for the results of this and other tables.

TABLE II

Endo-action alone of barley enzymes.

| | Reaction Time (hr.) | | | | | |
|-------------------------------------|---------------------|------|------|------|------|------|
| | 0 | 6 | 18 | 24 | 30 | 42 |
| Reduction : mg. per 100 mg. | | | | | | |
| Original glucan:- Dextrin | 0.30 | 0.47 | 0.84 | 0.97 | 1.03 | 1.32 |
| Oligosaccharides | - | 0.00 | 0.39 | 0.57 | 0.47 | 0.70 |
| Glucose | - | 0.00 | 0.56 | 0.43 | 0.50 | 0.52 |
| Dextrin characters:- | | | | | | |
| Recovery (mg. per 100) | 100 | 99.6 | 97.1 | 93.9 | 93.9 | 91.8 |
| Reduction (R_1 μ g. per mg.) | 3.00 | 4.72 | 8.65 | 10.3 | 11.0 | 14.4 |
| Complexity (1000/R) | 333 | 212 | 115 | 97 | 91 | 69 |

TABLE III

Joint Endo- and Exo-activities of enzymes from oats.

| | A : Reaction time (hr.) | | | | | | B : Reaction time (hr.) | | | | | |
|--|-------------------------|------|------|------|-------|-------|-------------------------|------|------|------|------|-------|
| | 0 | 6 | 18 | 24 | 30 | 42 | 0 | 6 | 18 | 24 | 30 | 42 |
| Reduction: mg. per 100 mg. | | | | | | | | | | | | |
| Original glucan:-- | | | | | | | | | | | | |
| Dextrin | 0.14 | 1.50 | 2.10 | 2.50 | 2.14 | 3.10 | 0.46 | 1.31 | 2.99 | 3.16 | 3.27 | 4.64 |
| Oligosaccharides | - | 0.38 | 2.12 | 2.38 | 2.54 | 4.88 | - | 1.09 | 3.53 | 1.65 | 2.54 | 3.41 |
| Glucose | - | 1.62 | 6.18 | 9.36 | 10.76 | 14.26 | - | 1.29 | 3.61 | 6.56 | 9.70 | 15.11 |
| Dextrin Characters:- | | | | | | | | | | | | |
| Recovery (mg. per 100) | 100 | 95.6 | 91.8 | 84.0 | 83.0 | 81.0 | 100 | 92.8 | 86.9 | 85.9 | 81.0 | - |
| Reduction (R ₁ / μg. per mg.) | 1.44 | 15.7 | 22.9 | 29.7 | 25.6 | 38.3 | 4.64 | 14.1 | 34.4 | 36.8 | 40.4 | - |
| Complexity (1000/R) | 714 | 64 | 44 | 34 | 39 | 26 | 215 | 71 | 29 | 27 | 25 | - |

A : fresh oats; B : old oat sample.

TABLE IV

Joint Endo- and Exo-activities of Enzymes from barley (Barley glucan).

| | A : Reaction Time (hr.) | | | | | | B : Reaction Time (hr.) | | | | | |
|--|-------------------------|------|------|----|-------|-------|-------------------------|---|-------|-------|-------|-------|
| | 0 | 6 | 18 | 24 | 30 | 42 | 0 | 6 | 18 | 24 | 42 | 48 |
| Reduction: mg. per 100 mg. | 0.37 | 2.53 | 2.83 | - | 2.31 | 2.31 | 0.46 | - | 2.89 | 2.95 | 3.29 | 3.35 |
| Original glucan:-- Dextrin | - | 1.66 | 2.35 | - | 7.18 | 23.86 | - | - | 17.91 | 18.83 | 24.76 | 26.61 |
| Oligosaccharides | - | 4.45 | 5.25 | - | 15.83 | - | - | - | - | - | - | - |
| Glucose | - | 84.2 | 74.9 | - | 67.2 | 63.8 | 100 | - | 75.2 | 68.4 | 57.2 | 57.0 |
| Dextrin Characters:-- | 100 | 30.0 | 37.8 | - | 34.4 | 36.2 | 4.60 | - | 38.4 | 43.1 | 57.5 | 58.8 |
| Recovery (mg. per 100) | 270 | 33 | 27 | - | 29 | 28 | 218 | - | 26 | 23 | 17 | 17 |
| Reduction (R ₁ Mg. per mg.) | | | | | | | | | | | | |
| Complexity (1000/R) | | | | | | | | | | | | |

A, normal enzyme; B, celite treated enzyme.

TABLE V

Comparison of Joint activity of Endo- and Exo- β -glucanases of barley with that of Endo-alone (Barley glucan).

| | Endo [#] + exo-: Reaction time (hr) | | | | | | Endo [#] alone: Reaction time (hr) | | | | | | | |
|---|--|--------------------|--------------------|--------------------|--------------------|--------------------|---|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|
| | 0 | 6 | 18 | 24 | 30 | 42 | 48 | 0 | 6 | 18 | 24 | 30 | 42 | 48 |
| Reduction: mg. per 100 mg. Original glucan :- Dextrin | 0.26 | 1.79 | 2.91 | 2.58 | 3.05 | 2.71 | 1.52 | 0.26 | 1.56 | 2.37 | 2.31 | 2.78 | 3.05 | 2.82 |
| Oligosaccharides + Glucose | - | 11.71 | 23.64 | 28.20 | 38.98 | 47.27 | 51.11 | - | 2.69 | 5.76 | 6.50 | 7.73 | 9.49 | 9.72 |
| Dextrin characters:- Recovery (mg. per 100). Reduction (R μ g per mg.) Complexity (1000/R) | 100 2.60 385 | 90.8 19.7 51 | 70.2 41.4 24 | 64.3 40.1 25 | 52.8 57.8 17 | 47.2 57.4 17 | 27.5 55.3 18 | 100 2.60 385 | 99.7 15.6 64 | 97.3 24.3 41 | 91.6 25.2 40 | 90.1 30.9 32 | 88.5 34.5 29 | 87.7 32.2 31 |

Endo-action (by viscometry) is the same in each series.

TABLE VI

Total reduction on various substrates (Barley enzymes[§])

| Substrate | Activity | Reaction time (hr.) | | | | | | | |
|----------------|--------------|---------------------|-------|-------|-------|-------|-------|-------|--|
| | | 0 | 6 | 18 | 24 | 30 | 42 | 48 | |
| Barley glucan | Endo- + Exo- | 0.26 | 13.50 | 26.55 | 30.78 | 42.03 | 49.98 | 52.63 | |
| | Endo- alone | 0.26 | 4.25 | 8.13 | 8.81 | 10.51 | 12.54 | 12.54 | |
| glucan dextrin | Endo- + Exo- | 3.45 | 21.71 | 37.51 | 44.54 | 46.81 | 52.17 | 54.43 | |
| | Endo- alone | 3.45 | 17.18 | 28.20 | 38.07 | 41.74 | 52.20 | 54.84 | |
| Laminarin | Endo- + Exo- | 1.69 | 18.61 | 35.81 | 42.30 | 48.90 | 61.38 | 61.38 | |
| | Endo- alone | 1.69 | 17.81 | 35.81 | 42.40 | 47.80 | 56.86 | 61.00 | |
| Cellodextrin | Endo- + Exo- | 10.31 | 20.10 | 25.47 | 26.99 | 30.32 | 36.67 | 36.67 | |
| | Endo- alone | 10.31 | 14.86 | 19.16 | 22.44 | 26.68 | 30.32 | 30.32 | |

§ Endo-action is the same in series.

TABLE VII

Substrate "complexity" and Enzyme activity.

| | Original Substrate | | In first 6 hr., per hr. | | | | | |
|---------------------------|-----------------------------------|------------------------|--------------------------------------|---|----------------|---------------|---|----------------|
| | Reduction (R_1 μ G/mg.) | Complexity (1000/R) | Increased reduction (μ G/mg) | | | Scissions | | |
| | | | Endo- Exo- | + | Endo- alone | Endo- Exo- | + | Endo- alone |
| Barley - β - glucan | 2.6 | 385 | 20.4 | | 6.6 | 7.8 | | 2.6 |
| Laminarin | 16.9 | 59 | 28.2 | | 26.9 | 1.7 | | 1.6 |
| glucan dextrin | 34.5 | 29 | 30.4 | | 22.9 | 0.9 | | 0.7 |
| Celłodextrin | 103 | 10 | 16.3 | | 7.6 | 0.16 | | 0.07 |
| Oat- β - glucan (A) | 3.3 | 295 | 8.7 | | 5.4 | 2.6 | | 1.6 |
| Oat- β - glucan (B) | 16.9 | 50 | 21.6 | | 3.8 | 1.3 | | 0.22 |

TABLE VIII

Enzymolysis of Endo-action "resistant dextrin"*

| | Endo- + Exo : Reaction time (hr.) | | | | | Endo-alone : Reaction time (hr) | | | | |
|---|-----------------------------------|------|------|------|------|---------------------------------|------|------|------|------|
| | 0 | 6 | 18 | 30 | 42 | 0 | 6 | 18 | 30 | 42 |
| Reduction : mg. per 100 mg. | | | | | | | | | | |
| Original Dextrin : Dextrin | 3.00 | 4.36 | 3.84 | 4.36 | 4.36 | 3.00 | 4.24 | 4.00 | 4.16 | 3.92 |
| Oligosaccharides + glucose | - | 1.04 | 3.04 | 3.84 | 3.84 | - | 0.80 | 1.40 | 1.80 | 2.16 |
| Dextrin characters:- | | | | | | | | | | |
| Recovery (mg. per 100) | 100 | 96.8 | 89.6 | 57.2 | 56.8 | 100 | 98.4 | 94.2 | 92.8 | 92.0 |
| Reduction (R ₁) (μg. per mg.) | 30.0 | 44.7 | 42.9 | 76.2 | 76.6 | 30.0 | 43.1 | 42.4 | 44.8 | 42.6 |
| Complexity (1000/R) | 33 | 22 | 23 | 13 | 13 | 33 | 23 | 24 | 22 | 24 |

* Reaction time 30 hr.; yield on original glucan 80%

TABLE IX

Enzymolysis of Joint-action "resistant dextrin"[‡]

| | Endo- + Exo- : Reaction time (hr) | | | | | Endo-alone : Reaction time (hr.) | | | | |
|--|-----------------------------------|------|------|------|------|----------------------------------|------|------|------|------|
| | 0 | 6 | 18 | 30 | 42 | 0 | 6 | 18 | 30 | 42 |
| Reduction : mg. per 100 mg. | | | | | | | | | | |
| Original dextrin: Dextrin | 4.36 | 4.96 | 4.96 | 4.96 | 4.96 | 4.36 | 4.48 | 4.48 | 4.52 | 4.52 |
| Oligosaccharides + glucose | - | 0.32 | 0.44 | 0.44 | 0.44 | - | 0.48 | 0.48 | 0.44 | 0.52 |
| Dextrin characters:- | | | | | | | | | | |
| Recovery (mg. per 100) | 100 | 99.2 | 99.2 | 99.2 | 99.2 | 100 | 99.6 | 99.2 | 99.2 | 99.2 |
| Reduction (R ₁ μg. per mg.) | 43.6 | 50.0 | 50.0 | 50.0 | 50.0 | 43.6 | 44.9 | 45.2 | 45.6 | 45.6 |
| Complexity (1000/R) | 23 | 20 | 20 | 20 | 20 | 23 | 22 | 22 | 22 | 22 |

‡ Reaction time 30 hr., yield on original glucon 70%

Comparison of Joint-activity of Endo[#] and Exo- β -glucanases of Barley with that of Endo-alone[#] (Oat glucan A).

| | Endo- + Exo- : Reaction time (hr). | | | | | Endo- alone : Reaction time (hr). | | | | | | |
|--------------------------------|------------------------------------|-------|-------|--------|-------|-----------------------------------|------|------|-------|-------|-------|-------|
| | 0 | 6 | 18 | 30 | 42 | 48 | 0 | 6 | 18 | 30 | 42 | 48 |
| Reduction: mg. per 100 mg. | 0.33 | 1.41 | 2.53 | 1.41 | - | - | 0.33 | 1.91 | 2.54 | 2.82 | 3.21 | 3.10 |
| Original glucan : Dextrin | - | 3.98 | 26.98 | 75.01 | 87.98 | 87.98 | - | 1.69 | 11.56 | 21.15 | 26.68 | 33.84 |
| Oligosaccharides + Glucose | | | | | | | | | | | | |
| Dextrin characters:- | | | | | | | | | | | | |
| Recovery (mg. per 100) | 100 | 82.8 | 38.4 | 13.0 | - | - | 100 | 90.4 | 80.8 | 69.4 | 51.0 | 40.0 |
| Reduction (R μ g. per mg.) | 3.38 | 17.02 | 65.88 | 108.46 | - | - | 3.38 | 21.2 | 31.5 | 40.6 | 63.0 | 77.5 |
| Complexity (1000/R) | 295 | 58 | 15 | 9 | - | - | 295 | 47 | 31 | 24 | 15 | 13 |

Endo-action (by viscometry) is the same in each series.

TABLE XI.

Degradation of oat- β -glucan (B).

| | Endo- ^{##} + Exo- : Reaction time (hr.) | | | | | | Endo- ^{##} alone : Reaction time (hr.) | | | | | | | |
|---|--|-------|-------|-------|-------|-------|---|------|------|------|-------|-------|-------|-------|
| | 0 | 6 | 18 | 24 | 30 | 42 | 48 | 0 | 6 | 18 | 24 | 30 | 42 | 48 |
| Reduction: mg. per 100 ng. | | | | | | | | | | | | | | |
| Original glucan : Dextrin | 1.69 | 1.94 | 2.60 | 2.72 | 2.30 | 2.84 | 2.30 | 1.69 | 2.48 | 3.03 | 2.71 | 2.36 | 3.75 | 3.09 |
| Oligosaccharides + glucose | - | 12.71 | 16.02 | 30.16 | 42.57 | 54.34 | 54.90 | - | 1.51 | 9.92 | 11.98 | 14.28 | 20.45 | 27.77 |
| Dextrin characters:- | | | | | | | | | | | | | | |
| Recovery (mg. per 100) | 100 | 81.3 | 56.2 | 49.0 | 22.5 | - | - | 100 | 93.8 | 80.5 | 76.6 | 69.2 | 60.0 | 56.9 |
| Reduction (R ₁ μ g. per mg.) | 16.9 | 23.8 | 46.3 | 55.5 | 102.0 | - | - | 16.9 | 26.3 | 37.6 | 35.4 | 34.1 | 62.5 | 54.1 |
| Complexity (1000/R) | 59 | 42 | 22 | 18 | 10 | - | - | 59 | 38 | 27 | 28 | 29 | 16 | 18 |

^{##}-Endo-action (by viscometry) is the same in each series.

TABLE XII

Laminaribiase and Cellobiase activities of Barley enzymes.

| Enzyme | mg. hydrolysed per hr. per mg. enzyme | |
|--------------|---------------------------------------|------------|
| | Laminaribiase | Cellobiase |
| Endo- alone | 0.28 | 0.20 |
| Endo- + Exo- | 0.89 | 0.25 |

K E Y.

- Fig. 1. Endo-degradation of barley glucan: dextrin recovery, oat enzyme (A); barley enzymes (B and C).
- Fig. 2. Endo-degradation of barley glucan: comparison of increase in total reducing groups (RT) with proportion remaining as reducing groups in dextrin ($100R_D/R_T$), oat enzyme (o); barley enzymes (X and *).
- Fig. 3. Endo-degradation of barley glucan: approximation of the curve of Fig. 2 with the relation $\log RT = 1.4 - 1.8 R_D/R_T$.
- Fig. 4. Endo-degradation of barley glucan: comparison of total scissions with relative scissions ($100 S_D/S_T$) in dextrin, oat enzyme (o); barley enzymes. (X and *).

