ABSTRACT OF THESIS

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Title of Thesis: The Molecular Structure and Enzymic Hydrolysis of Polysaccharides.

A study has been made of the molecular structure and enzymic degradation of the starch components (amylose and amylopectin), glycogen, lichenin and related β-glucosans, and certain mannose-containing polysaccharides. Enzymic preparations from barley, malt, fungi, and algae have been used.

The observed increase in β-amylolysis limit of amylose caused by barley β-amylase and emulsin preparations, formerly attributed to Z-enzyme, has now been shown to be due to a trace of α-amylase. Continuous electrophoresis has been used to separate the Z-enzyme (α-amylase) from the β-amylase activity in the barley preparation. Dilute solutions of barley and malt α-amylase can also simulate Z-enzyme activity. Amylopectin and glycogen are slowly attacked, the rate being lower than that observed with amylose; there is also limited action on their β-dextrins. Viscometry has provided a rapid and sensitive method for detection of trace amounts of α-amylase. The α-amylase activity of Cladophora rupestris, unlike that of malt α-amylase, is increased by a low concentration of borate ions.

Fractionation of barley and malted barley preparations by fractional precipitation with ammonium sulphate, chromatography on alumina, or by continuous electrophoresis did not result in the complete separation of the β-glucosidase and β-glucosanase activities. However, indications were obtained that the β-1,3-, β-1,4-glucosidase, laminarinase and celloextrinase activities were due to distinct enzymes. Glucono-1,4-lactone causes complete inhibition of the β-glucosidase and β-1,4-glucosanase activities of malted barley with a retention of 30% β-1,3-glucosanase activity. The fungus, Rhizopus arrhizus, selectively produces a β-1,3-glucosanase which together with the malt β-1,3-glucosanase has been shown to be specific for the hydrolysis of β-1,3- or β-1,4-glucosidic linkages attached to a 3-substituted glucosyl unit.

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Use other side if necessary.
The fungal enzyme degrades lichenin to oligosaccharides, the major product being $4^2-\beta$-glucosyl laminaribiose. The results together with chemical data indicate that the polysaccharide consists mainly of $\beta$-cellotriosyl units joined by 1,3-linkages.

The reserve polysaccharide from *Peranema trichophorum*, paramylon, has been characterised as a $\beta$-glucosan probably containing glucose units joined by 1,3-linkages.

A critical examination of acid hydrolysates from laminarin have failed to show the presence of D-mannose.

The enzymic degradation of yeast glucan has been examined but the results did not enable the minor structural linkages to be characterised.

Malted barley and *Rhizopus arrhizus* preparations contain enzymes capable of hydrolysing mannose-containing polysaccharides, with the exception of yeast mannan, to their constituent monomers. The "mannanase" activity of malted barley has not been separated by fractional precipitation with ammonium sulphate or acetone.
STUDIES ON THE MOLECULAR STRUCTURE AND
ENZYMIC DEGRADATION OF POLYSACCHARIDES

- by -

William L. Cunningham, B.Sc.

Thesis presented for the degree of Doctor of Philosophy

University of Edinburgh

July, 1961
TO
MY PARENTS
AND
JAN
I wish to express my gratitude to Dr. D.J. Manners for his advice and guidance throughout the course of this work. I wish to thank Professor E.L. Hirst, C.B.E. for his interest in this work and also for providing laboratory facilities.

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<table>
<thead>
<tr>
<th>CONTENTS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acknowledgements</td>
<td>1</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>11</td>
</tr>
<tr>
<td>Index of Figures</td>
<td>v</td>
</tr>
<tr>
<td>Section I General Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Section II General Methods</td>
<td>14</td>
</tr>
<tr>
<td>Section III Studies on the Enzymic Degradation of Starch-Type Polysaccharides</td>
<td></td>
</tr>
<tr>
<td>Part 1 Introduction</td>
<td>29</td>
</tr>
<tr>
<td>Part 2 The Action of Barley $\beta$-Amylase and $\alpha$-Enzyme</td>
<td>36</td>
</tr>
<tr>
<td>Methods and Materials</td>
<td>38</td>
</tr>
<tr>
<td>Results</td>
<td>40</td>
</tr>
<tr>
<td>Discussion</td>
<td>52</td>
</tr>
<tr>
<td>Part 3 $\alpha$-Enzyme Activity of Almond Emulsin</td>
<td>55</td>
</tr>
<tr>
<td>Materials</td>
<td>57</td>
</tr>
<tr>
<td>Results</td>
<td>67</td>
</tr>
<tr>
<td>Discussion</td>
<td></td>
</tr>
<tr>
<td>Part 4 Observations on $\alpha$-Amylases from Barley, Malt, and Human saliva</td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>70</td>
</tr>
<tr>
<td>Materials</td>
<td>72</td>
</tr>
<tr>
<td>Results</td>
<td>73</td>
</tr>
<tr>
<td>Discussion</td>
<td>84</td>
</tr>
<tr>
<td>Part 5 Effect of Ions on $\alpha$-Amylase Activity of Cladophora rupestris</td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>87</td>
</tr>
<tr>
<td>Results</td>
<td>89</td>
</tr>
<tr>
<td>Discussion</td>
<td>93</td>
</tr>
<tr>
<td>Summary</td>
<td>94</td>
</tr>
<tr>
<td>Section IV Studies on the Preparation of Specific $\beta$-Glucosanases</td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>96</td>
</tr>
<tr>
<td>Part 1 Fractional Precipitation by Ammonium Sulphate</td>
<td></td>
</tr>
<tr>
<td>Methods and Materials</td>
<td>101</td>
</tr>
<tr>
<td>Results</td>
<td>102</td>
</tr>
<tr>
<td>Discussion</td>
<td>103</td>
</tr>
<tr>
<td>Part 2 Adsorption Chromatography</td>
<td></td>
</tr>
</tbody>
</table>
## Contents continued

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Methods and Materials</strong></td>
<td>104</td>
</tr>
<tr>
<td><strong>Results</strong></td>
<td>106</td>
</tr>
<tr>
<td><strong>Discussion</strong></td>
<td>107</td>
</tr>
<tr>
<td><strong>Part 3 Electrophoresis</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Methods and Materials</strong></td>
<td>109</td>
</tr>
<tr>
<td><strong>Results and Discussion</strong></td>
<td>110</td>
</tr>
<tr>
<td><strong>Part 4 Selective Inhibition</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Introduction</strong></td>
<td>111</td>
</tr>
<tr>
<td><strong>Materials</strong></td>
<td>112</td>
</tr>
<tr>
<td><strong>Results</strong></td>
<td>114</td>
</tr>
<tr>
<td><strong>Discussion</strong></td>
<td>118</td>
</tr>
<tr>
<td><strong>Part 5 Selective Production of Enzymes by Fungi</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Introduction</strong></td>
<td>120</td>
</tr>
<tr>
<td><strong>Methods and Materials</strong></td>
<td>121</td>
</tr>
<tr>
<td><strong>Results</strong></td>
<td>123</td>
</tr>
<tr>
<td><strong>Discussion</strong></td>
<td>125</td>
</tr>
<tr>
<td><strong>Summary</strong></td>
<td>126</td>
</tr>
<tr>
<td><strong>Section V The Molecular Structure of Lichenin and Related ( \beta )-Glucosans</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Introduction</strong></td>
<td>127</td>
</tr>
<tr>
<td><strong>Part 1 Studies on Lichenin</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Materials</strong></td>
<td>131</td>
</tr>
<tr>
<td><strong>Results</strong></td>
<td>132</td>
</tr>
<tr>
<td><strong>Discussion</strong></td>
<td>143</td>
</tr>
<tr>
<td><strong>Part 2 The Molecular Structure of the Reserve Polysaccharide from Peranema</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Introduction</strong></td>
<td>146</td>
</tr>
<tr>
<td><strong>Materials</strong></td>
<td>147</td>
</tr>
<tr>
<td><strong>Results</strong></td>
<td>148</td>
</tr>
<tr>
<td><strong>Discussion</strong></td>
<td>150</td>
</tr>
<tr>
<td><strong>Part 3 Studies on Laminarin</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Results</strong></td>
<td>151</td>
</tr>
<tr>
<td><strong>Discussion</strong></td>
<td>152</td>
</tr>
<tr>
<td><strong>Part 4 Studies on Yeast Glucan</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Results and Discussion</strong></td>
<td>155</td>
</tr>
<tr>
<td>Summary</td>
<td>156</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Section VI</td>
<td>Studies on Mannanases</td>
</tr>
<tr>
<td>Introduction</td>
<td>158</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>161</td>
</tr>
<tr>
<td>Results</td>
<td>162</td>
</tr>
<tr>
<td>Discussion</td>
<td>166</td>
</tr>
<tr>
<td>Summary</td>
<td>167</td>
</tr>
</tbody>
</table>
# INDEX OF FIGURES

1. Methylation Method. .................................................. 4
2. Periodate Oxidation. .................................................. 5
3. Reduction and Hydrolysis of Periodate-oxidised Polysaccharide 5
4. Barry's Method. ......................................................... 6
5. Hydrolysis of Agarose. ................................................. 9
6. Phosphorolysis of Amylopectin or Glycogen. ...................... 10
7. Transglucosylation of Maltose. ...................................... 11
9. Determination of Formaldehyde. .................................... 19
10. Determination of Periodate Reduction. .......................... 19
11. Estimation of Protein Nitrogen (Biuret Method). ............... 24
12. Molecular Structures of Amylopectin and Glycogen. .......... 32
13. Protein content and β-Amylase Activity of Alumina Column Fractions. (Barley β-Amylase) 45
14. Relative Activities of Electrophoretic Fractions. (Barley β-Amylase) 54
15. Effect of Enzyme (Emulsin) Concentration on Fall in A.V. of Amylose-Iodine Complex. 59
16. Effect of pH on Z-Enzyme Activity of Emulsin. ................ 59
17. Stabilising effect of Calcium Ions on the Z-Enzyme Activity of Emulsin. 61
18. α-Amylolysis of Amylose. .......................................... 71
19. α-Amylolysis of Glycogen. .......................................... 71
20. Effect of pH on α-Amylase activities of Barley and Malt. .... 76
21. Protein Content of Alumina Column Fractions (Malt). .......... 106
22. Protein Content of DEAE Cellulose Column Fractions (Malt). 106
23. Relative Activities of Electrophoretic Fractions (Malt; β-Glucosidases and β-Glucosanases). 110
24. Enzymic Hydrolysis of Cellobiose and Effect of Gluconolactone 115
25. Effect of Gluconolactone on the β-Glucosanases of Barley .... 115
26. Effect of Gluconolactone on the β-Glucosanases of Malt ....... 116
27. Periodate Oxidation of Lichenin. .................................. 128
28a,b. Periodate Oxidation of Products of Lichenin Enzymic Hydrolysate. 138
29a. Analysis of 4'-Glucosyl Laminaribiose. ......................... 139
29b. Action of Laminarinase on Lichenin. ............................ 139
30. Peranema ............................................................... 146
31. Infra-red spectrum of Paramylon. ................................. 149
Polysaccharides are high-molecular weight polymers, composed of one or more monosaccharide types which are united by glycosidic linkages; they occur in quantity in plants and animals as reserve materials and skeletal substances. Many plant exudates and mucilages are also largely polysaccharide in nature. Polysaccharides such as starch or inulin form the reserve carbohydrate material of most land-plants, and glycogen fulfils the same function in animals. Cereal starch is one of the principal articles of diet today, and is used on a large scale in the production and preparation of a variety of foodstuffs. The fundamental structural substance in land plants consists mainly of α-cellulose (a polymer of β-1,4-linked D-glucose units), in conjunction with hemicelluloses and lignin. Cellulose is a major raw material in many industries, including the manufacture of cotton goods, paper, explosives, plastics, jute and rayon. Some fungi contain chitin (a polymer of N-acetyl-D-glucosamine) in place of cellulose, together with glucans and other carbohydrates of relatively low molecular weight. Marine algae contain reserve and structural polysaccharides such as glucans, galactans, pentosans/
and polyuronides (1). Alginic acid, the intercellular polyuronide of the brown seaweeds, finds applications as an emulsifier and thickener in the food and cosmetics industries; and agar-agar, a galactan from species of *Gelidium* and *Gracillaria* is of considerable importance as a gel-forming agent especially for the preparation of culture media for the growth of microorganisms. Species of plants such as *Acacia*, *Astragalus*, and *Prunus* secrete gums either as a product of metabolism or as a result of a pathological condition. These gums (e.g. gum arabic and tragacanth) are commercially important in the manufacture of adhesives, foodstuffs, pharmaceuticals, and textiles (2).

Careful purification of polysaccharides is essential before any structural analysis can be performed, otherwise misleading results may be obtained. Methods for the isolation and purification of a wide range of polysaccharides include selective extraction of plant or animal tissues by water, dilute alkali or acid at various temperatures followed by fractional precipitation with alcohol or other organic solvents, ammonium sulphate (3), or complexing agents (e.g. Fehling's solution). Recently, use has been made of dimethyl sulphoxide as a polysaccharide solvent (4), cupriethylene diamine (5), and cetyl trimethylammoniumbromide (5) for the preferential precipitation of some polysaccharides.

Smith/
and his coworkers have shown that the protein concanavalin-A, will selectively precipitate certain polysaccharides (e.g. yeast mannan and glycogen from aqueous solutions (7), and Heidelberger and his coworkers have demonstrated the usefulness of immunochemical methods in the specific precipitation of polysaccharides (8).

Recently, progress has been made in the separation of polysaccharides by electrophoresis; in a Tiselius apparatus and with borate buffer, it is possible to separate glycogen, starch, and yeast mannan (9). The analytical paper electrophoresis apparatus has been developed into a small scale preparative method by the use of thick glass paper (10), and later, by columns of powdered glass (11).

The use of ion-exchange cellulose (DEAE cellulose) as an adsorbent for the efficient chromatographic fractionation of polysaccharide mixture has been demonstrated by Deuel and his coworkers (12). "Sephadex", a sulphated dextran polymer, has also been used for this purpose (13). Undoubtedly these methods will find wide application in the future, especially in the separation of acidic and neutral polysaccharides.

The homogeneity of the polysaccharide sample (i.e. the presence of molecules having identical chemical structure/)
Methylation of a linear polysaccharide (repeating linkage 1:4-glucosidic)

Methylation of a branched polysaccharide (R = chain of monosaccharide residues)

FIGURE 1.
but not necessarily the same molecular weight), may be tested by applying physico-chemical techniques, including infra-red spectroscopy (11), ionophoresis (10), ultracentrifugation (15), and differential thermal analysis (16). The immunochemical technique is also important (8b), and for starch-type polysaccharides, measurement of iodine binding-power by differential potentiometric titration may be used (17).

The structure of a purified polysaccharide may then be determined by a variety of chemical methods. For example, qualitative and quantitative analysis of complete acid hydrolysates will enable the component sugars to be identified. The development of the techniques of chromatography and electrophoresis have completely revolutionised the fractionation of such hydrolysates (18). The order of the monosaccharide residues and the anomeric configuration of the repeating glycosidic linkages in the polymeric chains can be conveniently elucidated by a study of the oligosaccharides formed by partial acid hydrolysis of the parent polysaccharide. As an example, Wolfrom and his coworkers made use of this method in proving the presence of a small proportion of \( \alpha-1,6 \)-glucosidic linkages in amylopectin (the branched component of starch) and glycogen, by the isolation of isomaltose (19) and panose (20) from partial acid hydrolysates.