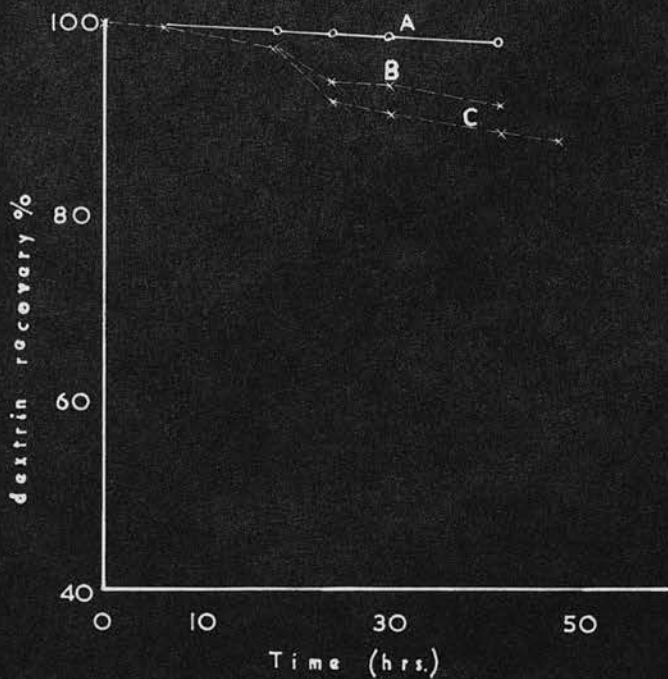


FIG. 1

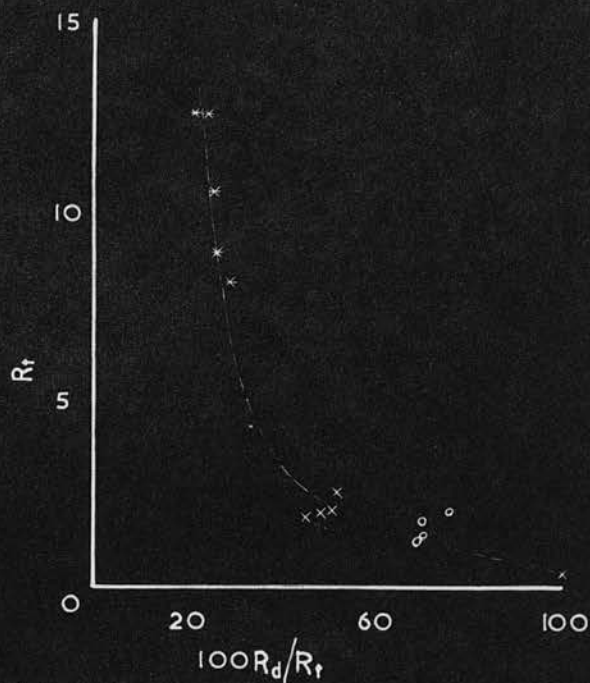


FIG. 2

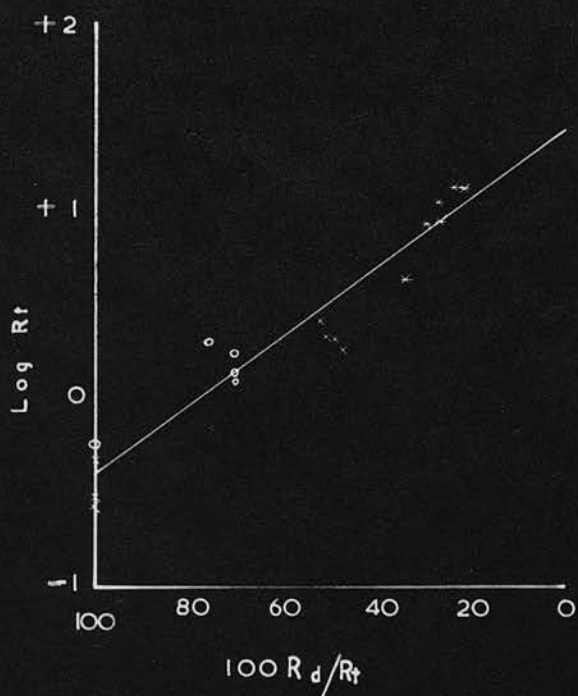


FIG. 3

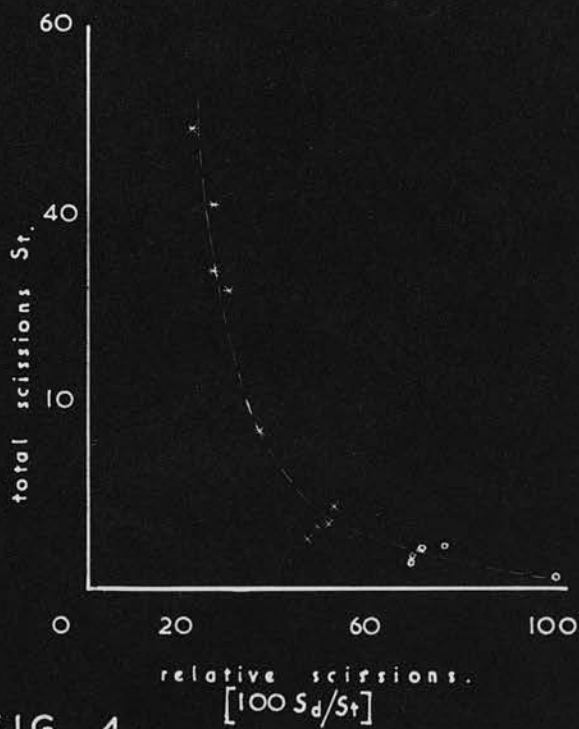
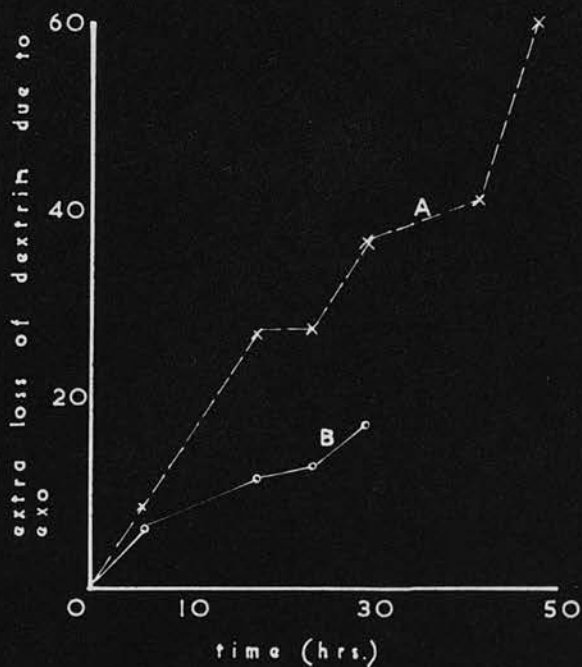
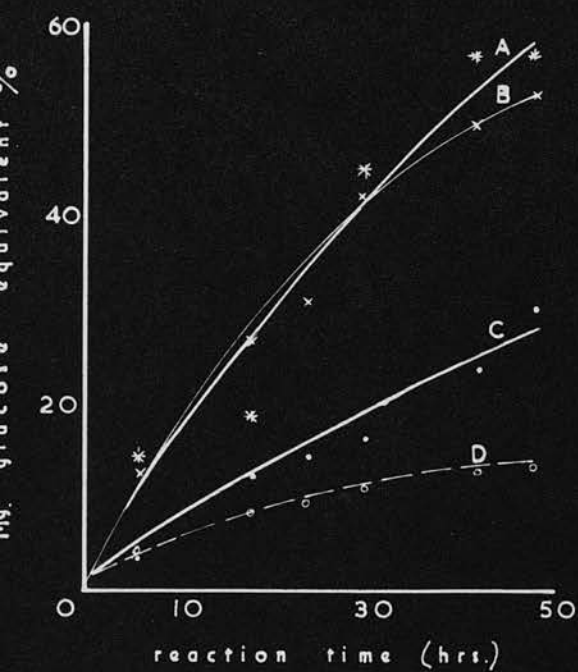
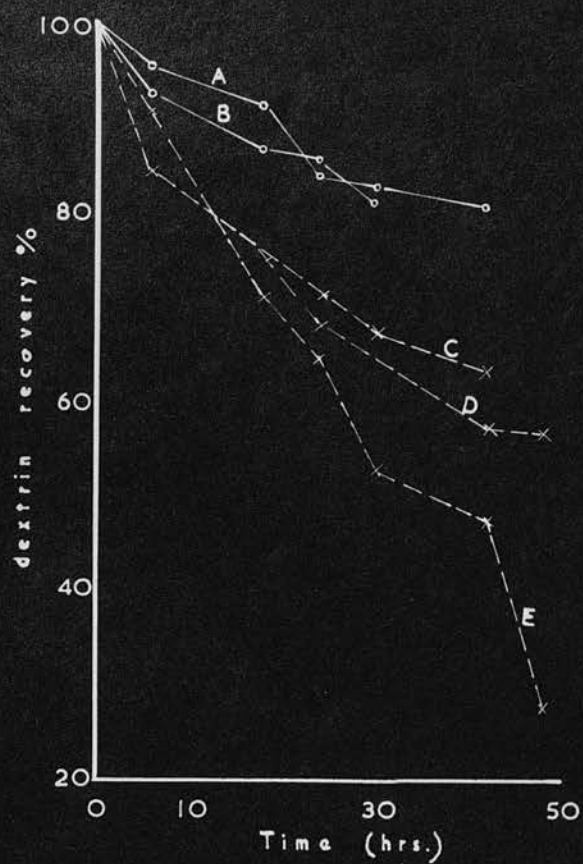
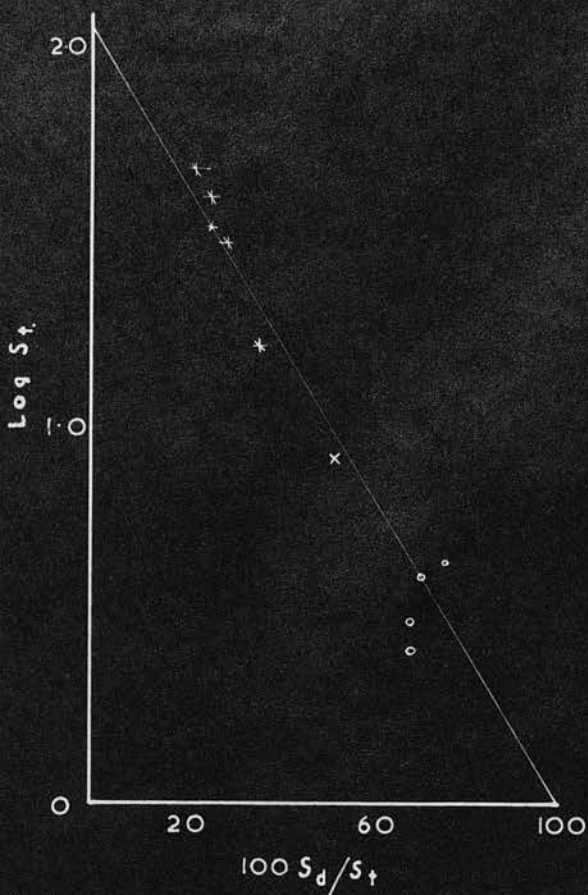


FIG. 4

K E Y.

- Fig. 5. Endo-degradation of barley glucan: approximation of the curve of Fig. 4. with the relation $\log S_T = 2.05 - 2.05 S_D/S_T$.
- Fig. 6. Joint-action (endo- + exo-) degradation of barley glucan: dextrin recovery, oat enzyme (A and B); barley enzymes (C, D and E).
- Fig. 7. Degradation of oat and barley glucan with barley enzymes: production of reducing groups, joint-action on oat glucan (A); endo-action alone on oat glucan (C); joint-action on barley glucan (B); endo-action alone on barley glucan (D).
- Fig. 8. Degradation of barley glucan: extra loss of dextrin due to exo-action, oat enzyme (A); barley enzyme (B).



K E Y.

- Fig. 9. Joint-action (endo- + exo-) degradation of barley glucan: comparison of increase in total reducing groups (R_T) with the proportion remaining as reducing group in dextrin ($100 R_D/R_T$), oat enzymes (o and \emptyset); barley enzymes (X, * and +).
- Fig. 10. Joint-action (endo- + exo-) degradation of barley glucan: approximation of the curve of Fig. 9. with the relation $\log R_T = 1.8 - 3.1 R_D/R_T$
- Fig. 11. Degradation of barley glucan dextrin with barley enzymes: production of reducing groups, Endo- + exo- (A); Endo- alone (B).

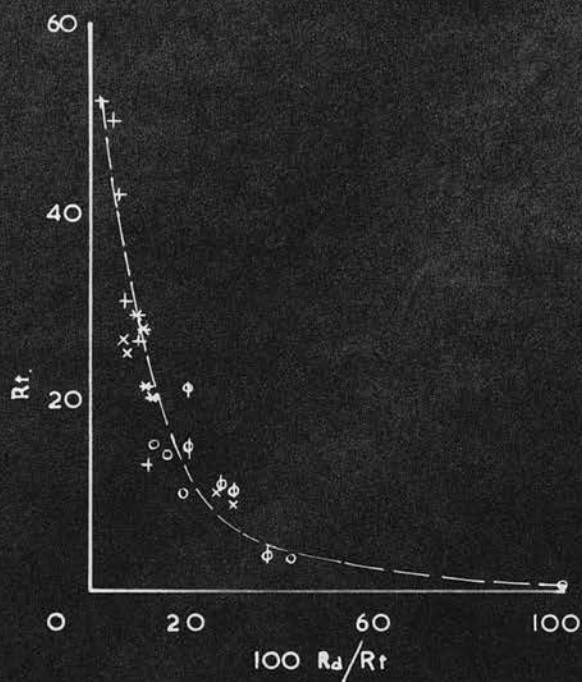


FIG. 9.

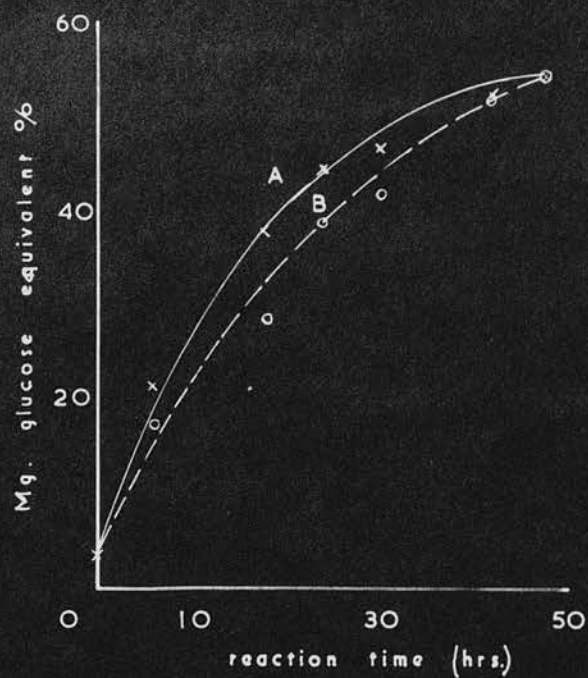


FIG. 11.

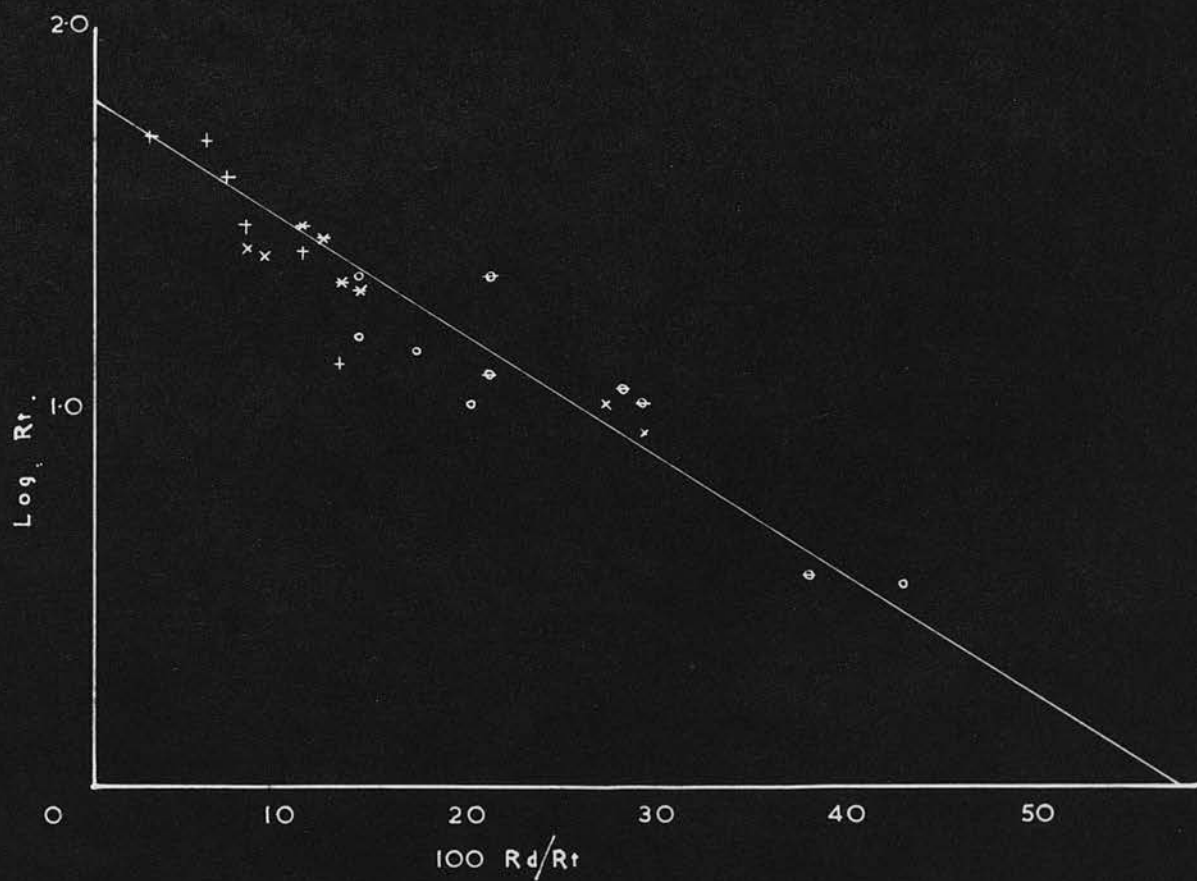


FIG. 10.