The complete hydrolysis of an exhaustively methylated polysaccharide and the subsequent separation, identification,
**Fig. 2. Periodate oxidation.**

$$\text{CH}_2\text{OH} \xrightarrow{\text{(Oxidation)}} \text{CH}_2\text{OH}$$

$$\text{OH} \xrightarrow{\text{(Hydrolysis)}} \text{CH}_2\text{OH}$$

**Fig. 3**

\[
\begin{align*}
\text{CH}_2\text{OH} & \quad \text{[II]} \\
\text{CHOH} + (n + 1) \text{CH}_2\text{OH} & \quad \text{HC}=\text{O} \\
\text{CHOH} & + (n + 1) \text{CH}_2\text{OH} \quad \text{HCO}_2\text{H} \\
\end{align*}
\]
and estimation of the monomers is the basis of the methylation technique (see Fig.1). The original procedures developed by Purdie and Irvine (21), Denham and Woodhouse (22), and Haworth (23) are still used today with only slight modification. This technique gives valuable information on the repeating linkages present, the average chain length (CL.), without, however, indicating the configuration of the linkages, the order of the structural units or the types of branching (i.e. simple or multiple) involved.

Oxidation with sodium or potassium meta-periodate has provided a useful semi-microtechnique in polysaccharide analysis (24) (Figure 2). The relationship between the reduction of oxidant, and the release of formic acid or formaldehyde during oxidation of a polysaccharide provides data on the repeating and inter-chain linkages. With some polysaccharides, the CL. and degree of polymerisation (DP.) can also be determined. Smith and his coworkers have extended the periodate oxidation procedure by reducing the polyaldehydic products catalytically or with sodium borohydride to the corresponding polyalcohols. The polyalcohols were then hydrolysed and the products identified. Any 1,3-linked hexose residues in a polymer will therefore appear after the final hydrolysis step as the free sugar (25), e.g. the presence of 1,3-linkages in yeast mannan established by
Barry's method.

**Figure 4.**
methylation studies was confirmed by the isolation of mannose units resistant to periodate oxidation (26). Polymers containing hexose units e.g. glucose (i) joined by 1,4-glucosidic linkages will be transformed by periodate oxidation into the dialdehyde (ii) which will give the polyalcohol (iii) on reduction. Hydrolysis of the polyalcohol (iii) yields erythritol and glycollic aldehyde (Figure 3). This method has proved of the highest importance not only in ascertaining the nature of linkages between sugar residues but also in determining the positions of periodate-stable and periodate-oxidisable residues.

In a study of laminarin, Barry (27) found that periodate oxidation affected only the non-reducing terminal sugar unit. Treatment of the oxidised polysaccharide with phenylhydrazine acetate brought about a rapid separation of glyoxalosazone and a recoverable polysaccharide, the CL of which had been shortened by one glucose unit (Figure 4). Stacey and his coworkers have investigated nigeran, an α-glucosan, and by subjecting it to one Barry degradation, found that no polymeric material remained; this indicated that there were no successive α-1,3-linkages adjacent to the α-1,4-linkages (28).

The enzymic degradation of polysaccharides is a recent and important technique since it can provide information regarding the fine structure of the polysaccharide which may
be otherwise unobtainable by less specific chemical methods. The action of a purified hydrolytic enzyme (polysaccharase) on a polysaccharide usually follows one of two patterns; either random degradation occurs with the production of oligosaccharide fragments varying in DP., or the degradation is stepwise giving saccharide units which are constant in DP. These activities are usually named endo- or exo-, respectively.

The majority of carbohydrate-metabolising enzymes catalyse a reaction between a donor carbohydrate substrate and an acceptor substrate containing a hydroxyl group:—

Equation 1.

\[
\text{DOR} + \text{HOA} \rightleftharpoons \text{DOA} + \text{ROH}
\]

Donor Acceptor

With an endo-type polysaccharase, D and R denote polymeric chains of glycosidic units and A a hydrogen atom, so that the products are oligosaccharides or polymeric chains smaller than the starting material. For stepwise degradation, water is again the acceptor substrate, and R is either a mono- or disaccharide residue. It must be noted that in Equation 1, the position of equilibrium is usually directed towards degradation.

In the field of starch enzymology, the \(\alpha\)-amylases function as endo-polysaccharases, \(\beta\)-amylase as an exo-polysaccharase yielding the disaccharide maltose (29) and
amyloglucosidase as an exo-polysaccharase producing only the monosaccharide glucose (30).

The course of hydrolysis of a polysaccharide can be followed by various methods e.g.

(i) change in optical rotation,
(ii) increase in reducing power,
(iii) decrease in viscosity, turbidity, or sedimentation constant of the polysaccharide,
(iv) a loss in iodine staining power of iodophilic polysaccharides,
(v) chromatographic analysis for oligosaccharides.

The random hydrolytic action of salivary α-amylase on the -1,4-linkages in amylose, (the linear component of starch), is shown by a sharp decrease in the viscosity of the substrate with concomitant production of only a few reducing groups (31), whereas the stepwise hydrolytic action of amyloglucosidase on the same substrate, rapidly liberates reducing groups with a slow and regular decrease in viscosity. Thus, use can be conveniently made of methods (ii) and (iii) to distinguish between the two action patterns.

Polysaccharase specificity is best determined by investigating the degradation of oligo- and polysaccharides of known structure before use is made of the enzyme in structural studies on related polysaccharides. The number of oligosaccharides which are known to contain more than one type of linkage is limited. As more become available from improved chemical syntheses and newly-discovered
FIGURE 5
transglycosylase reactions, the specificity of some polysaccharases may subsequently have to be modified.

A few examples of structural analysis of polysaccharides by enzymic methods will now follow:

1. The main repeating glycosidic linkages of linear polysaccharides can be determined by examination of the degradation products after enzymic action. In this way, the identification of laminari-biose, -triose, -tetraose, and pentaose from *Ochromonas malhamensis* after the action of an endo- -glucosidase characterised the main repeating linkage as a \( \beta-1,3 \)-glucosidic linkage (32).

2. The selective hydrolysis of a particular bond in a polysaccharide containing more than one type of linkage, will provide information on the sequence of monosaccharide units and the position of other types of linkage. An example of this was the use, by Araki and Arai, of an agar digesting bacterium *Pseudomonas kyotoensis*, to characterise the linkages in agar (33). Selective hydrolysis of the \( \beta \)-linkages occurred with the production of neoagarobiose (3-0-\( \alpha -(3,6\)-anhydro-\( \mathrm{L}\)-galactopyranosyl)-\( \mathrm{D}\)-galactose); whereas with acid hydrolysis, the \( \alpha \)-linkages were broken to give agarobiose (4-0-\( \beta -(\mathrm{D}\)-galactosyl)-3,6-anhydro-\( \mathrm{L}\)-galactose) (Figure 5). The presence of \( \alpha-1,6 \)-linkages in amylopectin has been confirmed by the isolation of
Phosphorolysis of amylopectin or glycogen.

— Linear chain of $\alpha$-1:4-linked glucose residues.

$\uparrow 1:6$-linkage. A, B, C: Types of unit-chain.

$R$: Free reducing group. ..... Extent of phosphorolysis.

$X$: ca. 6 glucose residues.

FIGURE 6.
isomaltose (34) and 6-α-maltosyl maltotriose (35) from the products of α-amylolysis of waxy maize starch.

3. A particular enzyme may degrade a polysaccharide in such a way as to leave the molecule open to further attack by another enzyme. Thus, the combined action of phosphorylase and amylol 1,6-glucosidase on amylpectin or glycogen (branched α-1,4-glucosans) in the presence of inorganic phosphate will produce glucose 1-phosphate and glucose. Phosphorylase alone will catalyse the end-wise degradation of the exterior chains of branched α-1,4-glucosans, giving glucose 1-phosphate (ca. 40 per cent.) and a limit dextrin. Amylo-1,6-glucosidase has no action on the intact molecule, but will hydrolyse the 1,6-inter-chain linkages exposed in the limit dextrin by phosphorylase action so that the polysaccharide is then susceptible to further phosphorolysis (Figure 6). The amount of glucose liberated is a measure of the number of 1,6-linkages in the original polysaccharide and since there are equal numbers of non-reducing end-groups and inter-chain linkages, this enables the CL to be estimated (36). The results so obtained agree closely with those from chemical analysis of the same samples.

Enzymic degradation of an immunologically specific polysaccharide may result in the destruction of this specificity. Observations by Heidelberger and his coworkers indicated that although a number of glycogens of widely
Equation 2.

\[ G-O-X + \text{Enzyme-H} = \text{Enzyme-G} + H-O-X \]

\[ \text{Enzyme-G} + H-O-R = \text{Enzyme-H} + G-O-R \]

---

Figure 7.
different distribution were precipitated by antipneumococcal horse sera, the reactivity with the antisera disappeared as soon as the corresponding phosphorylase or \( \beta \)-amylase limit dextrins were used. The action of phosphorylase and \( \beta \)-Amylase had therefore removed the immunologically active sights (8c).

In the preceding examples, only the hydrolytic activity of the polysaccharases was considered. However enzymes may, in certain circumstances, possess synthetic activity. Almond emulsin slowly hydrolys the \( \beta \)-glucans, laminarin and yeast glucan (37), but when incubated with concentrated solutions of glucose, \( \beta \)-linked disaccharides are formed (38), so that "enzymic reversion" is possible in concentrated solutions of the \( \beta \)-glucans. Extracts of \textit{Aspergillus niger} also possess synthetic properties (39).

Further artefacts may arise during polysaccharase action in two additional ways. First, some preparations contain transglucosylase impurities. Transglucosylase enzymes catalyse the transfer of a glycosyl unit (G-) from a carbohydrate substrate (G-O-X) to a suitable acceptor compound (H-O-R), usually carbohydrate in nature. The reaction is shown in Equation 2; it is believed that an intermediate glucosyl-enzyme complex is formed (40).

In the example shown opposite (Figure 7), Pan and his coworkers found that \textit{Aspergillus niger} extracts converted
maltose into a mixture of panose (6-α-glucosyl maltose) and glucose (41). The same workers have also determined the transglucosylase activities of various amylase preparations (42). Oligosaccharide formation from cellobiose has also been observed with β-glucosidase preparations from barley (43), marine algae (44), and fungi (45).

Secondly, abnormal "synergic" actions may be observed when a mixture of two purified polysaccharases is used in structural analysis. When R-enzyme and α-amylase were allowed to degrade glycogen, amylopectin and panose, the end-products included substantial amounts of glucose; neither enzyme acting separately liberated glucose from any of the substrates (46). The specificity of one enzyme has therefore been altered by the presence of the other.

Enzymic techniques, therefore, provide a suitable and convenient method for the structural analysis of polysaccharides such as α- and β-glucans. With the advent of improved purification procedures such as column chromatography and electrophoresis, and the selective production of polysaccharases by moulds and bacteria, the number of purified polysaccharase preparations will increase, so that, in the future, still more complex polysaccharides will be analysed.
Scope of the Present Work

The present studies have been concerned with the molecular structure and enzymic degradation of the starch components (amylose and amylopectin), lichenin and related \( \beta \)-glucans, and certain mannose-containing polysaccharides.

Various methods have been applied to the attempted preparation of specific polysaccharidases from heterogeneous enzyme systems, particularly extracts of malted barley, including ammonium sulphate fractionation, selective inhibition, electrophoresis, and adsorption chromatography.

A comparative study has been made of the degradation of the \( \alpha \)-glucosans, amylose, amylopectin and glycogen, by preparations of \( Z \)-enzyme and \( \alpha \)-amylase.

A number of potential sources of mannanase activity have been examined and the action of preparations from malted barley on oligosaccharides and polysaccharides containing mannosidic linkages has been studied.

The reserve polysaccharide of \textit{Peranema} has been isolated and characterised as a \( \beta \)-glucan.
FIGURE 8.

CONCENTRATION of GLUCOSE (µg)

A.V. at 490 nm
Section II.

GENERAL METHODS.

The following general methods have been used throughout this work.

1. **Estimation of Reducing Sugars**

   (a) Reducing sugars were estimated with the Somogyi (1952) reagent (47) calibrated against glucose, mannose, cellobiose, and maltose. The concentration of the sugar solutions was determined polarimetrically. It was found that the ratio, mg. sugar : titre difference from blank (in mg. 0.01N sodium thiosulphate), was $0.285 \pm 0.01$ for the monosaccharides and $0.530 \pm 0.01$ for the disaccharides for different preparations of the reagent. The reagent was stored at 37°.

   (b) The colorimetric method of Smith and coworkers (48) was used for the estimation of semi-micro amounts of sugar. 5% Phenol and "Analar" sulphuric acid were suitable reagents for the calibration of glucose and gave reproducible results (Figure 8). From the DP. of an oligosaccharide, the observed glucose content could be converted to an estimated amount of oligosaccharide, so that further calibrations were unnecessary. The correction amounts to:

   $$\text{Corrected weight (mg.)} = \text{Glucose found (mg.)} \times (0.9 + \frac{0.1}{\text{DP.}})$$
(c) Glucose was estimated in the presence of cellobiose with the Philips and Caldwell reagent (49). Half the recommended amount of potassium iodate was used in the preparation of the potassium iodide-iodate solution since the blank titre would have otherwise been inconveniently high. Calibration curves were prepared for varying amounts of glucose in the presence of fixed amounts of cellobiose.

2. **Deproteinisation**

(a) Enzyme digests containing large amounts of protein were deproteinised prior to estimation of reducing sugars by the addition of zinc sulphate hexahydrate (5%; 0.5-1.0 ml.) followed by an equivalent amount of barium hydroxide solution (ca. 0.3N). These solutions were adjusted so that 4.7 - 4.8 ml. baryta gave a faintly alkaline solution with 5 ml. of the zinc sulphate solution. The resultant precipitate was removed by centrifugation and the sugar content of the supernatant solution was estimated (50).

(b) Small amounts of protein were removed by heating the solution in a water-bath at 100° for 5 - 10 minutes. The coagulated protein was then removed by centrifugation.

3. **Acid Hydrolysis of Carbohydrates**

(a) **Complete Acid Hydrolysis**

Concentrations of di-oligo-, and polysaccharides were determined by complete acid hydrolysis and estimation
of the monosaccharide content. The carbohydrates used in the present work were completely hydrolysed by heating at 100° for 2 - 2.5 hr. with 2N sulphuric acid. Control experiments showed that neither glucose nor mannose were destroyed under these conditions. Acid hydrolysates were neutralised to phenolphthalein with dilute sodium hydroxide solution and then made slightly alkaline before estimating the reducing sugar content with the Somogyi reagent. The original amount of di-or polysaccharide (mg.) was equivalent to 0.95 x glucose found (mg.) or 0.90 x glucose found (mg.), respectively.

(b) Partial Acid Hydrolysis

This was effected by heating the carbohydrate at 100° for 1 hr. with 0.33N sulphuric acid. Acid hydrolysates were examined by paper chromatography after neutralisation with barium carbonate or barium hydroxide followed by centrifugation, deionisation with Amberlite 1 R - 4 B (OH) and 120 (H) or with Biodeminrolit (CO₃) (51) ion-exchange resins, and concentration in vacuo at 40 - 50°.

4. Paper Chromatographic Methods

(a) Qualitative

Descending paper chromatograms were prepared at room temperature with Whatman No. 1, 4, or 3 MM paper. Ascending chromatograms were prepared with Whatman 3MM paper.
Solvents and Sprays

Solvent 1. Ethyl acetate-pyridine-water (10:4:3 v/v)
2. n-Butanol-pyridine-water (6:4:3 v/v)
3. Ethyl acetate-acetic acid-formic acid-water (18:3:1:4 v/v)

The Rg values (i.e. Rf relative to D-glucose) of the various carbohydrates in these solvents, is recorded in the text.

Spray reagent 1. Aniline oxalate (52).
After spraying the dried paper with a saturated solution of aniline oxalate in aqueous ethanol, the spots were developed by heating in an oven (100° – 180°) for a few minutes.