K E Y.

- Fig. 12. Degradation of laminarin with barley enzymes:
production of reducing groups, endo- + exo- (x);
endo- alone (o).
- Fig. 13. Degradation of celloextrin with barley enzymes:
production of reducing groups, endo- + exo- (A);
endo- alone (B).
- Fig. 14. Degradation of barley and oat glucan with
barley enzymes: dextrin recovery, endo-action
on oat glucan (A); endo-action on barley glucan
(B); joint-action on oat glucan (C); joint-
action on barley glucan (D).

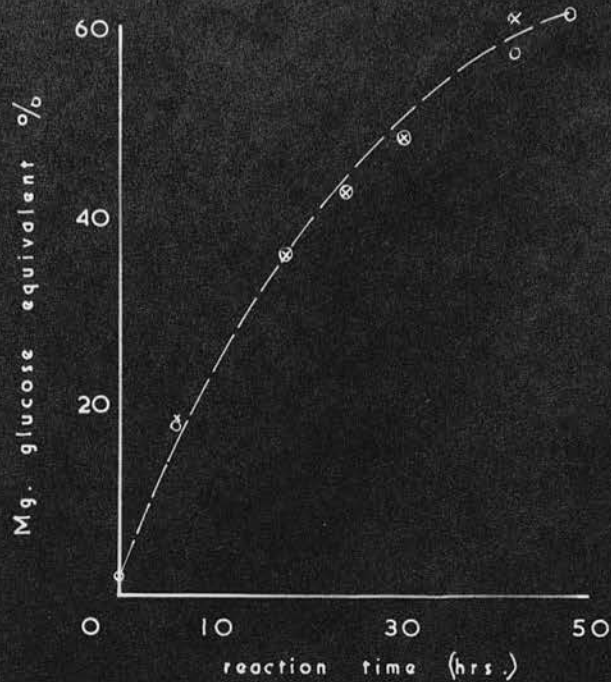


FIG. 12 .

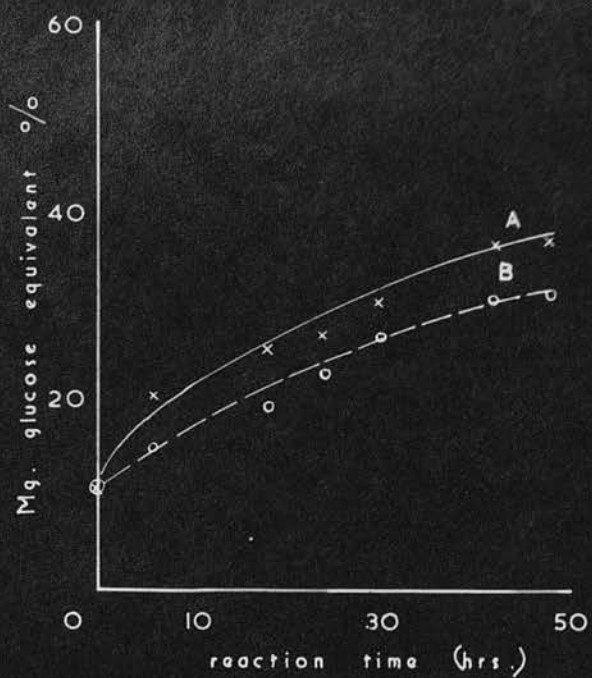


FIG. 13 .

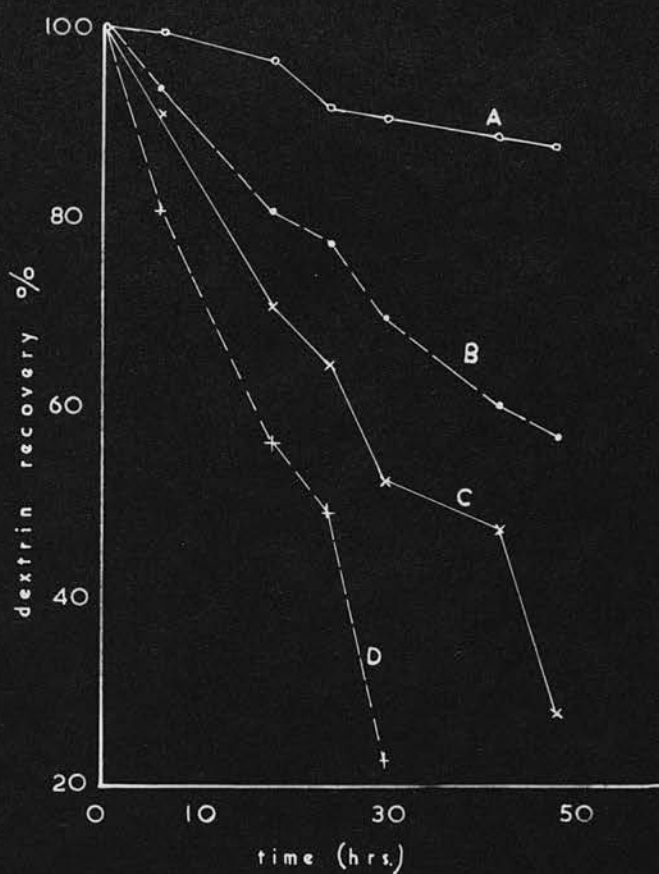


FIG. 14 .

DISCUSSION

Endo-degradation of barley β -glucan.

The work of Preece and Hoggan (loc.cit.) clearly established that the endo- β -glucanase action, in its early stages, involves attack on the glucan chains at points remote from the ends, resulting in the production of progressively smaller molecules, so that a linear relationship is obtained between reciprocal specific viscosity and reducing power. Prolonged endo-action, however, leads to the production in increasing amounts of glucose and chromatographically-mobile oligosaccharides. It seems unwise to consider that the viscosity/reducing power relationship would persist over prolonged periods and truly express endo-action throughout. The results in Tables I and II show that when the level of reducing groups in a conversion mixture exceeds 0.1% glucose equivalent, oligosaccharides and glucose are produced in measurable quantities, thereafter, any correlation of reciprocal specific viscosity with reducing power and enzyme activity becomes invalid. Hence, arises the necessity of evolving some criterion of endo-activity, other than reduction and the solution viscosity.

The recovery of high molecular material, by precipitation with alcohol, should approach 100% even after relatively prolonged endo-action because experience has shown that the limit of precipitability with alcohol, under the conditions employed here, approaches decasaccharide

level, with the molecules of the size used here at least $2^4 - 2^5$ scissions would be necessary to render a molecule non-precipitable. The concept of approximately 100% recovery is well borne out by the results of Tables I - III where the recovery for 42 hr. reaction with oat enzyme, and for 18 hr. reaction with barley enzyme was never less than 98% and 97% respectively. Even with the definitely more active enzyme preparation used in the experiments of Table V, (see also Fig. 1) the recovery after 48 hr. of reaction was still 88%.

Realizing the limitations of the device employed to represent "complexity" it was not possible to determine the actual size and average chain length of the dextrin. It was also particularly recognised that with smaller molecules reducing power is not a precise measure of molecular size, especially when both 1.3- and 1.4- types of linkages are involved. The function $1000/R_1$, where R_1 is the glucose equivalent in μg per mg. was employed merely as a rough approximation of "molecular complexity" of the glucan and its degradation product, referred to as "glucan dextrin". By no means, it is suggested that the "complexity" figures accurately represent the number of glucose residues in the dextrin molecule; the concept is employed in a broad sense to express that the calculated figures represent a decrease in the size of dextrin, remembering also, however, that 1.3- and 1.4- linked oligosaccharides give different values for reducing power, the glucose equivalents for cellobiose and laminaribiose

by the Somogyi method (loc.cit.) being different.

It is apparent from the results of Table V that the glucose equivalent of the mixture of dextrans steadily increases, showing clearly a corresponding decrease in the average "complexity" until a point is reached where the glucose equivalent becomes constant and the "complexity" for the mixture of dextrans approaches 30 units. In spite of the fact that the actual amount of the dextrin in the reaction mixture is steadily decreasing, its complexity remains substantially constant over a period of 18 hr. It is obvious that at this stage, a dextrin resistant to truly endo-attack is being produced. The endo-action-resistant-dextrin is no longer smoothly susceptible to degradation and therefore accumulates. This dextrin must then be split slowly to produce materials which are near the limit of non-precipitability i.e. decasaccharide level. This is, actually proved by the results of Tables VIII and IX, where a dextrin resulting after 20 hr. of endo-action on glucan was fairly rapidly degraded by joint-action and very slowly indeed by renewed endo-action; it also follows that the resistant nature of this dextrin as shown in the results of Table V is real, and its accumulation is not due to the inactivation of the enzymes during prolonged action but entirely due to its own chemical structure. The conclusion cannot be escaped that a prolonged endo-glucanase action is of multiple type: a dextrinising type in the early stages and a saccharifying type in the later. The multiple activity

may well be concerned not only with the difference of scissions of β -1.3- or β -1.4 linkages as independent action, but also with scissions at points determined by the relative positions of the two types of linkages. A similarity of β -endo-glucanase activity with α -amylase activity may be drawn, at least, in the sense that both the enzymes or enzyme systems involve rapid dextrinisation and slow saccharification.

For an individual undegraded glucan molecule dextrinisation must precede saccharification, but with the innumerable molecules present in the reaction from the start (the glucan preparation would be mixture of molecules of various sizes and what is observed by the viscosity-reducing power relationship is an average), there would inevitably be an overlap between the saccharifying and dextrinising activities of the endo- β -glucanase system. Since it is reported by Hopkins (1946) that α -amylase has greater affinity for the bigger molecules, it may reasonably be expected that during the earlier stages of a prolonged endo-action, dextrinisation would inevitably predominate over saccharification. Trace quantities of glucose and oligosaccharides should be formed, therefore, almost from the start, though in the results of Tables I and II they become measurable only after 6-18 hr. interval; this may be due to the lower activity of the enzyme preparation used in the early experiments. So far, it has not been possible to prepare endo- β -glucanases without contamination

with disaccharases (specific laminaribiase or cellobiase or a general one), and possibly oligosaccharases. Therefore, it cannot be said whether glucose is produced only by the hydrolysis of di- and probably oligo-saccharides or whether it is also formed directly by continued endo-action. However, this aspect of enquiry does not much affect the present discussion.

In a prolonged endo-action the total reducing groups (RT) increase, while the proportion remaining as reducing groups in the dextrin (100 RD/RT) decreases (see Fig. 2); the curve does not conform to any simple law, but perhaps the best relation approximates to $\log \text{RT} = 1.4 - 1.8 \text{ RD/RT}$ (Fig.3). This relation, however, cannot be stressed; a fresh relation might need to be obtained for each sample of the glucan used. The relation has, nevertheless, some utility in showing the overall pattern of degradation. Thinking in terms of scissions (RD/R_0 ; RT/R_0) rather than absolute reducing power does not lead to any more precise relationship (Fig. 4 and 5). It appears, as if an attempt to find a smooth relation of this type is to expect a pattern of degradation simpler than the existing one. It might well be the case that in the early stages the relation RD/RT is a smooth one, tending towards a constant value (70% for oat enzyme, 50% for barley enzyme), characteristic of the particular balance of enzyme function (dextrinisation and saccharification) in each enzyme preparation. Consequently, the curves of Fig. 2 and 3 would vary not

only with the substrate preparation but also with the enzyme. Nevertheless, the real advantage of pointing a general pattern of behaviour cannot be ignored. Here again the application of reducing power of the dextrin in developing the concept of "scission" as the ratio of reducing power at a particular time by initial reducing power suffers from the same limitations as the concept of the "complexity" of the dextrans. Nevertheless, it serves reasonably well in giving a first approximation of what is happening.

Perusal of the results recorded in Tables I, II and V shows that oat endo-enzyme preparation gives a smaller proportion of glucose and oligosaccharide reducing groups relative to dextrin reducing groups $(RT-RD)/RD$ than do the barley preparations, suggesting a deficiency of the enzyme activity responsible for saccharification. Similar differences occur in different preparations from the same barley. It cannot be stressed on the basis of these results that oat enzymes are deficient in saccharifying enzyme; the chief value of this observation lies in pointing out that the balance of dextrinising and saccharifying enzymes varies with each individual enzyme preparation even from the same sample of barley, and with enzyme preparations from different cereals.

The mechanism of endo-degradation is complex, involving several enzymes or enzyme activities, dextrinisation and saccharification being two major activities; probably the two types of linkages, β -1.3- and β -1.4-, are

preferentially involved in saccharification and dextrinisation. The balance of the two types of activities in the endo- β -glucanase system varies with each preparation and the source material.

Endo- β -glucanase system has much in common with malt α -amylase e.g. a general resistance to temperature inactivation and to inactivation by such reagents as phenylmercuric salts, and in the enormous increase in activity during malting etc. (Preece and Hoggan, loc.cit.). The potentialities for dextrinisation and saccharification are also common to α -amylase and endo- β -glucanase. It is usually agreed that α -amylase is one enzyme involving different actions, though the saccharifying and dextrinising functions of endo- β -glucanase do not run parallel. Preece and Shadaksharswamy (1949) have, however, shown that for the malt enzyme, the dextrinising function of α -amylase is more sensitive to temperature inactivation than is saccharification; thus indicating that the two functions may not necessarily run parallel in each α -amylase preparation. At this stage it is not possible to say that the dextrinising and saccharifying functions of the endo- β -glucanase are two separate enzymes or two activities of the same enzyme. The general parallelism of behaviour of α -amylase or the α -amylase system and endo- β -glucanase or endo- β -glucanase system cannot be doubted with the difference that the former attacks only one type of linkage (α -1.4) while the latter may split both β -1.3- and β -1.4- types of linkage.

Degradation of barley β -glucan by joint-action.

Comparison of the curves representing production of reducing groups in endo-action and joint-conversion (Fig.7), clearly bears out that in presence of exo-enzyme there is great acceleration in the production of reducing groups. Because of the presence of disaccharases in the glucanase system the production of glucose starts right from the beginning (Tables III and IV), and therefore the difference in the rate of production of reducing groups in joint-action and endo-action conversions can hardly be taken as a measure of true exo-activity. There is no way yet of allowing for this difficulty. Perhaps, the most precise indication of the influence of exo-activity is given by the extra loss of recoverable dextrin occurring in presence of exo-action (Fig. 6, 8 and 14). That there is a great acceleration in presence of exo-action in eliminating high-molecular matter can only be explained on the assumption that the comparatively long chains produced in the earlier stages of reaction are rapidly shortened by exo-action and that certain disaccharides (laminaribiose and cellobiose) and some chromatographically mobile higher oligosaccharides representing "residues" from such action are being produced; certain of these "residues" may be susceptible of further degradation.

On account of the presence of disaccharases and other limitations discussed elsewhere, any correlation of excess reducing groups produced by exo-action with the enzyme activity must be attempted with great caution, and

such a correlation should not be stressed too much for it will give, at the best, only an approximation of what is contributed by the exo-activity. The overall pattern seems to be similar to that by endo-action alone; Fig. 9 shows that as total reducing groups increase, the proportion provided by the dextrin decreases (compare with Fig. 2 and 3). The curve again does not conform to any simple relation, but shows some approximation to the relation $\log RT = 1.8 - 3.1 RD/RT$ (Fig.10). Some scatter around the curves is inevitable, especially when even endo-action alone gives scatter (Fig. 2 and 3). Despite the fact that oat enzyme is deficient in exo-activity (Preece et al., 1954; Preece and Hoggan, loc.cit.), there is hardly any reason to suggest that the pattern of degradation by oat enzyme and barley enzyme differ more than do the overall reactions by two enzyme preparations from the same source.

"Resistant dextrin" produced by the endo-action alone of barley enzymes on barley glucan after 24 hr. of reaction has a complexity of some 30 units while the complexity of similar dextrin produced by the joint action of endo- and exo- enzymes is about 18 units. Since these figures are based on an arbitrary scale and do not, in any way, indicate actual complexity, the difference shows merely, in a broad sense, that the endo-action dextrin is more complex than the joint-action dextrin. The two conversions of Table V are quite comparable, since the endo-action by viscometry, and the substrate concentration in the two cases are equal. Evidently, the two dextrans

are related to each other; the endo-action dextrin is resistant to endo-attack but has a portion susceptible to exo-attack (Table VIII). The difference in the complexity figures of the two dextrans (12 units) represents that part of the endo-action dextrin which is susceptible to exo-action. The presence in the dextrin of a block or blocks resistant to endo-action but susceptible to exo-action is beyond any shadow of doubt. This clearly bears out that the dextrin chain, as well as the β -glucan chain, has such arrangements of 1.3- and 1.4- linkages as permit the existence of blocks preferentially resistant to one type of enzyme action, and that there is a certain portion in the glucan (at least in barley glucan) which is resistant to both exo- and endo-action; this represents the final dextrin recoverable after 48 hr. of reaction. Any suggestion that single 1.3- and 1.4- linkages are repeated regularly throughout the entire chain of the glucan must be completely rejected.

Endo-activity is the fundamental mode of disruption of the β -glucan molecule, which is further accelerated by exo-action; the pattern of degradation in the two cases is the same. It follows that the glucan degradation is essentially an endo-conversion accelerated by exo-action, just as happens in the joint-action of α - and β -amylases on starch.

Degradation of laminarin and cello-dextrin.

Fig. 12 and 13 show the rate of production of reducing groups during the enzymic degradation of laminarin



and cello-dextrin. Because of the different initial reducing power of β -glucan, laminarin and cello-dextrin, direct comparison of the rates of reducing group production in two cases is not possible as an indication of the relative susceptibility to attack of β -1.3- and β -1.4-linkages. Such an impossibility is borne out by the results of Table VII which shows that the initial rate of scission for joint-action decreases with decreasing complexity; this would also be broadly true for endo-action if oat glucan (B) and barley glucan were transposed. The outstanding point of interest emerges from the enzymolysis of laminarin where the production of reducing groups by the action of endo-enzymes alone and the joint action of endo- and exo-enzymes is the same. It follows therefore, that whilst an endo- β -1.3-ase is present exo- β -1.3-ase is absent. The endo-enzymes alone produce smooth degradation; if action had been continued beyond 48 hr. period, glucose alone would have been almost the only product detectable in the reaction mixture.

Degradation of cello-dextrin with both the enzymes is slow; however, the rate of the joint-action exceeds that of endo-action alone, by an amount which is substantially constant throughout the entire period of reaction (Fig. 13). In view of the small initial complexity, the slow reaction is not unexpected. This suggests that the undoubted exo-1.4-ase has very feeble activity against shorter chains. The possibility of transglycosylation during the preparation of cello-dextrin in producing

artificial structural anomalies in impeding the reaction cannot be ignored. The presence of exo-1.4-ase and endo-1.4-ase is undoubted, however; an accurate comparison between their relative activities, though very desirable, is not possible.

Degradation of barley β -glucan dextrins.

Production of reducing groups by endo-action alone and by the joint action of endo- and exo-enzymes from β -glucan dextrin (produced by joint-action) is shown in Fig. 11. Because of the necessarily different initial molecular complexity of the original glucan and the dextrin, direct comparison of the activities is impossible. Initial complexity of this joint-action dextrin is 30 units on the arbitrary scale, so that in its preparation the "resistant dextrin" stage has not been quite reached; initial complexity of joint-action-resistant-dextrin is nearly 20 units (see Table IX). Fig. 11 shows that prolonged endo-action is as effective in producing reducing groups from the dextrin as joint-action. This may be taken as evidence of the decreasing effectiveness of exo-1.4-ase as chain length decreases, or may also be due to the exhaustion of available 1.4-linkages. Comparison of Fig. 11, 12 and 13 shows that mixed linkages are present in the dextrin.

The characters of "resistant dextrins" are given in Tables VIII and IX. Table VIII shows that the dextrin resulting from prolonged endo-action was fairly susceptible to joint-action but was only very slowly

degraded by renewed endo-action; the joint-action soon degraded this dextrin to a complexity of ~ 13 units which is very similar to the complexity of joint-action dextrin. Table IX shows that a joint-action dextrin underwent little, if any, degradation by either type of enzyme action. The presence of a resistant nucleus in the dextrans and consequently in the glucan can scarcely be doubted.

Degradation of oat glucan.

It will not be reasonable to assume an identity of chemical structure of "oat lichenin"- a β -glucan from oat prepared by the method of Morris (loc.cit.) - and the glucan prepared by the method of Preece and Mackenzie (loc.cit.). At this stage no more can be assumed, than that both the products contain β -1.3- and β -1.4- linkages, the proportion and arrangement of the two types of linkages in the oat glucan and the product of Morris, may or may not be identical. However, preliminary enzymic studies of oat glucan have given oligosaccharides similar to those obtained from barley (identification was based on RF values and electrophoresis). There also does not seem to exist any reason to presuppose that the glucans from oat and barley have fundamentally a different chemical structure, though there are certainly differences in detail. The rate of degradation, whether by joint-action or by endo-action alone, of oat glucan (A) (Table X) which is close to barley glucan in reducing power, is far greater than the rate of degradation of the barley product

(Table V), after 30 hr. of joint-action, high molecular material was no longer recoverable from oat glucan by alcohol precipitation. There is no evidence for the presence in the oat glucan of such "resistant dextrans" as are obtained from the corresponding barley product. (Tables V, VIII and IX).

Results of the same nature were obtained from the degradation of another sample of oat glucan (B) (see Table XI), which was near the complexity of a sample of laminarin used and of the barley glucan dextrin; accordingly it may be regarded as a dextrin. Since two different enzyme preparations were necessarily used in the experiments of Tables X and XI, the results in two cases cannot be regarded as strictly comparable, though the endo-action by viscometry was the same in each case. A smooth approach to complete degradation and the absence of "resistant dextrin" in the oat glucan is quite evident. The reaction in presence of endo-activity alone is retarded in both cases at dextrin "complexity" of nearly 15 units. This suggests, at least, slight difference in the chemical structure of oat glucan and barley glucan, in the sense that a "resistant nucleus" responsible for the production of resistant dextrans from the barley glucan is absent in the oat product.

If it is assumed that the oat glucan prepared according to the method of Preece and Mackenzie (loc.cit.) has 1.3- and 1.4- linkages in 3:7 ratio which has been reported by Peat et al. (1957) to be the case in oat lichenin,

a similar glucan prepared from oats by the method of Morris (loc.cit.) - a plausible explanation for the complete disappearance of high molecular material from the reaction mixture may be given. It would be quite reasonable to expect that greater proportion of 1.4-linkages in the molecule would provide more centres for the attack of α -1.4-ase which is mainly though indirectly responsible for the rapid disappearance of dextrin (see Table V).

Laminaribiase and Cellobiase.

The results of Table XII show that both endo- and joint-action-enzyme preparations contain both laminaribiase and cellobiase; laminaribiase activity suffers substantial loss during the treatment of the enzyme with phenylmercuric nitrate while the cellobiase activity is nearly the same in joint-action enzymes and endo-action enzymes. It follows that there is present in the joint-action enzyme preparation at least, a disaccharase effective against β -1.3- linkages but not against β -1.4- linkages; this might well be regarded as laminaribiase. The possibility of a general disaccharase or a general β -glucosidase being present cannot be excluded. It could not be determined whether enzyme activity responsible for the observed hydrolysis of cellobiose is a specific cellobiase or a general β -glucosidase which hydrolysed both cellobiose and laminaribiose. The presence of a specific laminaribiase in barley enzyme preparations seems to be quite convincing.

CONCLUSIONS

On the basis of these and previous investigations it is now possible to postulate the presence of the following enzymes or enzyme systems in β -glucanase activity:

- (a) Endo-1.3-ase: dextrinising and saccharifying.
- (b) Endo-1.4-ase : dextrinising and may be
saccharifying.
- (c) Exo-1.4-ase : saccharifying.
- (d) Laminaribiase.
- (e) Cellobiase and/or general β -glucosidase.

The dextrinising and saccharifying actions even in the endo-action do not run parallel. No evidence was obtained for the partial inactivation of 1.3-ase during treatment with phenylmercuric nitrate, the rate of production of reducing groups from laminarin being almost equal in endo-action and joint-action conversions, indicating either the absence of exo-1.3-ase or that the 1.3-ase activity is of a general 1.3-glucosidase type attacking the glucan chains at random both at points remote from the ends as well as the linkages at the terminal ends. Endo-1.4-ase is undoubtedly present but it is relatively weak, whilst exo-1.4-ase is very active and can be completely inactivated by treatment with phenylmercuric nitrate. The presence of a specific disaccharase, laminaribiase, is indicated whilst a cellobiase and/or a general disaccharase is also present. The possibility of transglycosylation cannot be ignored and is worthy of further investigation. The influence of transglycosylation associated with these

enzymes was reported at very high concentrations of sugars and enzymes (Anderson and Manners, 1959) and does not seem to affect these results; however it might affect certain changes in the structure of oligosaccharides, which possibility is investigated in Section II.

The enzymolysis of the glucan under the conditions of joint-action is essentially an endo-action accelerated by exo-action, the latter being mainly responsible for the disappearance of the high molecular material recoverable by precipitation with alcohol and production of cellobiose. Endo-action alone can ultimately produce oligosaccharides whose appearance is accelerated by exo-attack on intermediate dextrin (see Preece and MacDougall, 1958); the oligosaccharides may or may not be of the same types or in the same proportions. Degradation of the barley glucan is not a smooth process; dextrans of substantially constant complexity, ie. resistant dextrans, accumulate during the reaction. The degradation of oat glucan on the other hand is smooth, there being no indication of the presence of resistant dextrans.

SECTION II

Qualitative study of oligosaccharides
produced in the enzymolysis of β -glucan.

INTRODUCTION

Chemical methods of investigating the structure of polysaccharides consist in degrading the polysaccharide or its methylated derivative into smaller fragments which are then identified by conventional methods : further degradation, formation of acetyl derivatives and chromatography. Methylation, acid hydrolysis, acetolysis and periodate oxidation are well recognised methods of analysis in carbohydrate chemistry.

Resemblance between structure of cellulose and lichenin from Iceland moss was first pointed out by acetolysis experiments of Karrer et al. (1923); acetolysis of both polysaccharides gave cellobiose-octa-acetate. Methanolysis of lichenin and cellulose was also carried out by Karrer and Nishida (1924), and 2 . 3. 6 tri-O-methyl-D-glucoside was obtained in both cases. Nevertheless, lichenin differs from cellulose in being soluble in hot water and more susceptible to enzymic attack. Meyer and Gurtler (1947) gave the first clear evidence of a chemical difference between lichenin and cellulose. Hydrolysis of methylated lichenin gave tetra-O-methyl-D-glucose and a mixture of 2.3.6- and 2.4.6-tri-O-methyl-D-glucose, indicating the presence of both β -1.4- and β -1.3- linkages. Periodate oxidation showed that Ca

27% of linkages were the 1.3- type. In the same year Blossonnas (1947) confirmed the relative proportions of 1.3- and 1.4- linkages, Chanda, Hirst and Manners (1957) studied hydrolysis of methylated lichenin and partial hydrolysis of lichenin; the presence of glucose, cellobiose, laminaribiose, and higher oligosaccharides in the acid hydrolysates of lichenin was shown by paper chromatography, while methylation studies revealed the presence of :

(a) tetra-O-methyl-D-glucose equivalent to an average chain length of 62 glucose residues; (b) 2.4.6 tri-O-methyl-D-glucose (Ca. 30%); and (c) 2.3.6. tri-O-methyl-D-glucose.

The presence of 30% β -1.3- and 70% β -1.4- types of linkages in lichenin was confirmed. No more than a trace of di-O-methyl-D-glucose was present, indicating the virtual absence of branch points. On periodate oxidation lichenin consumed 0.7 mole of periodate per anhydroglucose unit; the product obtained after acetylation, fractionation and deacetylation of lichenin also gave the same value for periodate consumption, indicating that no preferential removal of material containing larger proportion of 1.3- linkages had occurred and that lichenin was a homogenous^e glucan. Furthermore, treatment of periodate-oxidised lichenin with iso-nicotinhydrazide or thiosemicarbazide gave the corresponding polymer obtained by Barry et al. (1954), and analyses for nitrogen content gave values for the α -glycol content of lichenin similar to those previously obtained. This, again confirmed that lichenin was chemically homogenous^e. Peat et al. (1957) studied

the chemical structure of lichenin from Iceland moss and a similar glucan from oats (Avena sativa). The two "lichenins" were found to be similar in chemical structure. The relative proportions of 1.3- and 1.4- linkages (30:70) as found by Chanda et al. (loc.cit.) was confirmed. Evidence was also obtained for the presence of laminaribiose, cellobiose, cellotriose, 4-0- β -laminaribiosyl glucose and 3-0- β -cellobiosyl glucose in the hydrolysates of both "lichenins". On the basis of these findings a structure of lichenin, in which repeating cellotriose units were linked with each other by 1.3- linkages, was proposed.

Barley glucan prepared by Preece and Mackenzie (1952), was chemically examined by Aspinall and Telfer (1954); the presence of equal proportions of 1.3- and 1.4- linkages was revealed. This was later supported by the work of Montgomery and Smith (1956) who state that Gilles et al. (1956) have obtained evidence for the alternate distribution of the linkages in β -glucan. Gilles, Meredith and Smith (1952), employing periodate oxidation determined that 1.4- and 1.3- linkages are present in equal numbers in the glucan. These workers reported that the polyaldehyde, obtained by periodate treatment, formed only glucose phenylosone, while the presence of consecutive 1.3- linkages in the molecule should have been revealed by the formation of osazones of one or more glucose oligosaccharides. It was concluded that the two types of linkages were alternately distributed

in the molecule.

The success of chemical methods, in investigating the structure of polysaccharides, is well established. In recent years enzymes have also been employed in the structural analysis of polysaccharides. Enzymic study not only provides information on the structure of the substrate, but also reveals the mode of enzyme attack, and this knowledge is of considerable help in understanding carbohydrate metabolism, though, it is realized that the experiments "in vitro" are not exactly comparable to the happenings in the natural cells of living systems; yet the information obtained from experiments "in vitro" can be applied, though with great reserve and caution, in understanding the mysteries of Nature.

The technique of paper electrophoresis has, in recent years, developed simultaneously with techniques of column and paper chromatography. Although paper electrophoresis is already extensively used in studying proteins, its potentialities in the carbohydrate field have been realized only recently. Introduction of borate buffers by Consden and Stanier (1952), Michl (1952) and Jaenicke (1952) was an important step taken in the development of electrophoretic methods for carbohydrates. Correlation of the mobilities of the sugars in borate buffers, with their stereochemistry, by Foster (1952) also proved a great help in the structural analysis of sugars by physical methods. Frahn and Mills (1956) successfully

employed electrophoresis in sodium bisulphite solution for determining the molecular weights of aldehyde sugars and oligosaccharides. Another important contribution in the field was from French and Wild (1953) who showed that when a straight line relationship is obtained for $\log \frac{1 - R_F}{R_F}$ values of sugars, the oligosaccharides concerned belong to an homologous series. By the application of electrophoretic and chromatographic methods it is now possible to obtain some information on the structure of oligosaccharides; these techniques are especially suited for the analysis of very small quantities.

Hoggan (1957) employing chromatographic and electrophoretic methods of analysis obtained evidence for the presence of glucose, laminaribiose, cellobiose, laminaritriose and two trisaccharides of the structures $G\beta 1 \rightarrow 3G\beta 1 \rightarrow 4G-OH$ and $G\beta 1 \rightarrow 4G\beta 1 \rightarrow 3G-OH$, in the enzymolysate of barley glucan. Nothing could be definitely concluded about the distribution of the linkages in glucan molecules, the alternate arrangement of linkages as suggested by Gilles et al. was, however, contradicted by the presence of laminaritriose.