Spray reagent 2. Alkaline silver nitrate (53).
The dried paper was dipped in a solution of silver nitrate in acetone (1 ml. saturated silver nitrate solution in 200 ml. acetone) and, after drying, sprayed with a solution containing equivalent amounts of 1N sodium hydroxide solution and methylated spirits. Background colour was removed by washing the paper in a saturated solution of sodium thiosulphate and then water.

(b) Quantitative

In the small scale estimation of a mixture of carbohydrates, known volumes of the mixture were applied with a micropipette to the starting line of a paper chromatogram (Whatman No. 1). Guide spots were also prepared. The chromatogram was developed with solvent 1. or 2. for a
suitable time and then air dried at room temperature. The guide strips were cut off and sprayed with spray reagents 1. or 2., so that sections of the unsprayed strip could be cut corresponding to the carbohydrate spots on the guide strip. Carbohydrate material was eluted with 20 ml. distilled water and the carbohydrate content of the filtered supernatant solution estimated by method 16.

(c) **Preparative**

Larger amounts of a carbohydrate mixture were separated by applying ca. 100 mg. to sheets of Whatman No. 3 MM paper and effecting the separation by descending flow (solvent 1.) in the machine direction of the paper. Guide strips were removed from the dried chromatograms and the positions of the carbohydrates determined. The sections were then cut out and the carbohydrate material eluted by pulping with a large volume of water. After filtration, the solutions were concentrated at 40 - 50° in a rotary evaporator and further purified with Biodeminrolit (CO$_3$) ion-exchange resin.

5. **Electrophoretic Separation of Sugars**

Separation of oligosaccharides was effected in an apparatus similar to that described by Foster (54) at 700 volts and 10 mA for 4 hours. The oligosaccharides were spotted on Whatman No. 1 paper as for normal descending chromatography,
**Figure 9.**

A.V. at 570 m\(\mu\)

Concentration of Formaldehyde (\(\mu\)g.)

**Figure 10.**

A.V. of Periodate (0.015M)

A.V. of Iodate (0.015M)

Periodate Reduced (m. Moles)
and prior to insertion in the apparatus, the paper was saturated with borate buffer (0.1M; pH 8.5) by spraying. The ionophoretograms were developed by spraying the dried paper with a solution of saturated aniline oxalate containing glacial acetic acid and thereafter heating at ca. 140° for a few minutes.

6. **Drying of Carbohydrate Samples**

Prior to estimation of purity, samples were dried in vacuo over phosphorus pentoxide, at 60° for several hours.

7. **Periodate Oxidation**

(a) **Determination of Formaldehyde**

An adaption of the method of Frisell, Meech, and Mackenzie (55) was used.

The aqueous carbohydrate solution (5 ml.; 2-5 mg.) was treated with sodium meta-periodate (3 ml.; 0.3M) and 0.2M- acetate buffer (10 ml; pH 3.6); distilled water was then added to a total volume of 25 ml. The reactions were carried out at room temperature and were usually complete in 30 min. A sample (0.1 - 0.5 ml.; 2 - 20 µg. formaldehyde) was treated with sodium sulphite solution (0.5 ml.; 26.5% w/v followed by chromotropic acid (10 ml.; 0.5 g. sodium salt dissolved in 50 ml. distilled water and 200 ml. 12.5M sulphuric acid). This was heated for 30 min. at 100° and the red colour of the reagents minimised by the addition of thiourea (2 ml.; 4.6% w/v) to the cooled solution. The colour was compared with a blank
in a Unicam SP 600 Spectrophotometer at 570 m\(\mu\). The blank was prepared by mixing together sodium meta-periodate (3 ml.; 0.3 M), 0.2 M-acetate buffer (10 ml.; pH 3.6), sodium sulphite (25 ml.; 26.5% w/v) followed by carbohydrate solution (5 ml.). 1 ml. of this solution was then treated with chronotropic acid (10 ml.) as above.

Calibration of the reagent was carried out using pure dry mannitol (0.5 mg./ml.), which yields two molar proportions of formaldehyde (Figure 9).

(b) **Determination of Formic Acid**

The formic acid released during sodium meta-periodate oxidation of carbohydrates was determined by titrating a sample with ca. 0.005N standard sodium hydroxide (carbonate-free) to an end-point at pH 5.8 (Pye Universal pH meter) after the destruction of excess periodate with ethylene glycol. The absorption of atmospheric carbon dioxide was prevented by bubbling nitrogen through the solution during titration.

(c) **Determination of Periodate Reduction**

This was followed spectrophotometrically by measuring the decrease in light absorption due to the periodate ion at the maximum of 223 m\(\mu\) and correcting for the light absorption of the iodate ion produced (56). Carbohydrate (10-30 mg.) was added to 0.015M sodium metaperiodate solution (10 ml.) and incubated in the dark at 30\(^\circ\). Aliquots were withdrawn and diluted 250 times. The optical densities of the resulting
solutions were measured on a Unicam SP 500 Spectrophotometer at 223 m\text{m}, and compared with those of the original solution of periodate (diluted 250 times) and of an equimolecular iodate solution (Figure 10).

8. Determination of DP. of Oligosaccharides

(a) Method of Acid Hydrolysis

The DP. of an oligosaccharide was obtained by measuring (i) the monosaccharide concentration liberated by complete acid hydrolysis, and (ii) the disaccharide equivalent, using equal amounts of the oligosaccharide solution. The DP. of an oligosaccharide with a $\beta$-1,4-glucosidic linkage at the reducing end would be:

$$\text{DP.} = \frac{\text{Wt. of glucose by acid hydrolysis (mg.)}}{\text{celllobiose equivalent (mg.)}} \times 180$$

(b) Periodate Oxidation Method

In this method, the formaldehyde released by periodate oxidation of the reduced oligosaccharide was measured (57). Oligosaccharide (2-5 mg.) and sodium borohydride (10 mg.) were reacted together in aqueous solution (5 ml.) for 2 - 2$^\frac{1}{4}$ hr. The sodium borohydride was then destroyed with excess 2N sulphuric acid. This solution was oxidised with sodium metaperiodate as in 7a above, and the formaldehyde released was then determined. Under the conditions used, two molar proportions of formaldehyde would be released by every molecule of oligosaccharide, so that

$$\text{DP.} = \text{Weight of oligosaccharide producing 2 moles formaldehyde}$$
9. **Iodine Staining**

When solutions of amylose, amylpectin and glycogen are stained with iodine, characteristic colours are produced by the polysaccharide-iodine complex. Unless otherwise stated, the following conditions were usually applied: polysaccharide solution (1-3 ml.) was introduced into a 25 ml. standard flask containing one drop 3N hydrochloric acid and iodine solution (1 ml.; 0.2% iodine in 2% potassium iodide) and the mixture diluted to 25 ml. The absorption value (A.V.) of this solution was examined in a Unicam SP 600 Spectrophotometer against an iodine-water blank in 1 cm. silica glass cells, at the wavelength of maximum absorption (\( \lambda_{\text{max}} \)) for the polysaccharide. The max. for amylose, amylpectin, and glycogen was usually 680, 540, and 420 m\( \mu \), respectively.

10. **Viscosity Determination**

The specific viscosity, \( \eta_{\text{sp}} \), of a dilute polymer solution is given by:

\[
\eta_{\text{sp}} = \frac{T - T_0}{T_0} = \frac{T}{T_0} - 1
\]

where \( T \) is the solution flow time and \( T_0 \) is the solvent flow time, in seconds. Since \( \eta_{\text{sp}} \) is concentration dependant, the ratio \( \eta_{\text{sp}}/C \) is termed the viscosity number, and by extrapolation of the viscosity number to infinite dilution, the limiting viscosity number or intrinsic viscosity \( [\eta] \) is obtained

\[
[\eta] = \lim_{C \to 0} \frac{\eta_{\text{sp}}}{C}
\]

where \( C = \) concentration in g./ml.
For the determination of \([\eta]\), a modified Ubbelohde viscometer was used. Measurements were carried out at constant temperature. All solutions were filtered through sintered glass (G 4) before analysis. The method involved placing the solvent (10 ml.) in the viscometer, determining the flow time, and then adding portions (5 ml.) of the polysaccharide solution and again measuring the flow times. Polysaccharide concentrations were always measured by method 3a.

Enzymic activity involving random hydrolysis may be determined by measuring the fall in viscosity of an enzyme-polysaccharide digest after incubation for various times:

\[
\text{Activity} = C^2 \frac{d}{dt} \frac{1}{\eta_{sp}}
\]

where \(t = \text{time of incubation,}
\text{and } c = \text{concentration in g./ml.}

Digests were prepared in Ostwald or modified Ubbelohde viscometers, and the viscosity at 25\(^\circ\) was measured at intervals. All solutions were filtered through sintered glass and the concentration of polysaccharide determined as previously.

11. **Estimation of Protein Nitrogen**

(a) **Kjeldahl Method**

The semi-micro method of Chibnall, Rees, and Williams (59) was used to determine the \(\alpha\)-amino \(N\) content of enzymic preparations. The solid sample was boiled overnight with
Figure II.

Estimation of Protein Nitrogen by Biuret Method

Protein Nitrogen (mg.)

A.V. at 550 m\(\mu\)
concentrated sulphuric acid (2 ml. "Analar", N-free grade) containing a catalyst (0.2g.; a mixture of anhydrous sodium sulphate, copper sulphate pentahydrate, and sodium selenate (N-free grade) in the ratio 80:20:1 w/w). The solution was transferred to a Pregl micro-distillation apparatus and 10N potassium hydroxide (10 ml.) run in; the liberated ammonia was reacted with standard hydrochloric acid (ca. 0.03N) and the excess determined by back-titration using Taschiro's indicator (60).

(b) Biuret Method

The method described by Robinson and Hogden (61) was as follows:

Aliquots were introduced into 10 ml. centrifuge tubes and the volume made up to ca. 9 ml. with 3% sodium hydroxide; copper sulphate pentahydrate (0.25 ml.; 20%) was then added and the volume adjusted to 10 ml. with the sodium hydroxide. A reagent blank was also prepared. The tubes were shaken for 1 min., and a further 15 min. allowed to elapse before centrifuging. A.V's of the supernatant solutions were compared with the reagent blank in a Unicam SP 600 Spectrophotometer at 550 μμ.

A commercial preparation of Lysozyme (13.2% N by Kjeldahl method) was used to calibrate the reagents (Figure 11); the calibration was checked by determining the N-content
of glycylglycine (found 10.5%; theoretical 10.6%).

In estimations of the protein N content of enzyme preparations, an equivalent volume of trichloroacetic acid (10%) was added to the solution of enzyme in 0.2M-acetate buffer of pH 5.6. The precipitate was analysed as above; the supernatant solution was discarded.

12. **Chromatographic Separation of Enzyme Preparations**

The enzyme preparation (100-300 mg.) was dissolved in McIlvaine buffer (3 ml.; pH 4.0) and adsorbed on to a column (2 x 20 cm.) of Brockmann alumina which had been previously equilibrated with the same buffer. The protein was eluted from the column with buffer of increasing pH; an exponential pH gradient (62) was effected by addition of 0.2M-disodium hydrogen phosphate solution to 0.2M-McIlvaines buffer of pH 4.0 contained in a mixing chamber; the addition of the phosphate solution was controlled by the rate of flow of liquid from the column. Fractions of the eluate (5 ml.) were collected automatically in a Towers Automatic Fraction Collector, designed to operate a siphon-relay circuit, and were stored at 0°C. Spectrophotometric examination (Unicam SP 500 Spectrophotometer) at 280 mμ (63) gave a measure of the protein concentration of these fractions.

13. **Continuous Electrophoresis of Enzyme Preparations**

This was carried out in a Shandon apparatus similar to
that described by Durrum (64).

It was found that Whatman No. 54 filter paper sheets (46 x 57 cm.; serrated edge) and 0.05M-phosphate buffer of pH 6.9 were preferred to the 3MM grade and 0.05M-veronal buffer of pH 8.6, in the separation of the enzyme preparations. A potential of ca. 900 volts was selected and excessive evaporation of the buffer, due to the heat developed in the paper, was prevented by operating at 0°. The liquid in the buffer trough was kept at a constant level to give an even flow of buffer through the paper curtain. Strips of 3MM filter paper were attached to the sides of the curtain to enhance the separation.

Method of operation: The paper curtain was inserted in the apparatus and the buffer allowed to saturate the whole area of the paper and wash it free of contamination. The proposed working potential was applied during this period. After equilibration had been established, a concentrated solution of the sample was applied to the centre of the curtain by permitting the narrow filter paper wick of the specimen container to make contact with the curtain. The eluate was collected in tubes, and stored at 0°.

Distribution of the fractions was determined by heating the curtain at 100° for 10 min., and staining the denatured protein with nigrosine solution (1% in 2% acetic acid), washing with water to remove excess reagent, and drying (65).
14. **β-Amylolysis**

A commercial sample of barley β-amylase (analytical grade; Wallerstein Company, New York) was used to determine the β-amylolysis limits of amylase, amylopectin, and glycogen. The activity of the preparation was 110 units/mg. determined by the method of Hobson, Whelan, and Peat (66) in which one unit of activity of β-amylase was defined as the weight of enzyme which liberated 1 mg. of maltose from the following digest after 30 min. incubation at 37°:

Substrate (25 ml.; 0.6% soluble starch in water) and 0.2M-acetate buffer (3 ml.; pH 4.6) were preheated to 35° and incubated with the enzyme solution (2 ml.). Aliquots (3 ml.) were withdrawn after 30 min., and the reducing power determined as maltose equivalents.

This preparation was known to contain a trace of Z-enzyme (67), so that digests were prepared with 0.2M-acetate buffer of pH 3.6 (amylose; 100 units/mg.) or 4.6 (amylopectin and glycogen; 25 and 50 units/mg., respectively); Z-enzyme is inhibited at pH 3.6 and in low concentration has little effect on amylopectin and glycogen. Samples were removed after 24 and 48 hr. incubation at 37° for estimation of maltose produced, the β-amylolysis limit being the percentage conversion into maltose. Polysaccharide concentration was found by method 3a.

15. **α-Amylolysis**
(a) The activity of the α-amylase preparations was determined by the method of Fischer and Stein (68) with the exception that water was used instead of the sodium chloride solution:

Substrate (25 ml.; 1% soluble starch was incubated with 0.2M-acetate buffer (3 ml.; pH 5.6) and enzyme solution (2 ml.) at 37° for 30 min. Aliquots (3 ml.) were examined for reducing power. One unit was defined as the amount of enzyme which liberated 1 mg. of maltose in 3 min.

(b) The method of Smith and Roe (69) was also used for α-amylase activity tests:

Substrate (2 ml; 0.3% soluble starch solution in 0.2M-acetate buffer pH 5.6, 0.005M with respect to sodium chloride) was incubated with enzyme (0.1 ml.). The solution was then washed into a 250 ml. standard flask containing water (ca. 100 ml.), 1.0N hydrochloric acid (3 ml.) and iodine solution (1 ml; 0.18% in 1.8% potassium iodide). A reagent blank was also prepared. The A.V's were compared at 620 μm with an iodine-water blank. The amylase activity was calculated from the expression:

\[ \text{Amylase units/100 ml.} = \frac{\text{A.V. reagent} - \text{A.V. digest}}{\text{A.V. reagent}} \times 600 \]

Toluene was used as an antiseptic in all enzyme digests.
SECTION III.

Studies on the Enzymic Degradation of Starch-Type Polysaccharides.

Part I.

A Review of the Structure and Enzymic Degradation of Starch-Type Polysaccharides.

Structure of Starch.

Starches are found in the higher plants as discrete granules which vary in size and shape according to the source (70). As previously indicated, natural starches consist of two discrete macromolecular components, amylose and amylopectin which can be conveniently separated by dispersing the starch in water and selectively precipitating the amylose as an insoluble crystalline complex by the addition of n-butanol or thymol; the amylopectin component is then recovered from the supernatant solution.

(a) AMYLOSE

This accounts for 20 - 30% of most starches and is generally considered to be a linear molecule of 1,4-linked glucose residues with a DP. ranging from ca. 100 to 6,000. As a result of periodate oxidation (71), enzymic degradation (72, 73, 74) and other analytical studies, it has been suggested that amylose may contain a small proportion
of structural anomalies or a low degree of branching (75). Amylose is characterised by giving an intense blue colour with iodine (λ max 680 μm).

(b) **AMYLOPECTIN**

Amylopectin constitutes the major portion of most starches and is virtually the sole component of waxy cereal starches, (e.g. waxy maize, waxy sorghum). It is a high molecular weight polymer, in which short chains of the amylose-type are inter-linked to form a highly branched structure, the CL. being ca. 24 glucose residues. The presence of inter-chain linkages of the 1,6-type has been suggested by the isolation of 2,3-di-O-methyl glucose (4%) from hydrolysates of methylated waxy maize starch (76), and the absence of significant amounts of glucose in acid hydrolysates of periodate-oxidised acorn amylopectin (77). As previously stated, evidence for assigning the α-configuration to the inter-chain linkage has been obtained from partial acid hydrolysis (p.4) and enzymic studies (p.9) on amylopectin. With iodine, amylopectin will give a purple-brown colour (λ max. 540 μm).

The isolation of small amounts of nigerose 3-O-α-glucosyl glucose) from partial acid hydrolysates (78), and the presence of glucose in the hydrolysates of the polyalcohol from periodate-oxidised amylopectin (79), may indicate the
presence of some 1,3-linkages. The nigerose could arise from "acid reversion" of the hydrolysis products (80), and the glucose from under-oxidation of the polysaccharide, respectively, and not from 1,3-(or 1,2-) linkages. The presence of phosphate ester groups (81), and fructose (31) in amylopectin may represent other structural anomalies.

Structure of Glycogen

The reserve polysaccharide of animals, glycogen (82), is stored principally in the liver and muscle; related polysaccharides are also found in sweet corn (83) and yeast (84). Glycogen, unlike starch, is soluble in water, and its solution gives a red-brown colour with iodine (λ max 420 - 490 μ). This polysaccharide is structurally similar to amylopectin and consists of short chains of α-1,4-linked glucose residues which are linked together to give a more highly branched structure with a CL. of ca. 12. Periodate oxidation results indicate that these inter-chain linkages are mainly of the 1,6-type (85); their α-configuration has been proved by the isolation of isomaltose (19a), panose and isomaltotriose (20a), the last indicating that some of the α-1,6-linkages may be adjacent. Various workers have suggested the presence of anomalies in the glycogen molecule such as α-1,3-glucosidic linkages (20a), periodate-resistant units (25), phosphate ester groups, and that fructose may be a minor constituent (86).
(a) 'Laminated' structure

(b) 'Comb' structure

(c) 'Tree' structure

**Key**

- Linear chain of α-1:4-linked glucose residues
- Inter-chain linkage
- A, B, C Types of chain
- R Free reducing group

**Figure 12**
Multiple-Branching in AMYLOPECTIN and GLYCOGEN.

Three types of structure have been postulated to explain the chemical and physical properties of these highly branched polysaccharides, viz. a "laminated" structure (87), a "comb" structure (88), and a multiply branched "tree" structure (89) (Figure 12). The last structure is now accepted as the correct form; it consists of three types of unit-chain composed of $\alpha$-1,4-linked glucose residues; A-chain, linked to the molecule only by a 1,6-linkage; B-chain, to which one or more A-chains are attached and which is attached to an adjacent chain by a 1,6-linkage; C-chain, other chains are attached to this chain which possesses the only reducing group (90). Interior chains lie between 1,6-branch points, and chains between branch points and the non-reducing ends are termed exterior chains. The average length of interior and exterior chains can be calculated from the CL. and $\beta$-amylolysis limit (see p. 27), e.g. exterior chain length (ECL.) = no. of glucose residues removed by $\beta$-amylase + 2.5, and interior length (ICL.) = CL. - ECL. - 1. It is found that the ICL. of amylopectin is approximately twice that of glycogen (see Table 1).

Enzymic degradation studies have provided evidence of multiple-branching in amylopectin and glycogen. Larner and his coworkers (91) observed that the action of phosphorylase followed by amylo-1,6-glucosidase caused the removal of
**TABLE I.**

<table>
<thead>
<tr>
<th></th>
<th>Amylose</th>
<th>Amylopectin</th>
<th>Glycogen</th>
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</thead>
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<td>Iodine colouration</td>
<td>Intense blue</td>
<td>Purple-brown</td>
<td>Red-brown</td>
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<td>$[\alpha]_D, H_2O$</td>
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<td>+212°</td>
<td>+198°</td>
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<td>$\lambda_{max}$ of absorption spectra (m$\mu$)</td>
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<td>Average chain length (glucose residues)</td>
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<td>10 - 14</td>
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<td>$\beta$-Amylolysis limit (%)</td>
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<td>55 ± 5</td>
<td>45 ± 5</td>
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<td>$\alpha$-Amylolysis limit (%)</td>
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<td>ca. 90</td>
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<tr>
<td>Interior chain length (glucose residues)</td>
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<td>ca. $10^7$</td>
<td>ca. $10^7$</td>
</tr>
</tbody>
</table>

* aqueous NaCl

Data from ref. 82, 93.
successive tiers of branch points (cf. p. 10). In the studies of Peat and his coworkers, $\beta$-amylase limit-dextrin of waxy maize starch was treated with R-enzyme, which hydrolyses the outermost 1,6-linkages in amylopectin but not in glycogen (92). The yield of maltose and maltotriose from the outer A-chain stubs was 12.8%. A "tree"-type molecule containing an equal number of A- and B-chains would have yielded 10.4%, whereas, "comb" or "laminated" structures would have given 20.8% or less than 0.1% maltosaccharides, respectively. Additional evidence for multiple-branching was the isolation of an oligosaccharide containing two branch points from the products of salivary $\alpha$-amylase action of amylopectin and glycogen (31).

Additional evidence for multiple-branching was the isolation of an oligosaccharide containing two branch points from the products of salivary $\alpha$-amylase action of amylopectin and glycogen (31).

A few of the general properties of amylase, amylopectin and glycogen are summarised in Table 1.

**Enzymic Degradation of $\alpha$-1,4-Glucosans.**

(a) $\beta$-Amylase

This enzyme, which occurs only in the higher plants e.g. soya bean, barley, wheat, catalyses the stepwise hydrolysis of alternate linkages in a chain of $\alpha$-1,4-linked glucose residues from the non-reducing end, with the liberation of $\beta$-maltose (29). $\beta$-Amylolysis is arrested on encountering a substituted glucose unit; thus action on amylopectin and glycogen ceases at 2-3 glucose units from the 1,6-linkage,
and will therefore yield maltose and a high-molecular weight limit dextrin with exterior chain stubs which are 2-3 glucose units in length (90a). Amylose containing no anomalous linkages will be completely degraded by β-amylase.

(b) **α-Amylase.**

A wide variety of animals, higher plants, moulds and bacteria are known to produce this enzyme; in many cases the enzyme has been highly purified and crystallised. α-Amylase catalyses the random hydrolysis of α-1,4-glucosidic linkages to yield α-dextrins (dextrinisation), which may be further broken down to α-maltose and maltotriose, (saccharification) (29). The end-products of α-amylase action on amylopectin and glycogen are maltose, maltotriose, and branched α-dextrins, since α-amylase can by-pass the α-1,6-linkages but cannot hydrolyse them. Amylose is completely degraded to maltose and maltotriose by α-amylase (31). The mode of action of different α-amylases will be discussed later.

(c) **Glucose-producing Amylases**

These enzymes hydrolyse terminal linkages at the non-reducing end of chains containing α-1,4-linked glucose residues. Glucamylase from *Rhizopus delemar* is such an enzyme (94); it also has the ability to by-pass α-1,6-linkages and to attack interior chains in branched α-glucosans.
Other glucose-producing amylases are amylglucosidase from
Aspergillus niger (95, 30), and the maltase from Clostridium
acetobutyricum (96); both enzymes can also hydrolyse termin-
al α-1,6-linkages.

(d) Debranching Enzymes

Isoamylase and R-enzyme catalyse the hydrolysis of
non-terminal 1,6-linkages in glycogen (97) and amylpectin
(98, 92), respectively, producing more-linear polysaccharides
of lower molecular weight. This is shown by the increase
in the β-amylolysis limits of the polysaccharides after pre-
treatment with the debranching enzymes.

Terminal α-1,6-linkages are hydrolysed specifically
by amylo-1,6-glucosidase obtained from rabbit muscle (36a);
it has no action on amylpectin or glycogen but can attack
their phosphorylase limit-dextrins by hydrolysing the
1,6-linkages attaching the remaining glucose residues of
the A-chains to the rest of the molecule.

In this section, a study has been made of the
enzymic degradation of α-glucosans (amylose, amylpectin
and glycogen) by various preparations containing α-amylase
(Z-enzyme).

Separation and purification of the Z-enzyme has
been attempted by salt fractionation; electrophoresis
and selective inhibition. The effect of various compounds
on the inhibition and activation of the enzyme has been studied.
PART 2.

The Action of Barley $\beta$-Amylase and Z-enzyme on Starch-Type Polysaccharides

Introduction.

$\beta$-Amylase was generally supposed to hydrolyse amylose completely to maltose; however, Peat and his coworkers observed that the action of crystalline sweet potato $\beta$-amylase, and highly purified soya-bean $\beta$-amylase on amylose at pH 4.8 ceased abruptly at ca. 70% conversion into maltose, a limit-dextrin of high blue-value (B.V) remaining (72). This incomplete hydrolysis of amylose, formerly attributed to the physical process of "ageing" (99), was more adequately explained by the presence of some structural anomalies in the polysaccharide. Since unpurified soya-bean $\beta$-amylase preparations caused complete hydrolysis of amylose, the presence of a second enzyme in soya-beans, designated Z-enzyme, was suggested.

Phosphorylase, like $\beta$-amylase, catalyses a step-wise degradation of starch polysaccharides, but attacks successive terminal $\alpha$-1, 4-glucosidic linkages giving glucose 1-phosphate. Phosphorolysis of amylose should therefore terminate at a limit similar to that of $\beta$-amylolysis.
This was indicated by the results of Hestrin (100) and Peat et al (74), using crystalline muscle phosphorylase and amorphous potato phosphorylase, respectively. It was therefore suggested (74) that amylose contained a small number of anomalous linkages which prevented both complete $\beta$-amylolysis and phosphorolysis. Z-Enzyme was shown to be neither a phosphatase nor an $\alpha$-amylase, and since emulsin (a mixture of carbohydrases, including $\beta$-glucosidases) could simulate Z-enzyme in effecting complete $\beta$-amylolysis of amylose, it was concluded that Z-enzyme was a $\beta$-glucosidase. The anomalous linkages could be $\beta$-glucosidic linkages joining single glucose residues to the main amylose chains.

Evidence of an arrest point for the $\beta$-amylolysis of natural amylose was also given by Hopkins and Bird (101) who suggested that the Z-enzyme action was explainable by a trace amount of $\alpha$-amylase in the $\beta$-amylase preparation. They also proved that active $\beta$-glucosidases in emulsin did not liberate glucose from amylose $\beta$-limit-dextrin so that Z-enzyme was distinct from $\beta$-glucosidase. In their comments on this communication, Peat and Whelan (102) emphasized that they could distinguish Z-enzyme from $\alpha$-amylase by pretreating an amylose with Z-enzyme or weak salivary $\alpha$-amylase before $\beta$-amylase action. The $\beta$-amylolysis limits were 98% and 89% for Z-treated and the $\alpha$-treated amylose, respectively.
The Z-enzyme appeared, therefore, to have exerted a true "debranching" action, and allowed complete \( \beta \)-amyloysis; it had not fragmented the molecule in random fashion to leave intact the barriers to \( \beta \)-amyloysis.

The absence of \( \beta \)-glucosidase-labile linkages in amylose was further proved by Neufeld and Hassid (103) who were able to separate Z-enzyme from the \( \beta \)-glucosidase, and laminarinase activities in almond emulsin, and by Baba and Kojima (104) who purified the \( \beta \)-glucosidase of apricot emulsin and eliminated \( \alpha \)-amylase. This purified preparation was ineffective in hydrolysing amylose \( \beta \)-limit-dextrin.

During studies carried out in this Department on the \( \beta \)-amyloysis of amylpectin (105), a relationship was found between the enzyme concentration of a \( \beta \)-amylase preparation known to contain Z-enzyme (67), and the \( \beta \)-amyloysis limit. Since this is characteristic of \( \alpha \)-amylases (106), it was possible that Z-enzyme might be a trace of \( \alpha \)-amylase, in spite of the observations of Peat and Whelan. This possibility has now been examined in detail.

**METHODS AND MATERIALS.**

(a) **Analytical Methods**

The general methods used were those described in Section 2. For viscometry, digests were prepared in an Ostwald or modified Ubbelohde viscometer, and the viscosity
at 25° was measured at intervals. Since the activity of an α-amylase is related to \( \frac{1}{\eta \sp{sp}} \) / dt, graphs of t against \( 1/\eta \sp{sp} \) were prepared (see p. 23). With identical substrate and enzyme concentrations, the effect of added reagents on the activity of the enzyme could be observed by comparison of the slopes produced. All solutions were filtered through sintered glass before analysis.

(b) **Enzyme Preparation**

Wallerstein barley β-amylase (Analytical grade) was used as a source of β-amylase; Z-enzyme was shown in earlier studies by Dr. I. D. Fleming, to be also present (67).

(c) **Substrates**

A number of starch-type polysaccharides were used in this work, several of which were prepared by previous workers.

(i) **Amylose**

Samples of amylose, prepared by Dr. A. Wright by the fractionation of potato starch with thymol and n-butanol, were used, together with amylose VIII prepared by Dr. K. Maung. Amylose β-dextrin was obtained by incubating amylose VIII (500 mg) with barley β-amylase (100 units/mg.) at pH 3.6 to a constant β-amylolysis limit of 72%. The digest was heated for 5 min. in a boiling-water-bath, cooled, filtered (G4 sinter), and the pH adjusted to 5.6 with aqueous sodium hydroxide. This solution was stored under toluene
at room temperature; the maltose present did not interfere with subsequent measurements.

(ii) Amylopectin

A commercial specimen of waxy maize starch (sample IV) was used. Amylopectin $\beta$-dextrin was prepared by Dr. I.D. Fleming, and corresponded to 47% of the original waxy maize starch IV.

(iii) Glycogen

Samples of glycogen from rabbit muscle (RM.III) and a case of human-liver storage-disease (SD.) were provided by Dr. A. Wright. Glycogen $\beta$-dextrin was isolated from a digest of *Acaris lumbricoides* glycogen and barley $\beta$-amylase by Dr. A.M. Liddle.

Results

(a) Selective Inhibition of $\beta$-Amylase in the Barley $\beta$-Amylase Preparation.