The purport of the present work is to study the chemical structures of the oligosaccharides produced during the enzymolysis of barley and oat glucans under the action of endo-enzymes and joint action of endo- and exo-enzymes, as well as to study the oligosaccharides produced during partial acid hydrolysis of the "resistant dextrins" from the barley glucan enzymolysates. Because of the resistant

nature of the "dextrins" to enzyme attack, acid hydrolysis was resorted to. It was hoped that the information would prove helpful in drawing certain conclusions about the distribution of linkages in glucan molecule, and the mode of enzymic attack.

The possibility of enzymic synthesis can not be overlooked during the degradation of such a substrate. During a study of barley enzymes, Manners (1955) found that enzymic hydrolysis of cellobiose in concentrated solutions was accompanied by the synthesis of other oligosaccharides, while in dilute solution cellobiose was completely hydrolysed to glucose. Later Anderson and Manners (1959), employing 5.4% (w/v) solution of cellobiose and high concentrations of enzymes from Spratt-Archer barley obtained synthesis of gentiobiose, laminaribiose, cellotriose, and $6^2\beta$ -glycosyl cellobiose in the conversion mixture. It was, however, found that barley enzymes were unable to synthesise oligosaccharides from dilute cellobiose solutions or solutions of glucose. Oligosaccharide formation in presence of enzymes from different strains of Aspergillus niger was reported by Barker et al. (1955) and Crook and Stone (1957) and from Aspergillus flavus by Giri et al. (1954).

It follows that in the use of enzymes for the structural analysis of glucan, conversion should be carried out at very low concentrations. Manners (loc.cit.) and Anderson and Manners (loc.cit.) reported transglycosylation

by barley enzymes, only under high concentration of cellobiose. Opportunity was therefore taken to determine the conditions under which synthesis does not occur.

EXPERIMENTAL

Transglycosylation.

Reaction mixtures containing 0.1%, 0.2% and 0.5% (W/V) cellobiose were prepared by mixing 8 ml. standard cellobiose solution with 1 ml. of acetate buffer (pH 5.0) prepared according to Palmer (1951), and 2 ml. of enzyme solution; enzymes treated with phenylmercuric nitrate and untreated enzymes were used. In each case enzyme activity, as measured by viscometry, was equal to the enzyme activity present in all glucan conversions of this series. The reaction mixture was incubated at 37°C. for 24 hr. and 48 hr. after which period the enzymolysates were analysed for identifying sugars by descending paper chromatography. The chromatograms obtained for 24 hr. and 48 hr. reaction periods with endo-enzyme preparations and joint-action enzymes were identical. Enzymic synthesis did not occur at all at 0.1% and 0.2% concentration; at 0.1% cellobiose was quantitatively converted to glucose while at 0.2% some unconverted cellobiose was left. Laminaribiose and other oligosaccharides (Plate A) were found to be synthesized at 0.5% after 24 hr. and 48 hr. incubation. It follows that in glucan conversions where the concentration of oligosaccharides does not exceed 0.2% cellobiose equivalent the oligosaccharides produced can safely be considered as

PLATE A.

Transglycosylation by barley enzymes.

| | |
|----------------|-------------------------|
| K | control |
| A ₁ | 0.1% cellobiose |
| A ₂ | 0.2% cellobiose |
| A ₃ | 0.5% cellobiose |
| 4 | higher oligosaccharides |
| C and 3 | cellobiose |
| L and 2 | laminaribiose |
| G and 1 | glucose. |

PLATE A

K

A1

A2

A3

4

products of degradation rather than of enzymic synthesis.

Enzymolysis of β -glucan.

The conversion mixtures were prepared as described in Section I and the enzyme activity, as measured by viscometry, was kept the same in all the experiments of this series. In order to ensure that the oligosaccharide concentration in each reaction mixture did not exceed 0.2% cellobiose equivalent, incubation was carried out at 37°C. for 24 hr., while in the enzymolysis of substrates under joint action of endo- and exo- enzymes, ie. the enzymes not treated with phenylmercuric nitrate, 0.25% substrate solution was used; more reducing groups are produced during the joint-action. After incubation, the enzymolysates were kept in boiling water for 3 minutes so as to inactivate the enzymes and then four volumes of alcohol was added to remove materials of high molecular weight. The liquor obtained after removing dextrin was concentrated and kept for analysis.

Acid hydrolysis of dextrans.

The dextrans obtained after 24 hr. action of endo-enzymes and joint-action enzymes were found to be resistant to further enzyme action, therefore, for studying their structure acid hydrolysis was resorted to. 50 mg. of test material (dextrin) was refluxed with 50 ml. of N/10 sulphuric acid for 1 hr. The hydrolysate was cooled and neutralised with barium carbonate suspension, the resulting precipitate of barium sulphate and unreacted barium carbonate

was removed and the clear solution was concentrated to small volume.

Fractionation of sugars.

Direct chromatography of the sugars in the concentrates from the enzymolyses was not possible because of the presence of buffer and excess of glucose. The concentrated solution was applied to a charcoal-celite column as described by Whelan (1953). Charcoal-celite columns were prepared by mixing equal quantities of acid-washed charcoal and celite 545 with water (322 ml. per 100 gm. of the mixture). The slurry was poured, 2 - 3 inches at a time, into a glass column which contained a piece of glass wool as support in the bottom. After allowing the column to settle it was washed with 500 ml. of water. The concentrated solution of mixture of sugars from an enzymolysis experiment was applied to the column, which was then exhaustively washed with water for removing the buffer and glucose; the remaining oligosaccharides were recovered by eluting the column with 2 litre of 30% (v/v) ethyl alcohol. The eluate was concentrated and applied in a streak on 3 m.m. Whatman No.1 filter paper which was irrigated by a descending flow of propanol-water (70 : 30) solvent for 5 - 6 days at room temperature. The position of a sugar spot on the chromatogram was located by spraying a test strip and then heating at 110°C. for 10 minutes, using aniline hydrogen oxalate as colouring reagent. Strips of chromatogram corresponding to each single spot on the test strip were cut and the sugar was

eluted from each strip by the method of MacLeod (1951). Eluates were concentrated and separately spotted onto another paper in the usual way. This chromatogram was irrigated with descending flow of the upper layer of n-butanol-acetic acid-water solvent (4:1:5 V/V) for 7 - 8 days at room temperature and the chromatogram was examined in the usual way. RF values of the spots on each chromatogram were calculated with reference to cellobiose. Ionophoresis.

(A) In sodium bisulphite. The method of Frahn and Mills (loc.cit.) was employed to determine the molecular weights of the oligosaccharides. Sugar corresponding to a single chromatographically mobile spot was eluted from unsprayed strips of chromatograms by the method of MacLeod (loc.cit.). The eluates were concentrated and mixed separately with equal volumes of 0.4 M solution of sodium bisulphite; each mixture was allowed to stand for $\frac{1}{2}$ hr. during which period the bisulphite formed an ionising complex with the oligosaccharide. The solution was applied to Whatman No.4 paper and electrophoresis was carried out for 6 hr., using 0.4 M sodium bisulphite solution as electrolyte at 40-50 milliamps and 8 Volts/cm. The paper was dried, dipped into a solution of anilinepicrate in acetone and heated at 100°C. Two spots separated by a faint streak were produced for each sugar; one spot represented electroendosmotic movement of uncharged sugar, whereas the other spot represented movement of a charged complex towards the anode. The distance between the two spots

being taken as measure of true mobility, Mg values for the oligosaccharides were calculated with reference to cellobiose (0.70). Mg values decrease regularly (not linearly) with increase in molecular size. Substituting experimental values in the following equation developed by Hoggan (loc.cit.), molecular weights of the oligosaccharides were calculated.

$(Mg + 7) (M.W. + 232.5) = 4863$, where Mg is the true mobility of the aldose and the M.W. is the molecular weight.

(B) In borate buffer. Foster (loc.cit.) correlated the mobilities in borate buffer of certain glucodisaccharides and of methyl derivatives of glucose with their stereochemistry. It was suggested that the complex formation was based mainly on three types of structure: (A) cis-hydroxyl groups at C₍₂₎:C₍₄₎; (B) cis-hydroxyl groups at C₍₁₎:C₍₂₎, and (C) hydroxyl groups at C₍₄₎:C₍₆₎. If (A) and (B) were possible in a methyl derivative or reducing moiety of glucodisaccharides, Mg values would be high; if (B) and (C) were possible Mg values would be moderate; if (A), (B), and (C) were not possible Mg values would be low. It was also found that if (A) were precluded there was a considerable fall in the Mg value, suggesting (A) to be of major importance in determining the mobility.

Applying this theory to β -1.4 linked gluco-oligosaccharides, it would be found that (A), (B) and (C) are not possible, consequently cellobiose, cellotriose etc. would be expected to give low mobilities, whereas in

β -1.3 linked gluco-oligosaccharides (A) and (B) would be possible, and consequently the Mg values for laminaribiose and homologous oligosaccharides would be moderate. It was experimentally observed that cellobiose does not move while laminaribiose moves towards anode; because of the presence of electroendosmotic flow, actual mobilities can not be measured. This criterion of mobility in borate buffer can also be applied in determining the type of linkage at the reducing end of an oligosaccharide - the sugars containing 1.3-linkages near the reducing group would move, while those having 1.4-linkages near the reducing group would be immobile or have very low mobility.

The oligosaccharides were eluted in the usual way and concentrated eluates were spotted onto a Whatman No. 4. filter paper by the method of Foster (loc.cit.) in which 0.2 M sodium borate solution (pH 10.0) was employed. Electrophoresis was carried for 3 hr. at 20 volts/cm. and 5 - 7 milliamps. The paper was dried and spots were revealed by spraying the paper with a solution of aniline oxalate acidified with glacial acetic acid.

Plate No. 1.

Barley glucan (Endo-action).

In this and other chromatograms of this series, K is control, Lam is laminaribiose, Cel is cellobiose; A, B, C, etc. are fractions from a previous chromatogram; and No. 1, 2, 3 etc. are various oligosaccharides.

| No. | RF. | $\log \frac{1-RF}{RF}$ | Mg. in bisulphite | Complexity | Mobility in borate buffer |
|-----|--------|------------------------|-------------------|------------|---------------------------|
| 1 | 0.1107 | 0.908 | 0.7 | di | ... |
| 2 | 0.0900 | 1.004 | 0.7 | di | ... |
| 3 | 0.0749 | 1.089 | 0.57 | tri | +++ |
| 4 | 0.0586 | 1.204 | 0.57 | tri | ++, -- |
| 5 | 0.0521 | 1.257 | 0.46 | tetra | +++ |
| 6 | 0.0385 | 1.397 | 0.46 | tetra | ++ [‡] , -- |
| 7 | 0.0316 | 1.485 | 0.38 | penta | +++ ^X |
| 8 | 0.0252 | 1.586 | Streak | ... | ++, -- |
| 9 | 0.0040 | 2.380 | Streak | ... | -- |

‡ compound spot

X contaminated with some non-mobile material

No 1

K

A

B

C

D

E

9

8

7

6

5

4

3

2

CEL

C

LAM

L

I

Plate No. 2.

Barley glucan (Joint Action : Endo + Exo).

| No. | RF | $\log \frac{1 - RF}{RF}$ | Mg in bisulphite | Complexity | Mobility in Borate. |
|-----|--------|--------------------------|---------------------|------------|------------------------|
| 1 | 0.1107 | 0.908 | 0.7 | di | ... |
| 2 | 0.0900 | 1.004 | 0.7 | di | . . . |
| 3 | 0.0747 | 1.092 | 0.57 | tri | + + +, |
| 4 | 0.0594 | 1.199 | 0.57 | tri | + +, - - |
| 5 | 0.0513 | 1.266 | 0.46 | tetra | + + +, |
| 6 | 0.0371 | 1.413 | 0.46 | tetra | + +, - - |
| 7 | 0.0288 | 1.528 | 0.37 | penta | + + [*] |
| 8 | 0.0153 | 1.808 | .. | ... | ++, - - |

* Contaminated with mobile material.

No 2

K

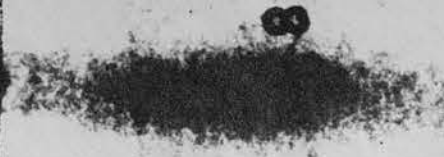
A

B

C

D

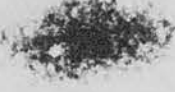
E



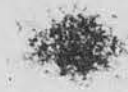
8



7



6



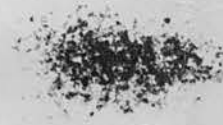
5



4



3



2



CEL

LAM



1

PLATE No. 3

Oat glucan (Endo action alone).

| No. | RF | $\log \frac{1-RF}{RF}$ | Mg. in bisulphite | Complexity | Mobility in Borate buffer |
|-----|--------|------------------------|----------------------|------------|------------------------------|
| 1 | 0.1071 | 0.921 | 0.7 | di | ... |
| 2 | 0.0900 | 1.004 | 0.7 | di | . . . |
| 3 | 0.0782 | 1.071 | 0.55 | tri | + + + |
| 4 | 0.0593 | 1.200 | 0.56 | tri | + +, - - |
| 5 | 0.0398 | 1.382 | 0.42 | tetra | + +, - - |
| 6 | 0.0303 | 1.505 | 0.30 | penta | + + +, |
| 7 | 0.0133 | 1.870 | 0.15 | . . | + +, [*] - - |
| 8 | 0.0098 | 2.004 | | . . . | + + + +, - |

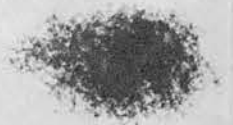
* Compound spot.

No. 3.

K A B C E FF



8
7

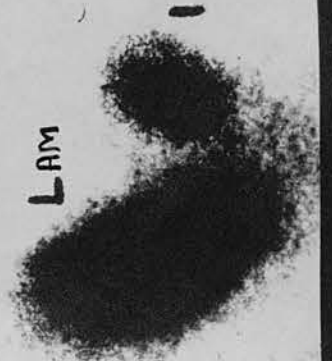


CEL



2

LAM



1

PLATE No. 4.

Oat glucan (Joint action : Endo + Exo).

| No. | RF | $\log \frac{1-RF}{RF}$ | Mg. in bisulphite | Complexity | Mobility in Borate buffer |
|-----|--------|------------------------|-------------------|------------|---------------------------|
| 1 | 0.1098 | 0.912 | 0.7 | di | ... |
| 2 | 0.0900 | 1.004 | 0.7 | di | ... |
| 3 | 0.0756 | 1.087 | 0.56 | tri | +++ |
| 4 | 0.0589 | 1.203 | 0.55 | tri | ++, -- |
| 5 | 0.0517 | 1.263 | 0.44 | tetra | +++ |
| 6 | 0.0418 | 1.360 | 0.40 | tetra | ++, - |
| 7 | 0.0315 | 1.487 | 0.40 | tetra | +, - |
| 8 | 0.0129 | 1.884 | 0.30 | penta | +, - |

No 4.

K A B C D E F G

8



7



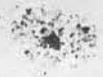
6



5



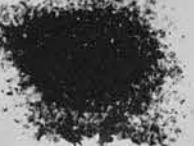
4



3



2



CEL



LAM



PLATE No. 5.

Acid hydrolysis of barley glucan dextrin.

A, endo-dextrin; B, joint-action dextrin.

| No. | RF | $\log \frac{1-RF}{RF}$ | Mg. in bisulphite | Mobility in Borate buffer |
|-----|--------|------------------------|----------------------|------------------------------|
| 2 | 0.0900 | 1.004 | 0.7 | -- |
| 3 | 0.0515 | 1.265 | 0.55 | -- |
| 4 | 0.0331 | 1.460 | 0.46 | ++, -- |
| 5 | 0.0240 | 1.610 | 0.46 | -- |

PLATE No. 6.

Acid hydrolysis of undegraded barley
glucan showing production of cellobiose
and laminaribiose.

1, laminaribiose; 2, cellobiose; and
3, 4 and 5, higher oligosaccharides.