1. Effect of inhibitors on $\beta$-amylase activity

Since the Wallerstein preparation contained both $\beta$-amylase and Z-enzyme, attempts were made to selectively inhibit the former activity. It is known that free sulphhydryl groups are essential for $\beta$-amylase activity (107), and the effect of various thiol-reactants was examined:
A number of mercury compounds and related thiol-reactants were preincubated with the β-amylase solution for 15 min. at 20° prior to determination of the β-amylase activities by the method of Hobson et al. (see p. 27).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (M)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mercuric chloride</td>
<td>$1.5 \times 10^{-5}$</td>
<td>100</td>
</tr>
<tr>
<td>Phenylmercuric acetate</td>
<td>$10^{-5}$</td>
<td>100</td>
</tr>
<tr>
<td>p-Chloromercuribenzoate</td>
<td>$10^{-5}$</td>
<td>100</td>
</tr>
<tr>
<td>Silver nitrate</td>
<td>$10^{-5}$</td>
<td>100</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>$10^{-2}$</td>
<td>100</td>
</tr>
<tr>
<td>Potassium permanganate</td>
<td>$10^{-2}$</td>
<td>100</td>
</tr>
</tbody>
</table>

2. **Effect of inhibitors on α-amylase activity**

Since there is evidence that thiol groups are not essential for the activity of α-amylases (108), the effect of the above inhibitors on the α-amylase activity of the Wallerstein preparation was then examined, using amylose β-dextrin as substrate, and iodine-staining as the method of analysis.

Digests containing amylose β-dextrin solution (0.46 mg./ml. by acid hydrolysis; 14 ml.) and β-amylase preparation (14 mg. in 0.5 ml. inhibitor solution) were
incubated at 35°. Samples (4 ml.) were withdrawn after 2.5 and 4.5 hr. and stained with iodine solution (0.2% in 2% potassium iodide solution; 1 ml.) and diluted with water to 25 ml. A.V.'s were measured at 560, 580, 600, 640 μμ, and the fall in A.V. calculated after correction for the appropriate blanks. The following results were obtained after 4.5 hr. incubation, and the inhibition (%) is based on the results at 600 μμ.

**TABLE III.**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (M)</th>
<th>560</th>
<th>580</th>
<th>600</th>
<th>640</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>61</td>
<td>64</td>
<td>67</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>Mercuric chloride</td>
<td>1.5 x 10⁻⁵</td>
<td>9</td>
<td>12</td>
<td>13</td>
<td>13</td>
<td>80</td>
</tr>
<tr>
<td>Phenylmercuric acetate</td>
<td>10⁻⁴</td>
<td>13</td>
<td>12</td>
<td>14</td>
<td>18</td>
<td>90</td>
</tr>
<tr>
<td>Phenylmercuric acetate</td>
<td>10⁻⁵</td>
<td>11</td>
<td>16</td>
<td>18</td>
<td>17</td>
<td>73</td>
</tr>
<tr>
<td>p-Chloromercuroibenzoate</td>
<td>10⁻⁵</td>
<td>56</td>
<td>59</td>
<td>62</td>
<td>65</td>
<td>7</td>
</tr>
<tr>
<td>Silver nitrate</td>
<td>10⁻⁴</td>
<td>6</td>
<td>9</td>
<td>11</td>
<td>13</td>
<td>84</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>10⁻²</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

Since the fall in iodine-staining power of the amylose β-dextrin is evidence for random hydrolysis, these results show the presence of a trace of α-amylase in the Wallerstein preparation. The presence of this contaminating α-amylase could account for the Z-enzyme activity reported previously (67).
From the above results, p-Chloromercuribenzoate (pCMB; $10^{-5}$M) is shown to be an efficient inhibitor of $\beta$-amylase activity with only slight inhibitory action on the $\alpha$-amylase (Z-enzyme) activity. This substance is, therefore, a better selective inhibitor than similar concentrations of the other mercury compounds.

3. **Effect of pH and mercuric chloride on the $\alpha$-amylase activity**

The presence of a trace of $\alpha$-amylase in the barley preparation was confirmed by measuring the change in viscosity of amylopectin $\beta$-dextrin in (i) the presence and (ii) absence of mercuric chloride. Peat *et al.* reported that Z-enzyme was inactive at pH 3.6 (72), so that if Z-enzyme is, in fact, a trace of $\alpha$-amylase, the barley preparation should have no effect on the viscosity of the amylopectin $\beta$-dextrin in a digest of pH 3.6.

Amylopectin $\beta$-dextrin (1% filtered solution; 10 ml.), 0.2M-acetate buffer (5 ml.), and barley $\beta$-amylase preparation (100 mg. in 5 ml. water) were mixed in a modified Ubbelohde viscometer and $\eta_{sp}$ was determined during 2 hr. incubation.
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>none</th>
<th>Mercuric chloride $1.5 \times 10^{-5}$ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>5.6</td>
<td>5.6</td>
</tr>
<tr>
<td>Time (min.)</td>
<td>1' sp</td>
<td>Time (min.)</td>
</tr>
<tr>
<td>5</td>
<td>5.17</td>
<td>5</td>
</tr>
<tr>
<td>30</td>
<td>5.31</td>
<td>20</td>
</tr>
<tr>
<td>40</td>
<td>5.38</td>
<td>50</td>
</tr>
<tr>
<td>60</td>
<td>5.55</td>
<td>65</td>
</tr>
<tr>
<td>70</td>
<td>5.60</td>
<td>115</td>
</tr>
</tbody>
</table>

Relative Activity ($x 10^{-3}$) 7.8 units 6.9 units.

At pH 3.6, there was no change in viscosity of the $\beta$-dextrin.

The results show the presence of $\alpha$-amylolytic activity which is inhibited at pH 3.6, but is active in the presence of $1.5 \times 10^{-5}$ M mercuric chloride. The relative effect of this inhibitor cannot be compared with the previous experiment since a branched rather than a linear substrate was used, and the method of analysis was viscometry compared with the iodine-staining method used previously.

(b) **Chromatography of Barley $\beta$-Amylase Preparation**

An attempt was made to separate the $\beta$-amylase and Z-enzyme activities by chromatography on alumina as described in method 12. The preparation (200 mg; 22,000 units) was dissolved in 0.2M-McIlvaine buffer (3 ml; pH 4.0) and eluted by applying an exponential gradient of pH. Forty fractions of 5 ml. volume were collected and analysed for the presence
Figure 13.
of protein and β-amylase (Figure 13). The β-amylase activity was determined by the method of Hobson, Whelan, and Peat.

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>-Amylase units/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 5</td>
<td>42</td>
</tr>
<tr>
<td>C 12</td>
<td>0</td>
</tr>
<tr>
<td>C 20</td>
<td>26</td>
</tr>
<tr>
<td>C 23</td>
<td>100</td>
</tr>
<tr>
<td>C 26</td>
<td>146</td>
</tr>
<tr>
<td>C 29</td>
<td>98</td>
</tr>
<tr>
<td>C 37</td>
<td>13</td>
</tr>
</tbody>
</table>

The recovery was ca. 49% i.e. 10,800 units.

The presence of Z-enzyme activity in the fractions was examined by incubating samples of the fractions with amylose and amylopectin; and observing their effect on the β-amylolysis limits of the polysaccharides. A typical digest contained polysaccharide (ca. 10 mg.), 0.2M-acetate buffer (2 ml.; pH 4.6 or 6.5), enzyme fraction (1.5-2 ml) in a total volume of 8 ml. Samples (2 ml.) were removed at intervals for reducing-power estimations. The results were as follows:
### Table IV

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Units*</th>
<th>Incubation (hr.)</th>
<th>pH 4.6</th>
<th>pH 6.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>22</td>
<td>24</td>
<td>85</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td></td>
<td>47</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120</td>
<td>96</td>
<td>96</td>
</tr>
<tr>
<td>B</td>
<td>10</td>
<td>24</td>
<td>75</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td></td>
<td>47</td>
<td>91</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120</td>
<td>96</td>
<td>93</td>
</tr>
</tbody>
</table>

* *β*-amylase units/ml. polysaccharide.

The fractions C 15 - C 26 and C 28 - C 40 were then combined to give fraction A (51 ml.; 112 *β*-amylase units/ml.) and (58 ml.; 69 *β*-amylase units/ml.). The effect of these fractions on the *β*-amylolysis limit of amylose VIII was observed:

* see above.
Chromatography on alumina had not separated the Z-enzyme and β-amylase activities since (a) although there was a slight increase in the β-amylolysis limit of amylose VIII on incubation at pH 4.6 with C 27, there was a significant increase in β-amylolysis limit at pH 6.5; (b) fractions C 16, and C 26 both caused an increase in the β-amylolysis limit of amylpectin IV from the true β-amylolysis limit of 57% to 63%. (c) fractions A and B both contained Z-enzyme activity since they caused the β-amylolysis limit of a amylose VIII to increase, at pH 4.6, with time of incubation;

(c) Effect of Dialysis on the Barley β-Amylase Preparation.

The stabilising effect of calcium and other ions on α-amylases has recently been investigated by Fischer and coworkers (109) who found that removal of calcium ions resulted in inactivation of the enzyme. It was therefore decided to observe the effect of dialysis on the barley preparation.

Barley β-amylase preparation (2 g.; 220,000 units; 0.47 protein N) was dialysed against several changes of distilled water for 72 hr. at 0°. The resulting solution was freeze-dried to a white amorphous powder (Fraction D; 6.43% protein N 180 mg.)

<table>
<thead>
<tr>
<th>β-Amylase units/mg.</th>
<th>Recovery (%)</th>
<th>Increase in Activity/mg. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wallerstein β-Amylase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction D</td>
<td>110</td>
<td>240</td>
</tr>
</tbody>
</table>
Dialysis had therefore removed the inorganic sulphate salts to give an enzyme preparation of higher specific activity, per mg., although 80% of the total activity had been lost, presumably by inactivation during dialysis.

The presence or absence of Z-enzyme in fraction D was determined by preparing a digest containing amylose VIII (10 mg. in 4 ml. water), 0.2M-acetate buffer (2 ml.; pH 4.6 or 6.5), and fraction D (230 β-amylase units; 0.96 mg. in 2 ml. water), and incubating at 37° for 24 and 48 hr.

<table>
<thead>
<tr>
<th>Incubation (hr.)</th>
<th>pH 4.6</th>
<th>pH 6.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>94</td>
<td>100</td>
</tr>
<tr>
<td>48</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Z-enzyme, present in the barley β-amylase preparation, had not been selectively inactivated by dialysis against distilled water since amylose VIII was completely converted into maltose after incubation for 24 hr. at pH 6.5, in contrast to previous results which showed only a 72% conversion into maltose at pH 3.6.

Results obtained in a related investigation by Dr. A. Wright (110), suggested that dialysis against a solution of 0.05M ethylenediaminetetra-acetic acid (EDTA) removed most of the Z-enzyme activity from a soya-bean β-amylase preparation; the action of the resulting preparation on amylose at pH 5.6 was incomplete after 23 hr. (c.f. 109).
(d) Electrophoresis of the Barley $\beta$-Amylase Preparation

**Qualitative**

A Shandon open-strip apparatus was used to examine the $\beta$-amylase preparation by electrophoresis in 0.05M-phosphate buffer of pH 6.9; cellulose acetate strips were preferred to Whatman No. 1 filter-paper strips as the support for the protein solution. The acetate strips were prepared for electrophoresis by equilibration overnight in the apparatus. A concentrated solution of fraction D (i.e. the dialysed $\beta$-amylase preparation) was applied to the starting line and the separation effected over 3.5 hr. (200 volts) at room temperature. The strip was then removed from the apparatus, heated at 100° for 20 min. before development with nigrosine solution (0.05% in 2% acetic acid) for 1 hr. Three bands were observed, one remaining stationary, one moving towards the anode, and the other to the cathode; at least three protein components were therefore present.

**Quantitative**

A Shandon apparatus (see p. 25) was used for the continuous electrophoresis of the $\beta$-amylase preparation. When 0.05M-phosphate buffer and a potential difference of 900 volts (10 mA) were used, fraction D (20 mg. in 1.5 ml. of 0.05M-phosphate buffer pH 6.9; 4,800 units) was satisfactorily separated. Development of the filter-paper curtain with nigrosine solution indicated three bands of protein, one
travelling vertically downwards (fractions E 23, E 24),
one travelling towards the cathode (E 20, E 21) and the other
migrating to the anode (E 25).

The fractions containing protein; viz. E 21 - 25,
were examined for enzymic activity with the following results:

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vol. of Fraction (ml.)</th>
<th>Units/ml.</th>
<th>Total Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>E 21</td>
<td>7</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>E 22</td>
<td>7</td>
<td>9</td>
<td>63</td>
</tr>
<tr>
<td>E 23</td>
<td>7</td>
<td>185</td>
<td>1295</td>
</tr>
<tr>
<td>E 24</td>
<td>7</td>
<td>21</td>
<td>147</td>
</tr>
<tr>
<td>E 25</td>
<td>7</td>
<td>ca.1</td>
<td>7</td>
</tr>
</tbody>
</table>

The recovery was therefore ca. 32%.

\[\text{\textbf{\textit{Z-enzyme activity}}}\]

The presence of Z-enzyme (\(\alpha\)-amylase) was detected
by examining the effect of the fractions on the iodine-staining
power of amylose \(\beta\)-dextrin. \(\beta\)-Amylase, alone, should have no
effect on this \(\beta\)-dextrin but when acting in conjunction with
Z-enzyme, there should be a rapid fall in iodine-staining
power. Amylose \(\beta\)-dextrin (0.8 mg./ml. by acid hydrolysis;
1.4 ml.) and fractions E 21 - E 25 (1 ml.) were incubated at 37°
for 20 hr. Samples (1 ml.) were stained with iodine solution.
A.V.'s were compared at 580 and 600 mμ.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Decrease in A.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E 21</td>
<td>0</td>
</tr>
<tr>
<td>E 22</td>
<td>0</td>
</tr>
<tr>
<td>E 23</td>
<td>65</td>
</tr>
<tr>
<td>E 24</td>
<td>86</td>
</tr>
<tr>
<td>E 25</td>
<td>81</td>
</tr>
</tbody>
</table>

To differentiate between true β-amylase and Z-enzyme action, similar digests were then incubated in the presence of pCMB (10^{-5}M) which would selectively inhibit β-amylase. A.V.'s were determined at 580, 600, and 640 mμ. The results shown were obtained at 600 mμ.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Fall in A.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E 23</td>
<td>3</td>
</tr>
<tr>
<td>E 24</td>
<td>4</td>
</tr>
<tr>
<td>E 25</td>
<td>13</td>
</tr>
</tbody>
</table>

From the above results, it appears that the Z-enzyme activity occurs mainly in fractions E 24 and E 25, and that the β-amylase activity is concentrated in fraction E 23.

**Action of electrophoresis fractions on the β-dextrins of amylpectin and glycogen**

The final proof that the β-amylase and Z-enzyme activities had been separated was obtained by incubating fractions E 23 - E 25 with the above β-dextrins. The action of Z-enzyme would cause a decrease in iodine-staining power.
of the amylopectin β-dextrin with a concomitant increase in percentage conversion into maltose \( (P_M) \).

Digests containing the β-dextrins (20 mg. amylopectin β-dextrin in 10 ml. water; 12 mg. glycogen β-dextrin in 4 ml. water), 0.2M-acetate buffer (5 ml.; pH 4.6), and fractions E 23 - E 25 (1 ml.) were incubated at 37°. Samples (1 - 3 ml.) were removed for analysis by iodine-staining \( (5^\circ \text{O m}^\circ) \) or reducing-power measurements.

<table>
<thead>
<tr>
<th>Amylopectin β-dextrin</th>
<th>Glycogen β-dextrin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age of Digest (hr.)</strong></td>
<td><strong>Decrease in A.V. (%)</strong></td>
</tr>
<tr>
<td>22</td>
<td>44</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fraction</th>
<th>E 23</th>
<th>E 24</th>
<th>E 25</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0 9</td>
<td>0 9</td>
</tr>
<tr>
<td>10</td>
<td>19</td>
<td>1 8</td>
<td>2 4</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>1 4</td>
<td>1 7</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The results show clearly that fraction E 24 has the highest Z-enzyme \( (\alpha\text{-amylase}) \) activity, and that it is possible to separate the β-amylase and Z-enzyme activities of the barley preparation by electrophoresis.

**Discussion**

Although Z-enzyme was known to be present in the Wallerstein β-amylase preparation \( (67) \), \( \alpha \)-amylase was not a
gross contaminant since (a) there was no effect on the iodine-staining power of amylopectin \( \beta \)-dextrin at 680 m\( \mu \) after 24 hr. incubation (111); (b) variation of enzyme concentration had no effect on the \( \beta \)-amylolysis limit of glycogen (111); and (c) the intermediate 50\% \( \beta \)-dextrin of amylose had the same molecular size as the original polysaccharide (106).

King and Manners (105), however, found that in digests containing 33, 66, 99, and 128 units of \( \beta \)-amylase per mg. amylopectin, the \( \beta \)-amylolysis limits were 64, 65, 68, 71, after 92 hr. incubation at pH 4.9 and 37\(^\circ\). This relation between enzyme concentration and \( \beta \)-amylolysis limit was characteristic of an \( \alpha \)-amylase (106), and suggested that there was perhaps a trace of \( \alpha \)-amylase, in contrast to the previous findings.

In the present work, the \( \beta \)-amylase component of the Wallerstein preparation was selectively inhibited with thiol-reactants and the effect of the preparation on the iodine-staining power of amylose \( \beta \)-dextrin, and the viscosity of amylopectin \( \beta \)-dextrin at pH 5.6 was then observed. In both cases, limited random hydrolysis occurred since there was a significant decrease in iodine-staining power and viscosity. There was no decrease in viscosity of a digest of pH 3.6, Z-enzyme being inactivated at this pH (74). These results, therefore, suggest that Z-enzyme action is in fact due to a
**Figure 14.**

Relative β-Amylase activity vs. Relative Z-Enzyme activity. Fractions 23, 24, and 25 are shown.

- **β-Amylase**
- **Z-Enzyme**

Fraction No.: 23, 24, 25

Fall in A.V. (%):

- 10
- 5
- 2
- 1
- 0

Relative Z-Enzyme activity:

- 40
- 30
- 20
- 10
- 5
- 0
trace of \( \alpha \)-amylase.

An attempted separation of the \( Z \)-enzyme and \( \beta \)-amylase activities in the \( \beta \)-amylase preparation by chromatography on alumina was unsuccessful. The results (Table IV) show that the conversion of amylose into maltose at pH 4.6 and 6.5 (ca. 90%) is much greater than that found with \( Z \)-enzyme free \( \beta \)-amylase preparations (ca. 70%). Since the random hydrolysis of one bond in the amylose \( \beta \)-dextrin gives a \( \beta \)-amyloysis limit of 85%, only a trace of \( \alpha \)-amylase would be required to give the observed \( \beta \)-amyloysis limit of about 90%.

Complete separation might be possible by chromatography on calcium phosphate gel (112) or ion-exchange cellulose (e.g. DEAE, TEAE (113)).

Inactivation of the \( Z \)-enzyme component by removal of the necessary stabilising ions by dialysis was not accomplished. Since there was complete conversion of amylose to maltose after 24 hr. at pH 6.5 (23 units/mg. polysaccharide), there was still a comparatively high concentration of \( Z \)-enzyme in the dialysed enzyme preparation (fraction D).

The barley preparation was successfully separated into the two active components by electrophoresis. Figure 14 gives an indication of the relative activities of three adjacent fractions; fractions E 25 was able to hydrolyse amylose \( \beta \)-dextrin in the presence of pCMB (which selectively inhibits \( \beta \)-amylase) and contained virtually no \( \beta \)-amylase, whereas fraction E 23 showed negligible action on amylose \( \beta \)-dextrin but
contained the major portion of the \( \beta \)-amylase. This separation could be improved by use of side electrodes and addition of propylene glycol or glycerol (64) to the eluant buffer.

The separation of these enzymes could probably be achieved by electrophoresis in a Tiselius preparative scale apparatus; unfortunately, this instrument was not available.

---

**Part 3.**

**The Z-Enzyme Activity of Almond Emulsin**

Since the Z-enzyme activity present in the Wallerstein \( \beta \)-amylase has been shown to be due to a trace of \( \alpha \)-amylase, it was decided to investigate almond emulsin, another source of Z-enzyme (72, 103), to see whether this Z-enzyme activity was again due to a trace of \( \alpha \)-amylase.

**Materials.**

(a) **Substrates**

These are described in Part I of this Section.
(b) **Enzyme Preparations**

(i) **Emulsin**

One sample of emulsin was prepared by Dr. F.B. Anderson, this is emulsin I. A second sample, emulsin II, was prepared by Tauber's method (114) as follows:

Sweet almonds (1 Kg.) were soaked in water overnight and the skins removed. The minced almonds were defatted with ether and the resulting oil-free powder dried at room temperature. Yield: 335 g. A portion of this powder (200 g.) was extracted with 33% ethanol (1600 ml.) for 10 min. at room temperature. The residue was filtered off, dried with acetone followed by ether and this crude almond powder (CAP) was stored at 0°. Yield: 185 g. The filtrate was cooled to 0° and the precipitate which formed was discarded before addition of 95% ethanol (800 ml.). The solution was centrifuged; the supernatant solution was removed and the precipitate dissolved in water and dialysed overnight at 0° against running tap-water before freeze-drying. Yield of emulsin: 5.0 g. (11.1% protein N).

In the preparation of digests, a weighed amount was added to the stated amount of liquid and any insoluble material was discarded.

(ii) **Fractionation of Crude Almond Powder.**

CAP (60 g.) was extracted with distilled water (400 ml.)
at room temperature for 30 min. The residue was separated and discarded. After cooling the supernatant solution to 0°, the precipitate which formed was centrifuged off to yield fraction A. The remaining solution was fractionated at 0° with saturated ammonium sulphate solutions (see Table V); all precipitates were dissolved in a small quantity of ice-cold water and dialysed overnight at 0° before being freeze-dried.

(iii) Crude Almond Powder α-Amylase

α-Amylase was extracted from CAP by the method of Kneen, Sandstedt and Hollenbeck (115) as follows:-

CAP (50 g.) was extracted for 60 min. at room temperature with a solution (400 ml.) containing 5 mg. calcium chloride per ml. The extract was brought to a pH of 6.0 and held for 15 min. at 70° before dialysing at 0° for 48 hr. against running tap-water. The precipitate was separated at the centrifuge and discarded; the supernatant solution was freeze-dried. Yield: 6.5 g. (8% protein N). The α-amylase activity was 0.0039 units/mg. (0.049 units/mg. N) measured by the method of Fischer and Stein.

Results

Action of Emulsin on Salicin and Amylose

Emulsin II (6 mg. in 50 ml. water) was stored at 20°, and samples removed at intervals for examination of the
action on the β-glucoside, salicin, and amylose.

(a) β-Glucosidase activity

The samples (10 ml.) were incorporated into digests with salicin (9 mg. in 3 ml. water) and 0.2M-acetate buffer (pH 5.6; 2 ml.). Relative activities were calculated from the amount of glucose released after 30 min. incubation.

<table>
<thead>
<tr>
<th>Time of treatment (days)</th>
<th>Activity</th>
<th>Decrease in activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.36</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>0.31</td>
<td>14</td>
</tr>
<tr>
<td>14</td>
<td>0.21</td>
<td>42</td>
</tr>
</tbody>
</table>

(b) α-Amylase activity

This was determined from the decrease in A.V. of the iodine-complex in a digest containing amylose solution (0.5 mg/ml.; 2 ml.), 0.2 M-acetate buffer (pH 5.6; 3 ml.) and emulsin sample (5 ml.). Samples (3 ml.) were removed after 4 hr. incubation, stained with iodine solution (1 ml.) and the A.V. measured at 640 μm.

<table>
<thead>
<tr>
<th>Time of treatment (days)</th>
<th>Fall in A.V. (%)</th>
<th>Decrease in activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.7</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>6.7</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>6.5</td>
<td>3</td>
</tr>
</tbody>
</table>

As a result of this experiment, the α-amylase activity was estimated by the method of Fischer and Stein and corresponded to 0.002 unit/mg. (0.017 units/mg. protein N).

It can be concluded that emulsin contains a trace of α-amylase which is distinct from the β-glucosidase activity.
Figure 15.

Figure 16.
since there is a difference in the stability of the two enzymes.

**Effect of Enzyme Concentration on the Iodine-Staining Power of Amylose**

Amylose (2 mg. in 4 ml. water), 0.2 M-acetate buffer (pH 5.6; 5 ml.), and emulsin II (20, 16, 12, or 8 mg.) were incubated at 37°, samples (2 ml.) being removed at intervals. After heating on a boiling water-bath for 5 min., coagulated protein was separated and aliquots (1 ml.) were stained with iodine solution. An EEL (Evans Electro-Selenium Ltd.) Colorimeter was used (608 filter) to measure the A.V's. The results were as follows (Figure 15)

<table>
<thead>
<tr>
<th>Enzyme concentration mg./mg. amylose</th>
<th>Fall in A.V. (%) 1.5</th>
<th>2.0</th>
<th>3.5</th>
<th>4.5 (hr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>30</td>
<td>36</td>
<td>55</td>
<td>64</td>
</tr>
<tr>
<td>8</td>
<td>20</td>
<td>26</td>
<td>45</td>
<td>52</td>
</tr>
<tr>
<td>6</td>
<td>14</td>
<td>19</td>
<td>35</td>
<td>41</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>11</td>
<td>20</td>
<td>27</td>
</tr>
</tbody>
</table>

There was a regular decrease in iodine-staining power of amylose as the enzyme concentration was increased.

**Effect of Emulsin on the β-Amylolysis Limit of an Amylose**

Amylose VIII (50 mg. in 10 ml. solution) was incubated with emulsin I (1% in 0.2 M-acetate buffer of pH 5.8; 10 ml.). Iodine-staining measurements were carried out at 680 m on samples (2 ml.) removed after 0 and 43 hr. incubation at 37°; the A.V. had fallen from 0.703 to 0.042. Enzyme action was terminated by heating, and the solution filtered through sintered glass (G 3), before the pH was lowered to
3.6 by addition of acetic acid, and the $\beta$-amylolysis limit of the residual polysaccharide determined. It was found to be 100%. In the absence of emulsin, the $\beta$-amylolysis limit was ca. 72%.

From the above results it can be seen that emulsin will (i) reduce the iodine-staining power of amylose, and (ii) hydrolyse amylose in such a way that complete $\beta$-amylolysis takes place.

**pH Activity Determination**

Further proof that emulsin contained an $\alpha$-amylase was provided by observing the action of emulsin on amylose $\beta$-dextrin in solutions of pH 4.6 - 7.6.

Digests were prepared containing amylose $\beta$-dextrin (2.4 mg. in 2 ml. solution), phosphate-citrate buffer (pH 4.6 - 7.6; 2 ml.), and emulsin I (15 mg. in 1 ml. water). Control digests (a) without enzyme, and (b) without $\beta$-dextrin were prepared. After 27 hr. incubation, samples (2.5 ml.) were withdrawn, heated, and centrifuged. Iodine solution (1 ml.) was added to samples (2 ml.) and the A.V's measured (640 m$\mu$) after dilution. The results are shown on figure 16.

Emulsin causes a decrease in iodine-staining power of amylose $\beta$-dextrin with optimum action at pH 5.9. This effect can be explained completely by the presence of trace amounts of $\alpha$-amylase.

**Effect of Various Ions on the Amylolytic Activity of Emulsin**

As already indicated (p. 47), calcium ions have a
Figure 17

Fall in A.V. (%) vs. Incubation (Hr.)

Graph showing the relationship between the fall in A.V. (%) and incubation (Hr.) with curves labeled (a), (b), (c), and (d).
stabilising effect on α-amylase which can be reversed by the addition of complexones such as EDTA. The effect of calcium sulphate, EDTA, or mercuric chloride, on the action of the α-amylase component of emulsin on various substrates was investigated.

(a) Iodine-staining measurements

(i) Action on amylase

Digests were prepared containing 0.4% amylase solution (5 ml.), emulsin I (0.5% in 0.2 M-acetate buffer of pH 5.6; 5 ml.), and calcium acetate (5 x 10^{-3} M; 0.5 ml.) or water (0.5 ml.). Digest (a) contained newly prepared enzyme and substrate and water; digest (b) contained enzyme pre-incubated at 37° for 40 hr.; digest (c) contained enzyme pre-incubated with calcium; and digest (d) contained pre-incubated enzyme added to calcium. Samples (2 ml.) were withdrawn at intervals, heated, and coagulated protein was removed. 1 ml. portions were used for A.V. measurements at 680 mu. The results, tabulated below, show that the calcium ions have a stabilising effect rather than an activating action (figure 17).

<table>
<thead>
<tr>
<th>Incubation (hr.)</th>
<th>5</th>
<th>6.5</th>
<th>16</th>
<th>21.5</th>
<th>40</th>
<th>47.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digest conditions</td>
<td>(a)</td>
<td>11</td>
<td>-</td>
<td>35</td>
<td>-</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>(b)</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(c)</td>
<td>-</td>
<td>4</td>
<td>-</td>
<td>29</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(d)</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>12</td>
<td>-</td>
</tr>
</tbody>
</table>

(ii) Action on amylase β-dextrin

Emulsin I solution (5%; 0.5 ml) was pre-treated with
water, mercuric chloride, EDTA, or calcium sulphate (0.5 ml.), for 30 min. at 20° before incubation with amylose β-dextrin (0.8 mg./ml; 14 ml.). Samples (2 ml.) were removed after 25 hr. and deproteinised by heating and removing coagulated protein at the centrifuge. 1 ml. portions were used for A.V. measurements at 580, 600, 640, 680 mµ.

### Digest conditions

<table>
<thead>
<tr>
<th>Wavelength (mµ)</th>
<th>580</th>
<th>600</th>
<th>640</th>
<th>680</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Water</td>
<td>56</td>
<td>60</td>
<td>65</td>
<td>69</td>
</tr>
<tr>
<td>(b) Mercuric chloride</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5 x 10⁻⁵ M</td>
<td>33</td>
<td>35</td>
<td>38</td>
<td>41</td>
</tr>
<tr>
<td>1.5 x 10⁻⁶ M</td>
<td>44</td>
<td>47</td>
<td>55</td>
<td>51</td>
</tr>
<tr>
<td>(c) Pretreated with calcium sulphate *</td>
<td>72</td>
<td>74</td>
<td>79</td>
<td>82</td>
</tr>
<tr>
<td>(d) Pretreated with EDTA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(i) alone *</td>
<td>23</td>
<td>24</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>(ii) diluted with water</td>
<td>27</td>
<td>28</td>
<td>31</td>
<td>32</td>
</tr>
<tr>
<td>(iii) diluted with calcium sulphate *</td>
<td>47</td>
<td>50</td>
<td>51</td>
<td>56</td>
</tr>
</tbody>
</table>

* Final concentration 2 x 10⁻¹ M

<table>
<thead>
<tr>
<th>Original</th>
<th>Max</th>
<th>640 mµ</th>
<th>Final</th>
<th>Max</th>
<th>580 mµ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The α-amylase component of emulsin shows a decreased activity in the presence of EDTA which can be reversed by the addition of calcium ions. Calcium ions again give an increased α-amylolytic action (probably due to their stabilising action) which seems to be independent of the substrate. Mercuric chloride (1.5 x 10⁻⁶ M) causes a slight inhibition as observed with barley Z-enzyme (116). A decrease in λ max of the iodine-polysaccharide complex is one characteristic of α-amylolysis.
(iii) **Action on glycogen**

Emulsin II Solution (0.5%; 4 ml.) was pre-treated with water, calcium acetate or EDTA (1 ml.) at 20° for 15 min. before addition to glycogen (0.94 mg./ml. of 0.2 M-acetate buffer of pH 5.6; 25 ml.). The action was followed by removing samples (2 ml.) for reducing-power or iodine-staining (480 µl) measurements.