No 5

K

A

B

5

4

3

2

CEL

LAM

No 6

K

G

5

4

3

2

1

CEL

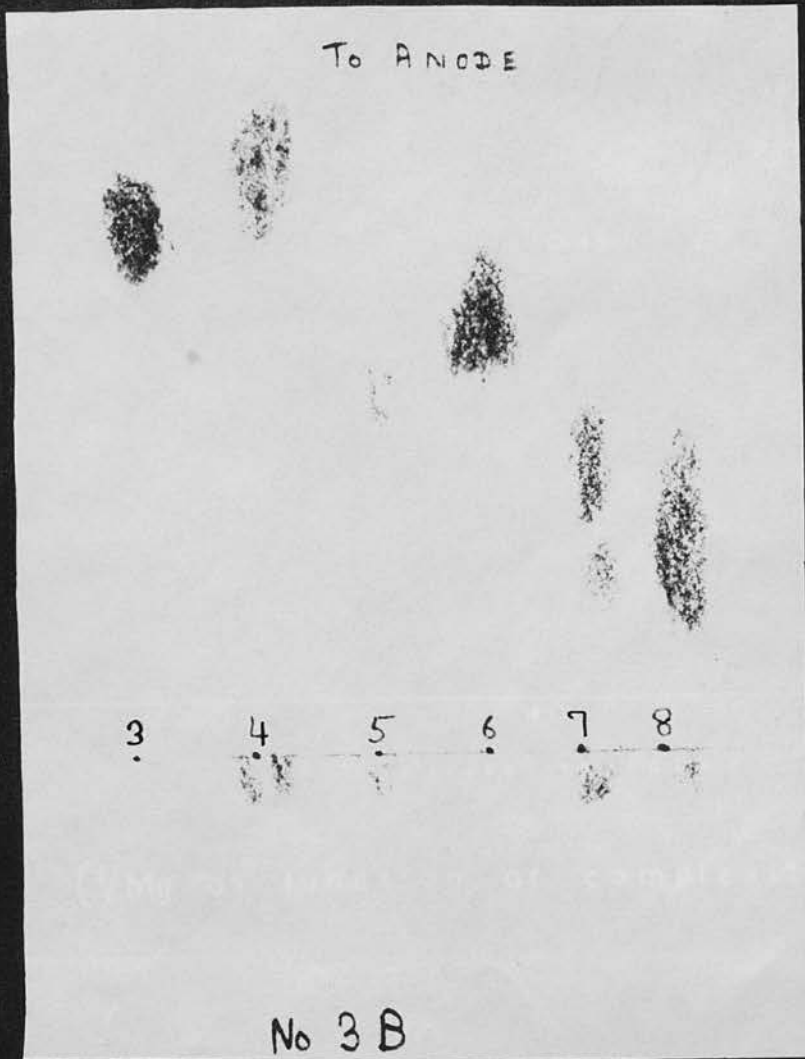
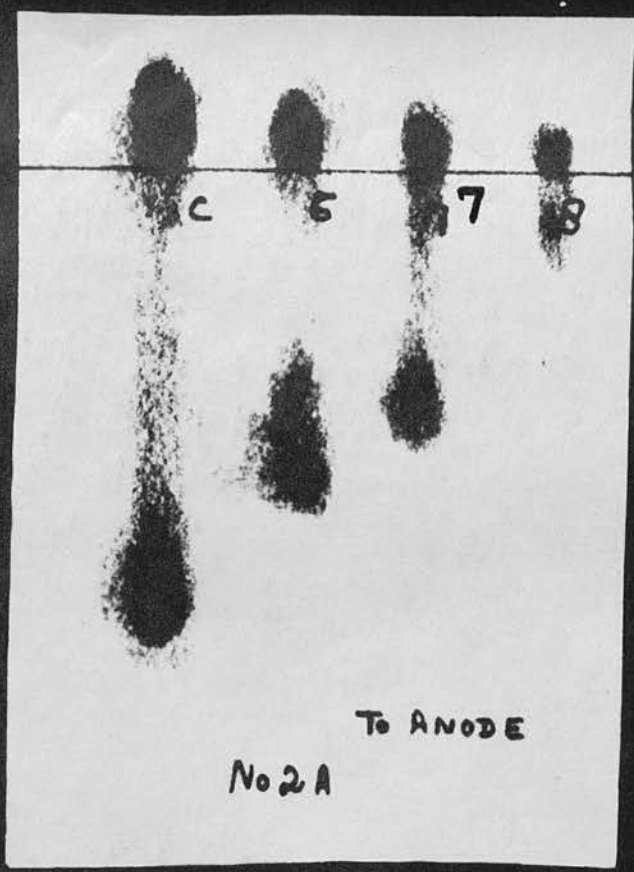
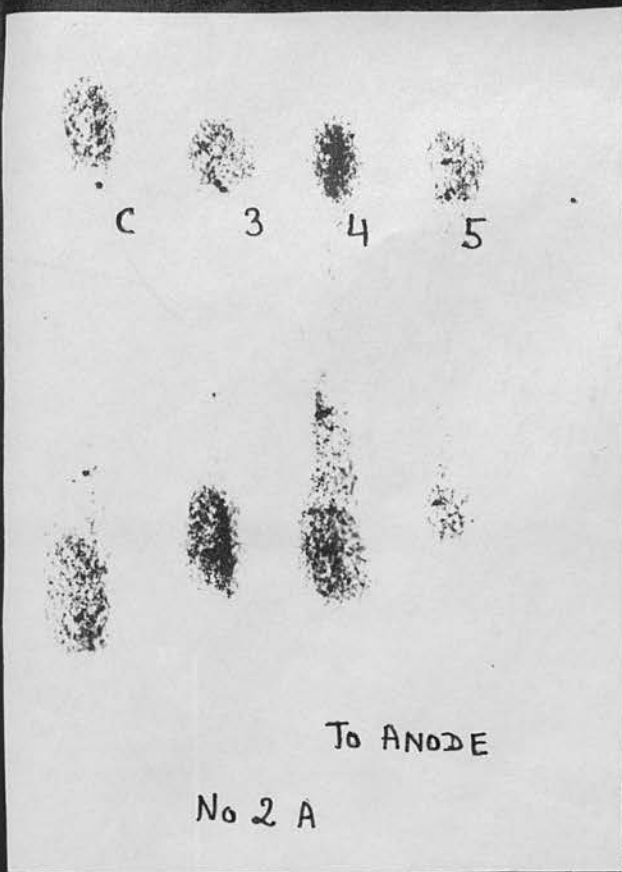
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PLATE 2A.

Ionophoresis of oligosaccharides of Plate 2 as bisulphite complexes. Mobilities and complexities of the oligosaccharides is given in Plate 2.

PLATE 3B

Ionophoresis of oligosaccharides of Plate 3 as borate complexes. Mobilities are expressed in Plate 3.



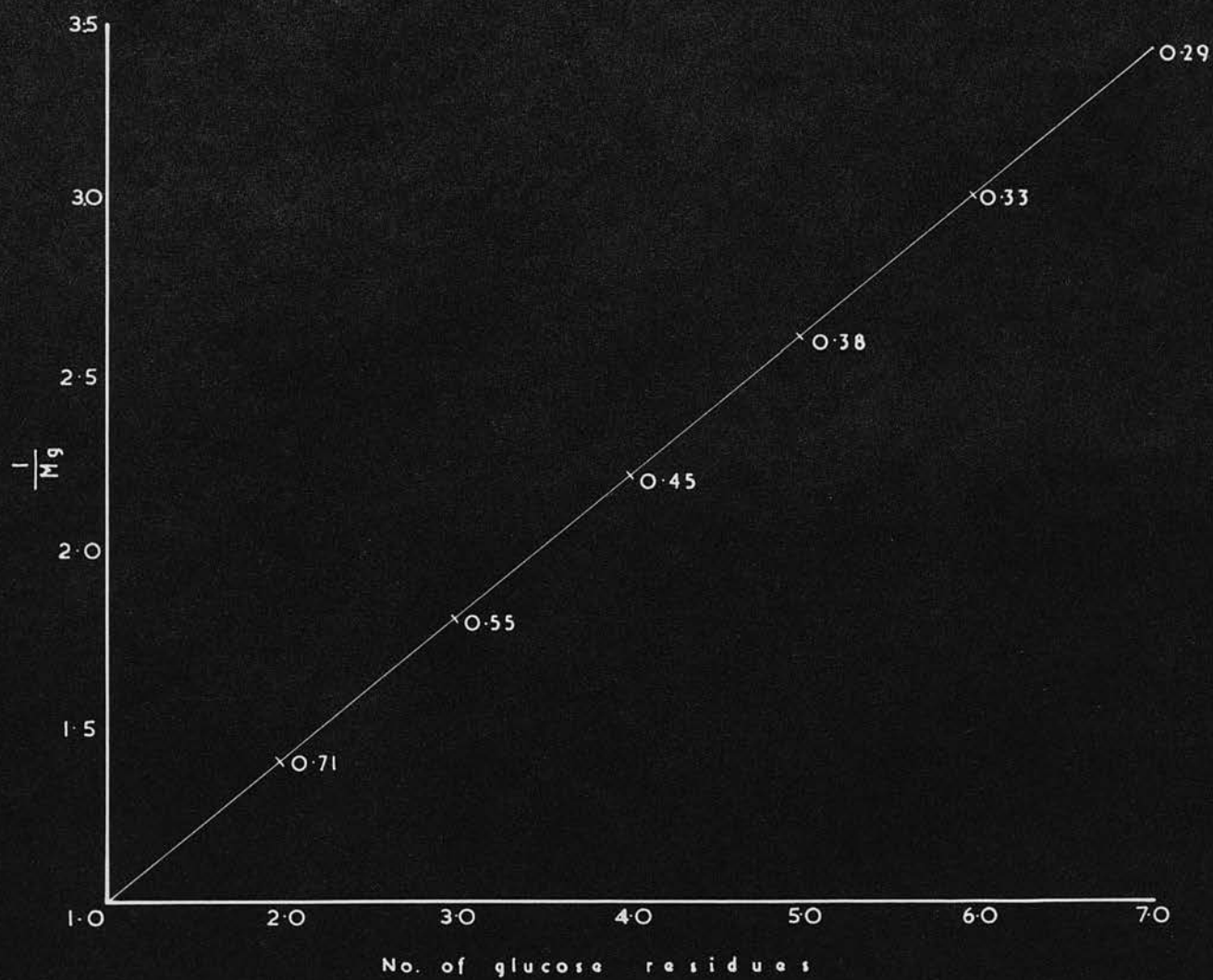


FIG. 15 ($1/M_g$ as function of complexity.)

K E Y.

Fig. 16 Relation of $\log \frac{1 - R_F}{R_F}$ to complexity,
and 17. points 2 - 11 correspond to compounds of
Table XIII; A is gentiobiose; B is maltose.

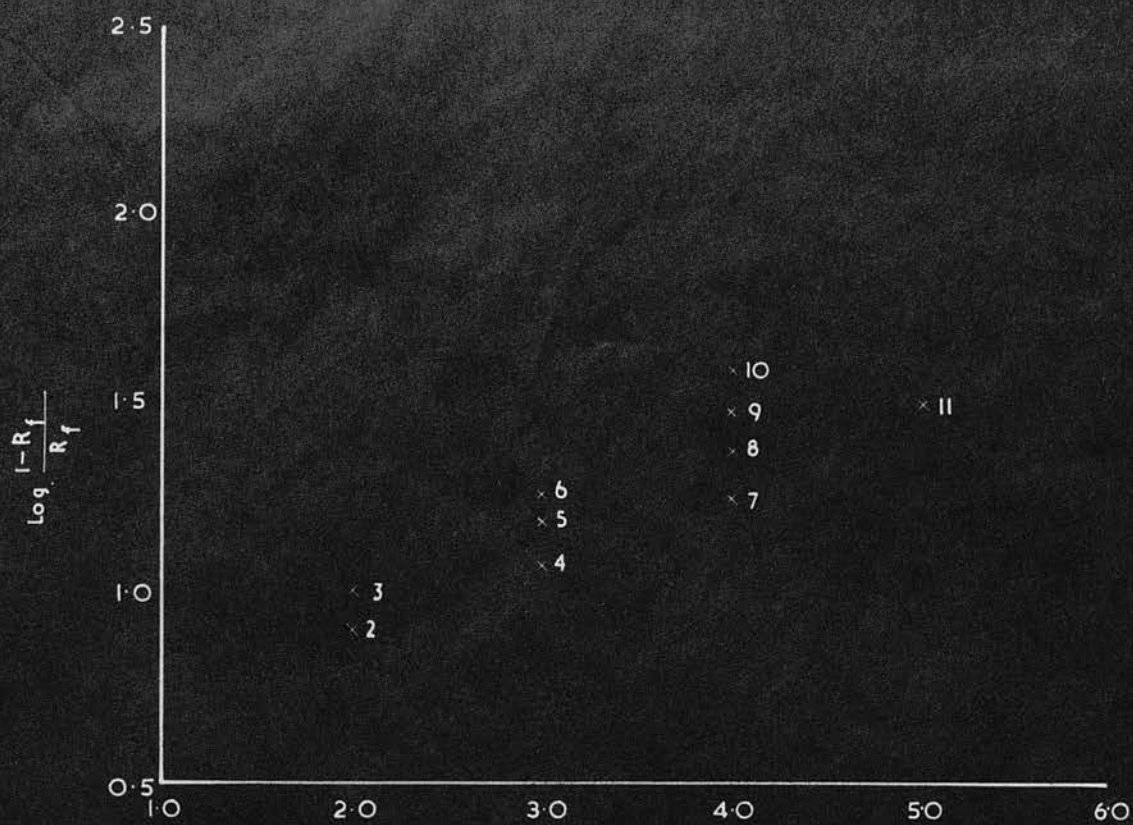


FIG. 16 relation of $\log \frac{1-R_f}{R_f}$ to complexity.

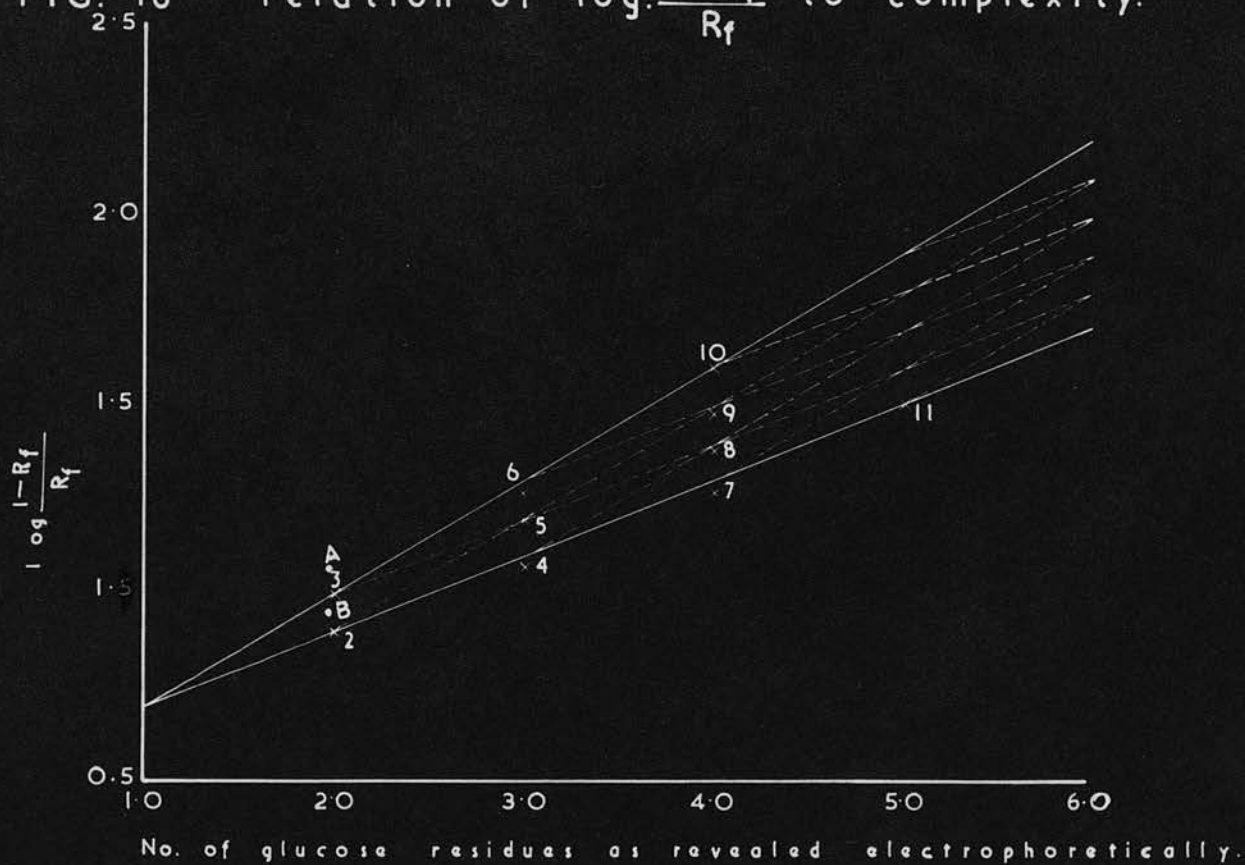


FIG. 17 (structural relationships.)