<table>
<thead>
<tr>
<th>Digest conditions</th>
<th>Decrease in A.V. (%)</th>
<th>PM</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Water</td>
<td>3 24 49</td>
<td>24 49</td>
</tr>
<tr>
<td>(b) pre-treated with calcium acetate *</td>
<td>8 28 36</td>
<td>2.6 4.4</td>
</tr>
<tr>
<td>(c) pre-treated with EDTA. *</td>
<td>0 3 6</td>
<td>0 0.9</td>
</tr>
</tbody>
</table>

* final concentration $10^{-4}$ M

Using glycogen as substrate, the results are similar to those of the previous experiments, although since glycogen is less iodophilic than amylose, the relative decrease in iodine-staining power is lower.

(b) **Viscosity measurements**

Amylose β-dextrin (13.5 mg), emulsin I (ca. 2%; 4.5 ml.) preincubated for 20 min. with water, mercuric chloride, calcium sulphate, or EDTA) were incubated in an Ostwald viscometer at 25°. The relative activities for two series of experiments are shown as follows:-
Relative \( \alpha \)-amylase units \( (x \times 10^{-3}) \)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration (M)</th>
<th>I</th>
<th>II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td></td>
<td>6.5</td>
<td>9.3</td>
</tr>
<tr>
<td>Mercuric chloride</td>
<td>( 1.5 \times 10^{-6} )</td>
<td>5.7</td>
<td>-</td>
</tr>
<tr>
<td>Calcium sulphate</td>
<td>( 2 \times 10^{-4} )</td>
<td>-</td>
<td>8.2</td>
</tr>
<tr>
<td>EDTA</td>
<td>( 2 \times 10^{-4} )</td>
<td>-</td>
<td>6.5</td>
</tr>
</tbody>
</table>

The sensitive analytical method of viscometry has also confirmed the previous conclusions i.e. there is a trace of \( \alpha \)-amylase in emulsin since there is a fall in viscosity of amylose \( \beta \)-dextrin.

The short time of incubation used (90 min.), has been insufficient to demonstrate the stabilising effect of calcium ions although the inactivating effect of EDTA can be clearly distinguished.

**Action of Emulsin on the \( \beta \)-Dextrins of Glycogen and Amylopectin**

Barley \( \beta \)-enzyme is able to hydrolyse glycogen and amylopectin \( \beta \)-dextrins to a slight extent (p. 51); digests were, therefore, prepared containing either amylopectin \( \beta \)-dextrin (30 mg. in 15 ml. of 0.2 M-acetate buffer of pH 5.8 or glycogen \( \beta \)-dextrin (20 mg. in 5 ml. of buffer), and 0.5% emulsin I (5 ml.)

The annexed results were obtained.

<table>
<thead>
<tr>
<th>Incubation (hr.)</th>
<th>(Fall in A.V. (%; 540 m( \mu ))</th>
<th>( P_M )</th>
<th>( P_M )</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td>26</td>
<td>4.2</td>
<td>-</td>
</tr>
<tr>
<td>30</td>
<td>-</td>
<td>-</td>
<td>2.4</td>
</tr>
<tr>
<td>48</td>
<td>35</td>
<td>7.8</td>
<td>3.7</td>
</tr>
</tbody>
</table>

The emulsin had hydrolysed twice as many bonds in
amylopectin $\beta$-dextrin as in the glycogen $\beta$-dextrin.

Experiments with CAP Fractions

The details of the fractions obtained as described on p. 56 & 57 are as follows:

<table>
<thead>
<tr>
<th>CAP Fraction</th>
<th>Saturation of Ammonium Sulphate (%)</th>
<th>Yield (mg.)</th>
<th>Protein N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>-</td>
<td>50</td>
<td>4.6</td>
</tr>
<tr>
<td>B</td>
<td>0 - 33</td>
<td>400</td>
<td>12.4</td>
</tr>
<tr>
<td>C</td>
<td>33 - 42</td>
<td>2800</td>
<td>11.9</td>
</tr>
<tr>
<td>D</td>
<td>42 - 51</td>
<td>650</td>
<td>7.3</td>
</tr>
<tr>
<td>E</td>
<td>51 - 100</td>
<td>400</td>
<td>11.1</td>
</tr>
</tbody>
</table>

$\alpha$-Amylase activity

Since unfractionated emulsin had been shown to contain $\alpha$-amylase, the CAP fractions were analysed for $\alpha$-amylase activity by observing their effect on the iodine-staining power of amylose.

Digests were prepared containing amylose VIII (4 mg. in 2 ml. water), and enzyme solution (4 ml. 0.2 M-acetate buffer of pH 5.6 containing 8 mg. of fractions B, C, D, and E, insoluble material being centrifuged off.) Samples (2 ml.) were deproteinised and the iodine-staining power measured in an EEL Colorimeter (608 filter).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Incubation (hr.)</th>
<th>Fall in A.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>65</td>
<td>30</td>
</tr>
<tr>
<td>C</td>
<td>51</td>
<td>67</td>
</tr>
<tr>
<td>D</td>
<td>98</td>
<td>99</td>
</tr>
<tr>
<td>E</td>
<td>77</td>
<td>98</td>
</tr>
</tbody>
</table>
This suggests that all fractions contain \( \alpha \)-amylase, the highest concentration occurring in fraction D.

The \( \alpha \)-amylase activity was then determined by the method of Fischer and Stein.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Units/mg. fraction</th>
<th>Units/mg. protein N.</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>0.0030</td>
<td>0.041</td>
</tr>
<tr>
<td>E</td>
<td>0.0047</td>
<td>0.042</td>
</tr>
</tbody>
</table>

Fractions B and C were also examined, but the time of incubation (60 mins.) used, did not allow significant amounts of maltose to be produced.

The digests used in the above estimations were incubated for a further 43 hr. and reducing power measurements carried out. An approximate estimation of A.V. of the polysaccharide-iodine complex was made by adding iodine-solution (0.5 ml.) to the remainder of the digest and measuring the resulting A.V. at 680 m\( \mu \).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Apparent maltose produced/ mg. fraction</th>
<th>A.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>0.09</td>
<td>1.36</td>
</tr>
<tr>
<td>C</td>
<td>0.07</td>
<td>1.90</td>
</tr>
<tr>
<td>D</td>
<td>0.55</td>
<td>0.153</td>
</tr>
<tr>
<td>E</td>
<td>0.63</td>
<td>0.430</td>
</tr>
</tbody>
</table>

These results show that although fraction D causes the most significant fall in iodine-staining power of starch, fraction E will cause a greater hydrolysis of starch to apparent maltose. This could be explained by the presence of a trace of maltase or \( \beta \)-amylase in addition to the \( \alpha \)-amylase.

**Maltase activity**

Maltase acting in conjunction with \( \alpha \)-amylase could
cause the previous unusual result. Paper chromatographic examination of digests containing maltose (2% in phosphate-citrate buffer of pH 5.9; 1 ml.) and fractions B, C, D, and E (20 mg. in 4 ml. water) did not, however, show proof of maltase activity after 40 hrs. incubation. Similar control digests with cellobiose, salicin, phenyl \(\beta\)-glucoside, and lactose, indicated that the enzyme solutions used were active by producing the expected monosaccharides.

Experiments with CAP \(\alpha\)-Amylase

Effect of pH on the Activity of CAP \(\alpha\)-Amylase

Soluble starch (0.1%; 5 ml.), McIlvaine buffer (3 ml; pH 4.6 - 7.6), and \(\alpha\)-amylase solution (0.020 units/ml; 2 ml.) were incubated at 37\(\degree\)C for 5 hr. Samples were removed, deproteinised and stained with iodine.

\[
\begin{array}{cccccccc}
\text{pH} & 4.6 & 5.1 & 5.6 & 6.0 & 6.3 & 6.7 & 7.1 & 7.7 \\
\text{Fall in A.V.} \text{ (\%)} & 11 & 27 & 31 & 38 & 40 & 43 & 41 & 37 \\
(680 \text{ mm})
\end{array}
\]

The \(\alpha\)-amylase has maximum activity in McIlvaine buffer of pH 6.7.

Discussion

The \(\beta\)-enzyme activity of almond emulsin can now be attributed to the presence of a trace of \(\alpha\)-amylase.
This is shown clearly by the fact that emulsin causes a decrease in the iodine-staining power and viscosity of an amylose $\beta$-dextrin solution, in addition to causing an increase in the $\beta$-amylolysis limit of an amylose from ca. 72 to 100%. The $\beta$-dextrins of amylopectin and glycogen are also hydrolysed to a small extent by emulsin, the $\alpha$-amylase component having a lowered affinity towards the more highly branched structure of glycogen $\beta$-dextrin. The ability of barley and emulsin Z-enzyme to attack glycogen $\beta$-dextrin and glycogen is in contrast to the results of Banks, Greenwood and Jones (117), who concluded that Z-enzyme was unable to attack glycogen.

In earlier experiments (106), emulsin had no action on Floridean or soluble starch, by reducing-power measurements, showing that the $\alpha$-amylase was not a major impurity.

Viscometry again provides a very rapid and sensitive method of analysis for a trace of $\alpha$-amylase, especially when amylose $\beta$-dextrin is used as the substrate.

A sample of emulsin which had been pretreated with calcium ions caused a larger decrease in the iodine-staining power of an amylose than a control preparation; calcium ions had, therefore, stabilised the $\alpha$-amylase component. This stabilising effect of calcium is a characteristic of $\alpha$-amylases. Several purified and crystalline $\alpha$-amylases from various sources are now known to be calcium-metalloproteins, and contain one or more calcium atoms per molecule. In addition,
calcium prevents the destruction of α-amylases in crude preparations by contaminating proteolytic enzymes (109). EDTA is able to reverse the stabilising effect by complexing with the added calcium ions, as well as decreasing the activity of an untreated sample, presumably by complexing with the metallic ions already present in the preparation.

The Z-enzyme activity of emulsin has been shown to be unrelated to the β-glucosidase activity by a comparison of their stabilities at 20°. This is in agreement with results reported by other workers (103, 101).

After extraction of emulsin from the oil-free almond powder, it was found that the residual CAP contained a further amount of α-amylase. Fractionation of the CAP water-extract with saturated ammonium sulphate solutions yielded two fractions which possessed approximately 2.5 times the α-amylase activity of the emulsin II preparation. There was an indication that one of the fractions, E, might contain a trace of β-amylase (c.f. 103) since it possessed greater saccharifying properties (measured as apparent maltose produced) than the neighbouring fraction D, although the dextrinising properties (measured by iodine-staining) were greater in fraction D.

The yield of α-amylase from the CAP was improved by heating the extract in the presence of stabilising calcium ions, to give a total of 25.3 units compared with ca. 4 units
by the above ammonium sulphate fractionation. This preparation also showed a slightly higher activity per mg. protein nitrogen than the most active fraction obtained by ammonium sulphate fractionation.

The presence of $\alpha$-amylase in almond emulsin may be compared with the results of Baba and Kojima (104) who separated an $\alpha$-amylase from apricot emulsin.

Part 4.

Observations on $\alpha$-Amylases from Barley, Malt, and Human Saliva.

Introduction.

The random hydrolysis of $\alpha$-1,4-glucosidic linkages, catalysed by $\alpha$-amylase, is at first relatively rapid, causing a sudden decrease in viscosity and iodine-staining power of the substrate, resulting in the formation of $\alpha$-dextrins (dextrinisation) with DP. values of 6 - 10 glucose residues. The achroic stage is usually reached, in the case of amylose, when 20% of the linkages have been hydrolysed, yielding linear dextrins; amylopectin, on the other hand, will yield branched $\alpha$-dextrins. The dextrinisation reaction is followed and overlapped by a much slower reaction (saccharification) in which the short-chain fragments are degraded into the smaller oligosaccharides maltose and maltotriose, and in some cases, glucose.

Specificity differences in the above stages have been reported by various workers during the hydrolysis of
Figure 18.

- Glucose residue
- Non-reducing end
- Reducing end
- α-1,4-linkage
- Linkage hydrolysed by salivary α-amylase
- M “ “ malt
- B “ “ bacterial

Figure 19.

Graph showing incubation in hours on the x-axis and optical density (OD) at 480nm on the y-axis. The graph compares two digestion processes, labeled Digest (a) and Digest (b).
amylose by α-amylases from different sources. Caldwell and her coworkers (118) have suggested that during the dextrinisation of amylose, the achroic stage is reached when 15 or 12% of the glucosidic linkages are hydrolysed by human salivary or fungal α-amylase, respectively, whereas with swine pancreatic α-amylase, this stage is reached when 23% of the linkages are broken. Hopkins and Bird (119) stated that with linear substrates, salivary α-amylase did not readily attack the first two, and bacterial and malt α-amylases the first five linkages from the non-reducing end, whereas salivary and malt α-amylases readily attacked the second and bacterial amylase the third linkage from the reducing end (figure 18). In certain cases, fission was said to take place nearer the non-reducing end, so that bacterial and malt α-amylases were able to hydrolyse maltotriose.

A dilute solution of salivary α-amylase has been shown to hydrolyse amylopectin and glycogen to maltose, maltotriose and a series of α-dextrins, the smallest being the pentasaccharide 6^3-α-maltosylmaltotriose; a more concentrated solution of the enzyme yielded a tetrasaccharide 6^3α-glucosylmaltotriose and was also able to hydrolyse maltotriose (31,120). This suggests that salivary α-amylase can attack α-1,4-glucosidic linkages close to the branch points in amylopectin and glycogen and should perhaps show a higher affinity towards a branched substrate than the malt α-amylase discussed previously.

In the following experiments, the action of dilute solutions of salivary, malt, and barley α-amylases on starch-type polysaccharides was investigated in an attempt to correlate
their effect with that of Z-enzyme from barley and emulsin.

**Materials**

**Enzyme Preparations**

(a) **Malt α-amylase**

A commercial preparation of malt diastase (Wallerstein Company, New York) was used as one source of malt α-amylase, with short incubation times to minimise the effect of β-amylase. A second sample of malt α-amylase was prepared by the method of Kneen *et al* (115).

Malted Ymer barley flour (50 g.; W. Younger and Co., Edinburgh) was extracted for 1 hr. at room temperature with 250 ml. water containing calcium chloride (0.5 g.). The extract was adjusted to pH 6.0 and held at 70° for 15 min. This solution was then dialysed for 30 hr. at 0° against running tap-water. The resulting precipitate was separated at the centrifuge and the supernatant solution freeze-dried. Yield: 1.1 g. (0.19 % N; 0.725 α-amylase activity units/mg. preparation; 382 units/mg. N).

(b) **Barley α-amylase**

This was also prepared by the above method. 50 g. Barley flour (Ymer; W. Younger and Co., Edinburgh). Yielded: 0.9 g. barley α-amylase (0.144% N; 0.005 units/mg. preparation; 1.14 units/mg. N).

(c) **Salivary α-amylase**

Freeze-dried salivary α-amylase which had an activity of 34 units/mg. was prepared by Dr. A. M. Liddle.

All α-amylase activity measurements were determined by the method of Fischer and Stein.
Results

Experiments with Barley $\alpha$-Amylase

L. Examination of Barley $\alpha$-Amylase Preparation for Contaminating Carbohydrases.

(a) Digests were set up containing the enzyme preparation (5 mg. in 0.5 ml. water; insoluble material being centrifuged off) and cellobiose, laminarin or maltose (2.0% in McIlvaine buffer of pH 5.9; 0.5 ml.). Paper chromatographic analysis after 24 hr. incubation at 37° indicated that no hydrolysis of the substrates had occurred i.e. cellobiase, laminarinase, and maltase were absent.