TABLE XIII

Summary of oligosaccharide characters ($\log \frac{1-RF}{R_F}$ values)

| No. | Complexity (Sulphite mobility) | Barley glucan (Endo) | Barley glucan (joint) | Oats glucan (Endo) | Oats glucan (joint) | Barley dextrin | Mean values for $\log \frac{1-RF}{R_F}$ |
|-----|--------------------------------------|----------------------------|-----------------------------|--------------------------|---------------------------|-------------------|--|
| 1 | Mono | - | - | - | - | - | (0.700)* |
| 2 | Di | 0.908 | 0.908 | 0.921 | 0.912 | | 0.912 |
| 3 | | 1.004 | 1.004 | 1.004 | 1.004 | 1.004 | 1.004 / |
| 4 | Tri | 1.089 | 1.092 | 1.071 | 1.087 | | 1.085 |
| 5 | | 1.204 | 1.199 | 1.200 | 1.203 | | 1.202 |
| 6 | | | | | | 1.265 | 1.265 |
| 7 | Tetra | 1.257 | 1.266 | | 1.263 | | 1.262 |
| 8 | | 1.397 | 1.413 | 1.382 | 1.360 | | 1.388 |
| 9 | | | | | 1.487 | 1.460 | 1.473 |
| 10 | | | | | | 1.610 | 1.610 |
| 11 | Penta | 1.485 | 1.528 | | | | 1.506 |

* From Fig. 3.

/ Compound 3 (cellobiose) was the reference compound for chromatographic measurements.

TABLE XIV

Tentative characterisation of oligosaccharides.

| No. | Complexity | Linkages | Nature | Barley β -glucan | | Oats β -glucan | | Barley glucandextrin (acid Hyd.) |
|-----|------------|-------------------------------|-------------------|------------------------|-------|----------------------|-------|----------------------------------|
| | | | | Endo- | Joint | Endo- | Joint | |
| 1 | Mono | - | glucose | ✓ ⁷ | ✓ | ✓ | ✓ | ✓ |
| 2 | Di | 3 ₁ ^f | Laminaribiose | ✓ | ✓ | ✓ | ✓ | - |
| 3 | | 4 ₁ | cellobiose | | | | | |
| 4 | Tri | 3 ₂ | Laminaritriose | ✓ | ✓ | ✓ | ✓ | - |
| 5 | | 3 ₁ 4 ₁ | Mixed | ✓ | ✓ | ✓ | ✓ | - |
| 6 | | 4 ₂ | Cellotriose | - | - | - | - | - |
| 7 | Tetra | 3 ₃ | Laminari-tetraose | ✓ | ✓ | - | ✓ | - |
| 8 | | 3 ₂ 4 ₁ | Mixed | ✓ | ✓ | ✓ | ✓ | - |
| 9 | | 3 ₁ 4 ₂ | Mixed | - | - | - | ✓ | ✓ |
| 10 | | 4 ₃ | Cellotetraose | - | - | - | - | ✓ |
| 11 | Penta | 3 ₄ | Laminari-pentaose | ✓ | ✓ | - | - | - |

See Table I. ^f Subscript represents number of linkages of given type present, eg. 3₂4₁ signifies two -1,3 and one -1,4 linkage. The order in which linkages occur is not here defined.
⁷ ✓ - present; - not detected.

TABLE XV.

Nature of mixed oligosaccharides

| No. x | Complexity | Linkages | Mobility in borate \bar{Z} | Structure |
|-------|------------|-----------|------------------------------|--|
| 5 | Tri | $3_1 4_1$ | ++ | $G-O-4G-O-3G-OH$; $G-O-3G-O-4G-OH$ |
| 8 | Tetra | $3_2 4_1$ | ++ | $G-O-4G-O-3G-O-3G-OH$, $G-O-3G-O-4G-O-3G-OH$ |
| 9 | Tetra | $3_1 4_2$ | + | $G-O-4G-O-4G-O-3G-OH$; $G-O-4G-O-3G-O-4G-OH$ $G-O-3G-O-4G-O-4G-OH$ |

x See table I \bar{Z} On occasion, appear to be a compound spot.

\bar{Z} mobile, -- immobile : in all cases except acid hydrolysates of dextrin mixtures were present.
part mobile and part immobile.

DISCUSSION

The results of series 1-5 are recorded in Tables XIII - XV. From the results of studies of the mobilities of oligosaccharides in bisulphate solution in an electric field, it is possible to group the oligosaccharides according to their complexity; extrapolation of the reciprocal Mg values (true mobility) of the oligosaccharides against their complexity gives a straight-line graph (Fig. 15). Applying this concept to the oligosaccharides produced in series 1 - 5, their complexities were ascertained.

Also, being given by French and Wild (loc.cit.) that $\log 1-RF/RF$ values of the oligosaccharides of a homologous series lie on straight line, $\log 1-RF/RF$ values of the sugars 2-11 of Table XIII were plotted against their molecular complexity (Fig.16). It is observed that points 2, 4, 7, and 11 lie on a straight-line and, therefore, may reasonably be taken to represent a homologous series; the line joining these points on extrapolation, joins unity axis at 0.70 (\equiv glucose). Since known samples of laminaribiose and laminaritriose give plots exactly corresponding to points 2 and 4 of Fig.16, the line joining these points must represent the homologous series of β -1.3-linked glucose oligosaccharides. Therefore, points 2, 4, 7, and 11 would represent laminari-biose, -triose, -tetraose, and -pentaose respectively.

Similarly, points 3, 6 and 10 can be joined with a straight line meeting the unity axis and the line for β -1.3- linked glucose oligosaccharides at exactly 0.70 (\equiv glucose). Since known samples of pure cellobiose give plots exactly sitting on point 3, this second line appears to represent β -1.4- linked glucose oligosaccharides; points 3, 6 and 10 would then correspond to cellobiose, cellotriose and cellotetraose respectively. Fig. 17 is obtained by joining points 2, 4, 7 and 11 with one line and points 3, 6 and 10 with another line, the two lines thus representing respectively, β -1.3- linked and β -1.4-linked glucose oligosaccharides.

In glucan conversions where the concentration of reducing groups exceeded 0.1%, glucose equivalent, a spot with the chromatographic mobility of gentiobiose was obtained (RF 0.079; $\log 1-RF/RF = 1.066$). Some samples of the β -glucan were contaminated with starch and gave a faint spot corresponding to maltose (RF 0.101; $\log 1-RF/RF = 0.0950$). The position of these spots in Fig. 17 clearly shows that they do not lie on either line representing β -1.3- or β -1.4-linkage, which is consistent with their having anomalous linkages viz.

β -1.6 and β -1.4. The gentiobiose must be a product of enzymic resynthesis (see Anderson and Manners, loc.cit.) while maltose came as a result of starch degradation. Neither of these spots was ever observed from the conversions of which the results are shown in Tables XIII and XIV.

Reference to Fig. 16 and 17 shows that compounds

5, 8 and 9 of Table XIII do not lie on either reference line but in between these two lines representing the β -1.4- and β -1.3-linked homologous series; these compounds must therefore contain both 1.3- and 1.4- types of linkages. By drawing parallel lines from appropriate points between the two reference lines, a lattice is obtained (Fig. 17); the positions of compounds 5, 8, and 9 in the lattice correspond to the expected position of theoretically possible oligosaccharides containing mixed linkages. Compound 5, which is a trisaccharide, corresponds to a compound having one 1.3- and one 1.4-linkage, compounds 8 and 9 seem to correspond to tetrasaccharides with, respectively, one 1.4- and two 1.3- linkages and one 1.3- and two 1.4- linkages.

On the basis of the findings of Foster (loc.cit.) that mobilities of oligosaccharides in borate buffer pH 10.0 under the influence of an electric field are related with their stereochemistry, it can be ascertained whether an oligosaccharide has the reducing group near the 1.3-linkage or the 1.4-linkage; compounds having reducing group at the 1.3- end will move towards the anode while those with the reducing group at 1.4- end will not move or will move only slightly. Accordingly, compounds 3, 6, and 10 which are tentatively identified as cellobiose, cellotriose and cellotetraose respectively should be immobile, while compounds 2, 4, 7, and 11 corresponding to laminari- biose, -triose, -tetraose, and-pentaose

respectively should move completely towards anode; these expectations were confirmed. In some cases of one oligosaccharide being contaminated with another, the nature of the contamination would also be revealed eg. a mixture of cellobiose and laminaribiose will give a mobile and an immobile spot; the mobile part would be laminaribiose, while the immobile part would be cellobiose.

Pure compounds containing both types^{of} linkages will have mobility depending on the disposition of two linkages; reducing group at the 1.4- end will be comparatively immobile while those having reducing group at 1.3- end will move towards anode. The relative position of a 1.4- link in respect to a reducing group in an oligosaccharide will also affect its mobility. In the case of the trisaccharide with one 1.3- and one 1.4- linkage, one part of it moves towards anode while some sugar is left behind at the starting point. This clearly reveals the presence of $\dots G^1 - O - {}^3G - OH$ and $\dots G^1 - O - {}^4G - OH$ isomers. The clear definition and separation of this compound on the chromatogram leaves no doubt that the possibility of its being contaminated with another compound, must be entirely rejected. Similarly, the presence of $\dots G^1 - O - {}^3G - OH$ and $\dots G^1 - O - {}^4G - OH$ isomers in the mixed tetrasaccharide has also been revealed. The mobile part of compound 8 which is a tetrasaccharide containing two 1.3- linkages and one 1.4- linkage, can be a mixture of two isomers:

(a) $G^1-O-^3G^1-O-^4G^1-O^3-G-OH$ and (b) $G^1-O-^4G^1-O-^3G^1-O-^3G-OH$, however, because of steric influence depending on the relative position of the 1.3- link, the two isomers would be expected to resolve into two mobile spots, (b) moving the faster; a compound spot was, in fact, observed. The mobile part of the tetrasaccharide with two 1.4- linkages must be expected to represent $G-O-^4G-O-^4G-O-^3G-OH$.

Table XV shows isomerism in the mixed oligo-:saccharides. Compound 5, on electrophoresis in borate buffer resolves into two spots, the mobile one as stated above representing the isomer $\dots 1.3G-OH$ while the immobile spot represent the isomer $\dots 1.4G-OH$. The compounds 8 and 9 are both mixture of isomers containing $\dots 1.4G-OH$ and $\dots 1.3G-OH$ residues. In all these cases the greater part of each sugar is mobile, which suggests that the isomers having reducing groups at the -1.3- end are in greater quantities. This observation is completely in agreement with the view that attack by endo-1.3-ase is favoured by the near presence of β -1.3- linkages. In some experiments, the mobile part of compound 8 resolves itself into two spots, indicating the presence of $\dots G^1.4G^1.3G-OH$ and $\dots G^1.3G^1.3G-OH$ isomers. Compound 9, which is a tetrasaccharide $-G^1-O-^4G^1-O-^4G^1-O-^3G-$, may be a mixture of more than one mobile isomer, but owing to the small mobilities this is difficult to ascertain.

The results recorded in Table XIV show that

the oligosaccharides produced during the endo-degradation of the barley glucan are, qualitatively, exactly the same as produced during its degradation under the joint action of endo- and exo-enzymes. In both the cases glucose, which was removed from the mixture by fractional adsorption on a charcoal-celite column and therefore not present on the chromatogram, was produced; cellobiose, laminari-
biose, -triose, -tetraose, -pentaose, a mixed trisaccharide and mixed tetrasaccharides were also produced. The presence of mixed tri- and tetra-saccharides confirms that the glucan is a mixed polysaccharide and not a mixture of two polysaccharides. The production of laminari-
biose, -triose, -tetraose, and -pentaose leaves no doubt that 1.3- linkages are present in the molecule in blocks; the number of glucose residues in a block of 1.3- can be upto five at least, though the presence of smaller and bigger blocks is not excluded.

It is noteworthy that cellobiose is produced during enzymolysis but not cellotriose or cellotetraose. Since the glucan has equal proportions of 1.4- and 1.3- linkages, there must be present somewhere in the molecule 1.4-linkages equivalent to the blocks of 1.3-. It follows that either endo-1.4-ase is very weak and cannot attack blocks of 1.4- or that 1.4- linkages are scattered all over. If endo-1.4-ase is weaker than endo-1.3-ase the dextrans produced during enzymolysis should be richer in 1.4- linkages and should on further degradation yield

cellobiose, cellotriose, and cellotetraose etc.

Since the dextrin is relatively very resistant to further enzyme action, its degradation can only readily be studied by partial acid-hydrolysis. The products of acid hydrolysis of endo-action and joint-action resistant dextrans are the same; glucose, cellobiose, cellotriose, and cellotetraose, and a mixed tetrasaccharide $-G^1-O^4-G^1-O^4-G^1-O^3-G-$ are produced in both cases. The absence of laminaribiose and its homologes is noteworthy. The production of cellotetraose suggests that upto three consecutive 1.4- linkages at least are present in it; however, the presence of smaller or larger blocks of 1.4- linkages is not excluded. The presence of mixed oligosaccharides and the absence of laminaribiose and its homologes in the acid hydrolysates of the dextrans indicates that both the dextrans are rich in 1.4- linkages, but isolated 1.3- linkages are also present. The possibility of laminaribiose, if produced at all, being preferentially hydrolysed by acid appears to be excluded by the fact that laminaribiose is found to be produced in the acid hydrolysis of undergraded β -glucan under exactly similar conditions (Plate 6).

It follows that the blocks of 1.4- and 1.3- types of linkages are present in the glucan chains; present evidence confirms the presence of upto three 1.4- and 1.3- consecutive linkages in the glucan molecules, though the possibility of longer and shorter runs is not excluded.

During enzymolysis, 1.3- linkages are preferentially attacked, leaving a dextrin richer in 1.4- linkages.

The oligosaccharides produced by the endo-action alone and the joint-action of endo- and exo-enzymes from barley on oat glucan, are nearly the same as from barley glucan; in addition to the usual oligosaccharides produced by the degradation of barley glucan, oat glucan under the joint-action of endo- and exo-enzymes gives a tetrasaccharide with two 1.4- and one 1.3- linkage. This is well in agreement with the view that oat glucan is probably richer in 1.4- linkages, which is exactly the case with oat-lichenin (Peat, et al., loc.cit.).

CONCLUSIONS.

The following conclusions emerge from the foregoing discussion:

- (1) That β -glucan, whether from oats or from barley, is a mixed polysaccharide containing 1.3- and 1.4- linkages, and not a mixture of two polysaccharides.
- (2) That 1.3- linkages occur in the original glucan in blocks of upto four at least; the possibility of the presence of larger or smaller blocks is not excluded.
- (3) That the barley glucan dextrin is relatively rich in 1.4- linkages which are present in blocks of upto three at least; the possibility of larger and smaller blocks is not excluded.

- (4) That isolated 1.3- linkages are also present in the barley glucan dextrins.
- (5) That during enzymolysis of the glucan 1.3- linkages are being preferentially attacked.
- (6) That the possibility of greater proportion of 1.4- linkages being present in oat glucan is supported.
- (7) That the isomerism in mixed oligosaccharides is confirmed.

GENERAL DISCUSSION

Degradation of β -glucan and other non-starchy polysaccharides (hexosans and pentosans) during malting of barley seems to play an important part during germination. There is sufficient evidence to suggest that the dissolution of the cell-walls of the endosperm takes place before amylolysis or proteolysis. Besides the problem having academic interest, practical reasons (malting) also demand greater information on this phenomenon. It must, however, be admitted that even the latest scientific techniques do not provide adequate means of investigating biochemical aspects of such phenomenon "in vivo". Therefore, one has to resort to carrying out experiments "in vitro". Too much stress must not be laid upon the results derived from experiments "in vitro", since what can happen under certain conditions in the laboratory need not necessarily happen in the natural kernel of the intact grain. Still, it is hoped the results of experiments "in vitro" do provide at least a first approximation of what happens in nature.

Advancement of knowledge in the field of enzymolysis of cereal hemicelluloses was greatly hampered by the non-availability of chemically well-defined substrates uncontaminated with other impurities, as well as by the non-availability of specific enzymes. Preparation of a β -glucan uncontaminated with starch and pentosan material, by Preece and MacKenzie (1952), thus presented

a turning point. This discovery paved the path for the work of Preece and Hoggan (1956). These workers found that further purification and fractionation of β -glucanase enzymes was not possible along the lines of fractional precipitation with ammonium sulphate at an optimum pH, but that progress could be made using the new substrate in conjunction with specific enzyme inactivators, the device used here.

Since Manners (1955) and Anderson and Manners (1959) observed transglycosylation during the hydrolysis of cellobiose in presence of barley enzymes, it became important that the possibility of enzymic synthesis in the degradation of β -glucan should be examined more thoroughly. It seems most unlikely that transglycosylation, even if it occurred at all in experiments described in Section I, would introduce any significant error in the measurement of reducing groups during the course of reaction, though the possibility of anomalous structures being introduced in the oligosaccharides produced during enzymolysis is worthy of attention. Anderson and Manners (loc.cit.) employed high concentrations of cellobiose and enzyme in their experiments; synthesis was not observed at lower concentrations of cellobiose. Special care was, therefore, taken in the present work, especially while carrying out experiments for the characterisation of oligosaccharides, that the conditions employed were those most unlikely to permit enzymic synthesis. The resultant oligosaccharides could, therefore, safely be considered as products of

degradation and not of synthesis, since conversions were carried out at concentration levels where synthesis has not been observed.

Rf values, and ionic mobilities of the sugars in borate and bisulphite buffers under electric potential (electrophoresis) form the basis of the structural investigation of the oligosaccharides. The physical methods of analysis may not be as reliable as the chemical methods, but the quantities of the oligosaccharides produced during the enzymolysis were so small that the more unequivocal chemical methods could not be applied. The evidence in favour of the assigned chemical structure of the sugars is circumstantial, but it is strong and convincing; nevertheless, confirmation by chemical methods would be desirable.

Enzyme activities of β -glucanase system.

Preece and Hoggan (loc.cit.) postulated that at least three enzymes are involved in the enzymic degradation of β -glucan. An exo- β -glucanase attacks the glucan chains at non-reducing ends liberating cellobiose with or without laminaribiose; endo- β -glucanase attacks at random at points remote from the ends, producing ultimately oligosaccharides and dextrans (high molecular material precipitable with alcohol); and a cellobiase hydrolyses cellobiose into glucose. On the basis of the results of Section I a more elaborate pattern of glucan degradation may be put forward.

The degradation of β -glucan is essentially an endo-action accelerated by exo-action which involves attack at penultimate 1.4- linkages at the non-reducing ends of the chains, resulting in the production of cellobiose and aiding in the disappearance of high-molecular material precipitable with alcohol (dextrin). Cellobiose and possibly laminaribiose thus produced are further hydrolysed to glucose by the action of disaccharases, one of which is a specific laminaribiase; a cellobiase and/or general β -glucosidase is also present.

Endo-action is complex, involving dextrinisation and saccharification, two aspects of action which do not run necessarily parallel. With barley enzymes, saccharifying potentiality is far more efficient than with oat enzymes. There may be variations in the potentialities of different enzyme preparations even from the same sources. Dextrinising activity is the result of endo-scissions of both 1.3- and 1.4- linkages, while the saccharifying activity mainly involves 1.3- linkages; the possibility of some 1.4- linkages also being attacked cannot be completely ignored, though endo-1.4-ase seems to be weaker than endo-1.3-ase.

The degradation of barley glucan both under the joint action of endo- and exo- enzymes and of endo-enzymes alone is not a smooth reaction; during enzymolysis, dextrans, relatively resistant to further enzyme action are being accumulated. In the case of the oat glucan enzymolysis reaches completion, at least, under the joint action of

endo- and exo-enzymes, after 42 hr. of reaction; high-molecular material precipitable with alcohol cannot be recovered from the oat reaction mixture at this stage under the present conditions of working.

Production of oligosaccharides in β -glucan degradation.

In Section II, evidence is given for the presence of glucose, laminaribiose, laminaritriose, laminaritetraose, laminaripentaose, cellobiose, and mixed oligosaccharides in the enzymolysates of the barley glucan; the mixed oligosaccharides were the two trisaccharides containing each one 1.3- and one 1.4- linkage, and tetrasaccharides with one 1.4- and two 1.3- linkages. Joint-action of endo- and exo-enzymes and that of endo-enzymes alone produces the same oligosaccharides, the only difference being in the more rapid production of the oligosaccharides under the joint-action. Preece and MacDougall (1958) had also shown that the appearance of oligosaccharides is accelerated by exo-attack on the intermediate pentosan dextrans. The enzymolysates of oat glucan contain a tetrasaccharide with one 1.3- and two 1.4- linkages, in addition to those obtained from the barley glucan.

The dextrans produced after 24hr. of reaction of the barley glucan with endo-enzymes alone and the joint action of endo- and exo-enzymes from barley, are relatively resistant to further enzyme attack. Therefore, their characters could only be revealed by studying the oligosaccharides produced during their partial acid-hydrolysis. Both the joint-action- and endo-action-

resistant dextrans produce the same oligosaccharides; glucose, cellobiose, cellotriose, cellotetraose and another tetrasaccharide with one 1.3- and two 1.4- linkages are produced; the absence of laminaribiose and its homologues is noteworthy. As has been stated already, the possibility that during acid hydrolysis of the dextrans, any laminaribiose, if produced at all, was preferentially hydrolysed to glucose is rendered unlikely by the production of laminaribiose from the undegraded barley glucan which contains an equal proportion of 1.3- and 1.4- linkages. The production from the dextrans of oligosaccharides containing mixed linkages shows that the dextrans are not linked by 1.4- linkages alone. The conclusion cannot be escaped that both the dextrans contain mixed linkages; 1.4- linkages are by far in greater proportion, 1.3- linkages being few and isolated.

Structure of the barley glucan and its pattern of enzymic degradation.

Having determined the general characters of β -glucanase system of barley, and the oligosaccharides produced during enzymolysis, now it seems possible to formulate a general outline of the arrangement of 1.3- and 1.4- linkages in the glucan chains and to present a more detailed pattern of the enzymic degradation of the substrate. Given the proportions of 1.3- and 1.4- linkages in the glucan to be 50:50 (Aspinall and Telfer, 1954), and given also that the barley glucan is not a mixture of

two polysaccharides but that the two types of linkages occur in the same molecule (see Gilles et al., 1956; and production of mixed oligosaccharides in Section II), three possibilities exist for the arrangement of the two types of linkages in the glucan: (i) Gilles et al. (loc.cit.) believed that the two types of linkages are arranged in a regular alternating way; (ii) Aitken et al. (1956) suggested that the linkages appear separately in distinct groups; (iii) Preece and Hoggan quoted by Preece (1957) held the view that both types of linkages are distributed generally throughout the molecule, but that certain regions are rich in 1.3- linkages while others are rich in 1.4- linkages.

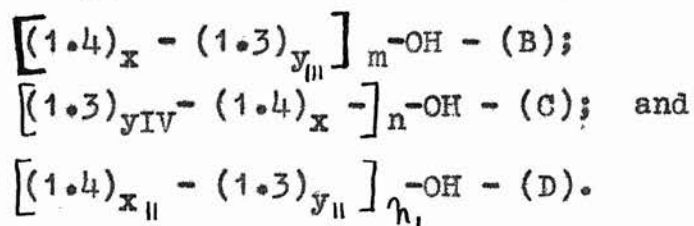
(i) Regularly alternating distribution of the linkages.

This view fails to explain the production of resistant dextrans, laminaritriose, and laminaritetraose during the enzymolysis of the glucan and production of cellotetraose during the acid hydrolysis of the dextrans. Therefore, the possibility of a regular repetition with alternate 1.3- and 1.4- linkages being present in the glucan chains is entirely rejected.

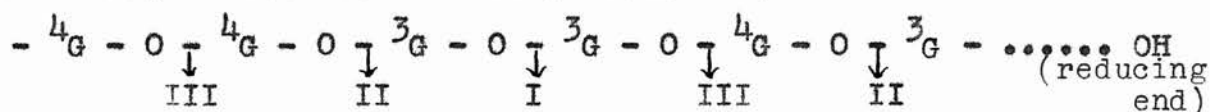
(ii) Distribution of the linkages in large, separate and distinct groups.

This assumption readily accounts for the production of laminaribiose and its homologes and the accumulation of resistant dextrans during enzymolysis of the glucan. Suppose the overall arrangement in the chains is $[-(1.4)_x - (1.3)_y]_n$, where $\sum x$ is

approximately equal to $\sum y$ and the reducing end of the chain is not defined; from the foregoing discussion of Sections I and II it follows that, principal endo-scissions must take place in the region $-(1.3)_y$. Accordingly, smaller fragments produced by the endo-action would have the structures: $\left[(1.3)_{y1} - (1.4)_x - (1.3)_{y1} \right]_n \text{-OH (A)}$, where the reducing group is at the 1.3- end. Further endo-degradation (strong endo-1.3-ase- and weaker endo-1.4-ase action) of these fragments would produce dextrans of the types:



It is evident that during the degradation, rapid decrease in molecular size is occurring and the relative disposition of 1.3- and 1.4- linkages is also changing both quantitatively and qualitatively; It is also expected that in such a reaction mixture, the production of (B) would be accompanied by production of laminaribiose and its homologes. From the evidence already discussed, it appears that oligo-saccharides with a 1.3- linkage near the reducing end are produced preferentially, those containing the 1.4- linkage near the reducing end are less abundant. This may be explained on the assumption that susceptibility to attack depends on the position of a linkage; the susceptibility can be represented for the endo-action as:



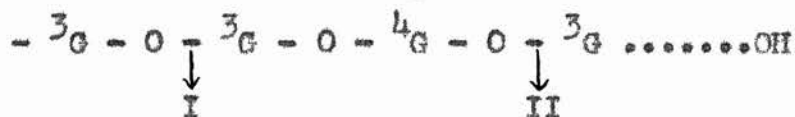
where the position I is most susceptible, II and III are relatively very resistant. In other words, in a chain where a reducing group is on the right, a 1.3- linkage having 1.4- linkage on its right and a 1.3- linkage on its left would be most susceptible; other 1.3- linkages would be fairly readily attacked, but all 1.4- linkages irrespective of their surrounding linkages would be fairly stable. It follows that there will ultimately be an accumulation of $(1.4)_x$ - material at the non-reducing end of the chain, while degradation of the $(1.3)_y$ - regions would still be possible. Under these circumstances, the accumulating endo-dextrin would show progressive increase in the proportion of 1.4- linkages. The degradation of the glucan under the joint-action of exo- and endo-enzymes is fundamentally an endo-conversion accelerated by exo-action. Here again the dextrans (B), (C), and (D) would arise but $(1.4)_x$ - residues present at their non-reducing ends would simultaneously be attacked by exo-1.4-ase, rapidly eliminating these segments as cellobiose, so that accumulation of 1.4- linkages in the dextrin would not occur; the joint-action dextrans might well be rich in 1.3- linkages. Accordingly, the endo-dextrin and joint-dextrin would differ profoundly in their chemical structure and the differences would be reflected in differences in their pattern of enzymic degradation (though this is so slow as not to produce useful results) and acid hydrolysis. In fact, both dextrans on acid hydrolysis behave quite similarly. Therefore, the existence

of large separate blocks of 1.3- and 1.4- linkages in the glucan seems to be excluded.

(iii) Distribution of linkages throughout the molecule.

If it is assumed that the distribution of the two types of linkage in the glucan involves short- perhaps variable runs - of the two types, and some parts of the chain are rich in 1.4- and some in 1.3- linkages, an attempt may be made to explain the observed facts.

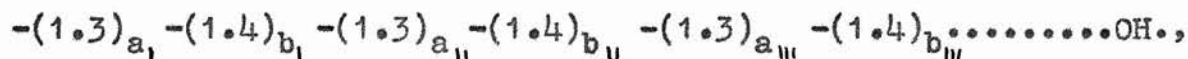
Endo-action alone would preferentially attack those parts of the molecule rich in 1.3- linkages. Laminaribiose, and its homologes, and cellobiose would be produced; cellobiose would come from the disruption of 1.3- linkages at I and II point in the following structure:



The degradation of regions relatively rich in 1.4- linkages would be only very slight because the endo-1.4-ase action is weak and readily susceptible 1.3- linkages of the type $- {}^3G - O - {}^3G - O - {}^4G - \dots\dots\dots OH$ are few, whereas, relatively stable 1.3- linkages of the type $- {}^4G - O - {}^3G - O - {}^4G - \dots\dots\dots OH$, and 1.4- linkages are present in greater proportion in the remaining fragments of the glucan. Therefore, the dextrans accumulating during endo-degradation would undoubtedly be rich in 1.4- linkages, but owing to the presence of the two types of blocks of varying size the discrepancy would not be so great as if only blocks of the two type were present. Laminaribiose and cellobiose would also be further hydrolysed to glucose.

In the case of joint-action, the 1.4- linked chains present near the non-reducing end of the molecule would largely be eliminated by exo-1.4-ase as cellobiose, but since the runs of 1.4- are short, exo-1.4-ase action would soon be impeded; resulting dextrans would, therefore, contain 1.4- and 1.3- linkages, whose relative proportion will not differ much from those of the endo-dextrans. Accordingly, the joint-dextrin and the endo-dextrin would be much more close to each other in chemical structure; their behaviour towards acid hydrolysis would be very much the same; in fact, this is found to be the case. The chemical structure of the dextrans may be represented as: $(1.3) - (1.4)_p - (1.3) - (1.4) - (1.3) - (1.4)_q \dots \dots \text{OH}$, where p and q are small, giving a molecule capable of yielding cellobiose and its homologes, at least up to cellotetraose level and certain mixed oligosaccharides; the presence of isolated 1.3- linkages would make the production of laminaribiose and its homologes impossible.

It is now possible to propose the following structure for the original barley glucan;



where $a_1, b_1, \text{etc.}$, are small numbers of value 1 to 5 or more and $\sum a = \text{approximately } \sum b$. This structure explains all the observed facts in a way which no other structure proposed before could. It must, however, be realised that this hypothesis depends very much on the view that steric factors - relative disposition of the types of linkage -

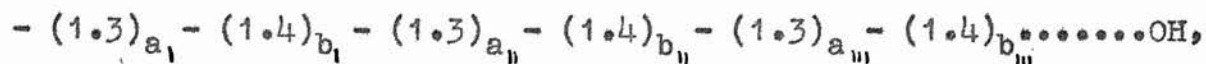
are involved in determining susceptibility of a linkage to enzyme attack.

Oat glucan.

The proportions of 1.3- and 1.4- linkages in oat glucan preparations used in the present work is not known. It might resemble the "oat lichenin" of Morris (1942), the structure of which has been recently investigated by Peat et al. (1957). It was reported that "oat lichenin" contains 1.3- and 1.4- linkages in 1:2 ratio and the types of linkage are dispersed in the molecule in regularly alternating fashion - $-4.4-3-4.4-3-4.4 \dots$. Such a structure does not allow for the enzymic production of laminaritriose and its homologes, the presence of which in oat glucan enzymolysates is undoubted. This structure does not also permit the presence of $-^3G-O-^3G-O-^4G \dots OH$ linkages, which are easily susceptible to enzyme attack, but it does permit the presence of $-^4G-O-^3G-O-^4G \dots OH$ linkages which are relatively resistant to enzyme attack. Accordingly, if this was the structure of oat glucan, the polysaccharide should have been resistant to enzyme action, which does not happen to be the case; oat glucan is smoothly degraded. Therefore, regularly alternating arrangement of the two types of linkage in the oat glucan cannot be accepted. Nothing more can be assumed than that the oat glucan is richer in 1.4- linkages than the barley glucan, which is consistent with the observed facts.

It has been shown that steric factors play an important part in determining the susceptibility of an

individual linkage to enzyme attack. Distribution of linkages generally throughout the glucan molecule would permit representing the glucan by the following structure which is very similar to that for the barley glucan:



where the value of a_1 , b_1 , etc. exceeds unity in most of the blocks, though their maximum value is still fairly small, especially 1.4- runs seem to be shorter than those in the barley glucan. It may well be that $a = \frac{1}{2} b$, but it still remains to be proved. Such a formula would explain the essential difference between barley and oat glucans; the former have isolated 1.3- linkages in certain regions of the molecule, these linkages, being of the $-^4\text{G}-\text{O}-^3\text{G}-\text{O}-^4\text{G} \dots \text{OH}$ type are relatively resistant to enzyme attack and account for the accumulation of resistant dextrans; whereas in the oat product most of the 1.3- linkages would be of $-^3\text{G}-\text{O}-^3\text{G}-\text{O}-^4\text{G} \dots \text{OH}$ type, thereby rendering the glucan to be less prone to yield resistant dextrans.

Nomenclature of enzymes.

Another interesting point emerging from the present work is that even endo-action itself involves more than one enzyme activity. It cannot be said whether these different activities are due to separate and distinct enzymes or they are just different aspects of the activities of one single enzyme. Therefore, it would be desirable to denote the enzymes of the β -glucanase system as endo- β -glucanases or the endo- β glucanase system; consequently the degradation of the substrate should be regarded as a

pattern of degradation, the details of which depend on the balance of functions present.

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