(b) Action of barley $\alpha$-amylase on glycogen

In order to detect any $\beta$-amylase impurity, a study was made of the amylolytic action of the preparation on glycogen in the presence and absence of pCMB at a final concentration of $5 \times 10^{-6} M$. This study was carried out prior to the freeze-drying of the preparation.

Barley $\alpha$-amylase solution (5 ml.) was preincubated at 20° for 10 min. with (a) water (5 ml.) or (b) pCMB (5 ml.; $5 \times 10^{-5} M$) before incorporation into a digest containing rabbit muscle glycogen, sample III (45 mg. in 20 ml. water) and 0.2M-acetate buffer (pH 5.6; 20 ml.). An enzyme control was also prepared. Samples were withdrawn for iodine-staining (2.5 ml.) and reducing-power (5 ml.) measurements. (Figure 19).

Digest conditions

<table>
<thead>
<tr>
<th>Incubation</th>
<th>$R.V.$ at 480 m$\mu$ (%)</th>
<th>$P_M$</th>
<th>$A.V.$ at 480 m$\mu$ (%)</th>
<th>$P_M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>75</td>
<td>1.0</td>
<td>77</td>
<td>1.3</td>
</tr>
<tr>
<td>2</td>
<td>54</td>
<td>-</td>
<td>49</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>45</td>
<td>4.9</td>
<td>38</td>
<td>4.7</td>
</tr>
<tr>
<td>4</td>
<td>41</td>
<td>-</td>
<td>27</td>
<td>6.1</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>-</td>
<td>5</td>
<td>7.0</td>
</tr>
</tbody>
</table>
The presence of β-amylase in the preparation would have been indicated by a significant difference in the above set of results, especially in the P_M values. Since this did not arise, it was considered that β-amylase was not present.

2. **Effect of Inhibitors and pH on the Action of Barley α-Amylase.**

(a) **Effect of mercuric chloride**

Digests were prepared containing soluble starch (0.1%; 20 ml.), 0.2M-acetate buffer (pH 5.6; 5 ml.), α-amylase (6 mg. in 4 ml.; insoluble material being centrifuged off), and water (1 ml.) or mercuric chloride (1 ml.). Samples (2 ml.) were removed and stained with iodine and the A.V.'s measured at 680 mλ.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Incubation (hr.)</th>
<th>Fall in A.V. (%)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1</td>
<td>16</td>
<td>29</td>
</tr>
<tr>
<td>Mercuric chloride</td>
<td>10^{-4}</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Mercuric chloride</td>
<td>10^{-5}</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Mercuric chloride</td>
<td>10^{-6}</td>
<td>14</td>
<td>15</td>
</tr>
</tbody>
</table>

Mercuric chloride had inhibited the barley α-amylase in a similar fashion to that of barley Z-enzyme (116). This inhibition is permanent since the treated α-amylase appears to cause no change in the iodine-staining power of the starch after a further incubation of an hour.

(b) **Effect of calcium ions and EDTA**

Trace amounts of α-amylase in almond emulsin (See p. 60) were stabilised by calcium ions and partially inhibited by EDTA. The action of a dilute solution of barley α-amylase on glycogen was therefore examined after preincubation of the
enzyme with the added reagents and the effects compared with those obtained previously.

$\alpha$-Amylase solution (4 mg. in 4 ml. water) was pre-incubated for 3 hr. at 37° with 0.2M-acetate buffer (pH 5.6; 5 ml.) and water (1 ml.) or calcium acetate ($3 \times 10^{-3}$M; 1 ml.) or EDTA ($3 \times 10^{-3}$M; 1 ml.). Glycogen (18 mg. rabbit muscle III in 20 ml. water) was then added and the digests incubated at 37°. An enzyme blank was also prepared. Samples (3 ml.) were removed for iodine-staining and reducing-power measurements.

<table>
<thead>
<tr>
<th>Incubation (hr.)</th>
<th>$p_M$</th>
<th>Fall in A.V. (%) at 480 m$\mu$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5.7</td>
<td>14.0</td>
</tr>
<tr>
<td>Calcium acetate*</td>
<td>7.9</td>
<td>21.6</td>
</tr>
<tr>
<td>EDTA*</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* final concentration $10^{-4}$M.

Calculated ions have increased the $\alpha$-amylolytic activity (which again probably acts as a stabiliser rather than an activator). Preincubation of the $\alpha$-amylase at 37° for 3 hr. with EDTA had succeeded in inactivating the amylolytic action completely. In a similar experiment with mercuric chloride, final concentrations of $10^{-4}$, $10^{-5}$, and $10^{-6}$M also gave complete inactivation.

(c) Effect of pH

This was observed by incubating soluble starch (0.1%; 5 ml.) with phosphate-citrate buffer (pH 4.1 - 7.1; 3 ml.) and $\alpha$-amylase (6 mg. in 2 ml. water; insoluble material being centrifuged off at 37° for 2.5 hr. Relative activities
Figure 20.
were determined by measuring the iodine-staining power of samples (3 ml.). The results can be seen on Figure 20.

The optimum pH of ca. 5.2 in agreement with that reported by other workers (29).

3. Effect of Barley $\alpha$-Amylase on the Limiting Viscosity Number of an Amylose

Barley $\alpha$-amylase (0.01%; 10 ml.) was incubated at 25° with amylose (271.6 mg., by acid hydrolysis, in 70 ml. water) and 0.2M-acetate buffer (pH 5.6; 20 ml.). Aliquots (25 ml.) were withdrawn at time intervals, heated to inactivate the enzyme, and the polysaccharide isolated as a complex by addition of n-butanol. The limiting viscosity number of the polysaccharide was determined by method 10, concentration being expressed in gm./ml.

<table>
<thead>
<tr>
<th>Time of Incubation (hr.)</th>
<th>Limiting Viscosity Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>204</td>
</tr>
<tr>
<td>1.5</td>
<td>143</td>
</tr>
<tr>
<td>3.0</td>
<td>105</td>
</tr>
<tr>
<td>9.5</td>
<td>95</td>
</tr>
<tr>
<td>19.5</td>
<td>83</td>
</tr>
</tbody>
</table>

From the relation,

\[
\text{No. of bonds hydrolysed} = \frac{[\eta]}{[\eta]_0} - 1 \quad (121)
\]

where \([\eta]_0\) = original limiting viscosity number, \([\eta]_t\) = limiting viscosity number after time, \(t\);

1.5 $\alpha$-1,4-glucosidic linkages were hydrolysed by the barley $\alpha$-amylase after incubation at 25° for 19.5 hr. This, therefore, agrees with the previous conclusions that such a trace of $\alpha$-amylase would suffice to cause an increase in the
\( \beta \)-amyloysis limit by randomly hydrolysing approximately one bond per molecule.

4. **Effect of Barley \( \alpha \)-Amylase on the \( \beta \)-Amyloysis Limit of Amylopectin and Glycogen.**

The effect of the above concentration of \( \alpha \)-amylase on amylopectin and glycogen was studied to observe if there was, in fact, an increase in the \( \beta \)-amyloysis limits.

Waxy maize starch (amylopectin IV; 191.4 mg.) or human-liver glycogen (240.9 mg.) from a case of glycogen storage-disease was dissolved in water (30 ml.) and incubated with 0.2M-acetate buffer (pH 5.6; 15 ml.) and \( \alpha \)-amylase (0.02\%; 5 ml.) at 25°. Aliquots (15 ml.) were removed at intervals and the \( \alpha \)-amylase inactivated by heating. The pH was adjusted to 3.6 with acetic acid and the \( \beta \)-amyloysis limits of the polysaccharides were then determined.

<table>
<thead>
<tr>
<th>Incubation (hr.)</th>
<th>( \beta )-Amyloysis Limits (%)</th>
<th>Amlopectin</th>
<th>Glycogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>57</td>
<td>46</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>62</td>
<td>47</td>
</tr>
<tr>
<td>22</td>
<td></td>
<td>62</td>
<td>48</td>
</tr>
</tbody>
</table>

There was a slight but significant increase in the \( \beta \)-amyloysis limits of both polysaccharides caused by pre-treatment with a low concentration of \( \alpha \)-amylase. This was probably limited in the case of glycogen, by the generally lowered affinity of \( \alpha \)-amylases for glycogen (122), as well as the low concentration of enzyme used.
5. Action of Barley $\alpha$-Amylase on the $\beta$-Dextrins of Amylose, Amylopectin and Glycogen.

This was investigated using a low concentration of $\alpha$-amylase.

Digests were prepared containing $\beta$-dextrin (amylose, 6.0 mg.; amylopectin, 15.6 mg.; glycogen, 19.0 mg. in 20 ml. water), 0.2M-acetate buffer (pH 5.6; 5 ml.), and $\alpha$-amylase (0.01%; 5 ml.). Samples (3 or 2 ml.) were removed for iodine-staining and reducing-power measurements.

<table>
<thead>
<tr>
<th>$\beta$-Dextrin</th>
<th>Incubation (hr.)</th>
<th>540 mU</th>
<th>680 mU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylopectin</td>
<td>7</td>
<td>8</td>
<td>17</td>
</tr>
<tr>
<td>Amylose</td>
<td>25</td>
<td>12</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>47</td>
<td>14</td>
<td>26</td>
</tr>
</tbody>
</table>

There was no significant change in reducing-power of the digest. The low concentration of $\alpha$-amylase had not effected either a large fall in A.V. of the amylopectin $\beta$-dextrin or an increase in reducing-power of the amylopectin and glycogen $\beta$-dextrins.

Experiments with Malt $\alpha$-Amylase.

1. Examination of Malt $\alpha$-Amylase Preparation for Contaminating Carbohydrases.

Incubation of the $\alpha$-amylase preparation (2.5 mg. in 0.5 ml. water) with cellobiose, laminarin, or maltose (2% in phosphate-citrate buffer of pH 5.9; 0.5 ml.) followed by
paper chromatographic analysis showed no cellobiase, laminarinase, or maltase activity after 24 hr.

2. Effect of Inhibitors and pH on the Action of Malt α-Amylase.

(a) Effect of mercuric chloride

The examination was carried out in a fashion similar to that of barley α-amylase (p. 74) using 0.4 mg. enzyme preparation, and soluble starch as the substrate.

<table>
<thead>
<tr>
<th>Incubation (hr.)</th>
<th>1</th>
<th>2</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fall in A.V. (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibitor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration (M)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>38</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>Mercuric chloride</td>
<td>1</td>
<td>2</td>
<td>97</td>
</tr>
<tr>
<td>10⁻¹</td>
<td>1</td>
<td>2</td>
<td>97</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>3</td>
<td>3</td>
<td>92</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>11</td>
<td>11</td>
<td>71</td>
</tr>
</tbody>
</table>

The inhibition appears to be complete after incubation for 1 hr., at 10⁻¹ M concentration.

(b) Effect of calcium ions and EDTA

Malt α-amylase (0.4 mg. in 1 ml. water) was preincubated with water (1 ml.), calcium acetate (1 ml.) or EDTA (1 ml.), and 0.2M-acetate buffer (pH 5.6; 5 ml), at 37° for 2 hr. before addition to rabbit muscle glycogen III (18 mg. in 20 ml. water). Iodine-staining and reducing-power measurements were carried out on samples (3 ml.) after 20 hr. incubation at 37°.

<table>
<thead>
<tr>
<th>Added Reagent</th>
<th>Pm</th>
<th>Fall in A.V. (%) at 480 mµ</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>8.2</td>
<td>66</td>
</tr>
<tr>
<td>Calcium acetate*</td>
<td>18.7</td>
<td>91</td>
</tr>
<tr>
<td>EDTA*</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* final concentration 10⁻¹ M.
(c) **Effect of pH.**

Starch (0.1%; 5 ml.), was incubated with phosphate-citrate buffer (pH 4.1 - 7.1; 3 ml.), and malt \(\alpha\)-amylase (0.4 mg. in 2 ml. water), at 37° for 15 min. Samples (1 ml.) were removed and stained with iodine solution, and the A.V.'s compared at 680 m\(\mu\). The results can be seen on Figure 20.

3. **Action of Malt \(\alpha\)-Amylase on the \(\beta\)-Dextrins of Amylose, Amylopectin and Glycogen.**

Digests were prepared as for barley \(\alpha\)-amylase (p. 78) using malt \(\alpha\)-amylase (0.001%; 5 ml.). The analysis was carried out in the same way to give the following results:

<table>
<thead>
<tr>
<th>Incubation (hr.)</th>
<th>(\beta)-Dextrin</th>
<th>Amylose</th>
<th>Amylepectin</th>
<th>Glycogen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fall in A.V. (%)</td>
<td>(P_M)</td>
<td>Fall in A.V. (%)</td>
<td>(P_M)</td>
</tr>
<tr>
<td>7</td>
<td>680 m(\mu)</td>
<td>540 m(\mu)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>76</td>
<td>49</td>
<td>50</td>
<td>3.3</td>
</tr>
</tbody>
</table>

From a consideration of the \(P_M\) values, malt \(\alpha\)-amylase is able to hydrolyse amylose \(\beta\)-dextrin at a greater rate than the \(\beta\)-dextrins of amylepectin and glycogen.

4. **Amylolytic action of Malt Diastase on Amylose**

(a) **Effect of inhibitors**

This experiment was carried out to compare the amylolytic properties of the commercial preparation and the above malt \(\alpha\)-amylase preparation.

\(\beta\)-Dextrin (5 mg.); 0.2M-acetate buffer (pH 5.6; 9 ml.),
0.01% diastase solution (0.5 ml.), and water or reagent (0.5 ml.) were incubated at 37° for 30 min.

The A.V. (640 mU) of a sample (3 ml.) was then determined.

<table>
<thead>
<tr>
<th>Added reagent</th>
<th>Concentration (M)</th>
<th>Fall in A.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>68</td>
</tr>
<tr>
<td>Calcium acetate</td>
<td>$2.5 \times 10^{-4}$</td>
<td>67</td>
</tr>
<tr>
<td>EDTA</td>
<td>$2.5 \times 10^{-3}$</td>
<td>66</td>
</tr>
<tr>
<td>Mercuric chloride</td>
<td>$1.5 \times 10^{-4}$</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>$1.5 \times 10^{-5}$</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>$1.5 \times 10^{-6}$</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>$10^{-5}$</td>
<td>51</td>
</tr>
<tr>
<td>p CMB</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The last observation shows the effect caused solely by the $\alpha$-amylase, and the following results are calculated on this basis.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration (M)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mercuric chloride</td>
<td>$1.5 \times 10^{-4}$</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>$1.5 \times 10^{-5}$</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>$1.5 \times 10^{-6}$</td>
<td>19</td>
</tr>
</tbody>
</table>

(b) **Effect of ions in the presence of pCMB**

The malt diastase was allowed to hydrolyse amylose $\beta$-dextrin in the presence of pCMB in order to inhibit the $\beta$-amylase activity and show the true effect of calcium ions and mercuric chloride.

$\beta$-Dextrin (4 mg. in 4 ml.), 0.2M-acetate buffer (pH 5.6; 7 ml.), 0.01% diastase solution (0.5 ml.), pCMB solution ($10^{-4}$ M; 1 ml.) and water or reagent (0.5 ml.) were incubated at 37° for 2 hr. Samples (3 ml.) were stained
with iodine solution and the A.V. measured at 630 m\u2032.

<table>
<thead>
<tr>
<th>Added reagent</th>
<th>Concentration (M)</th>
<th>Fall in A.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>(3 \times 10^{-4})</td>
<td>21</td>
</tr>
<tr>
<td>Calcium sulphate</td>
<td>(3 \times 10^{-5})</td>
<td>56</td>
</tr>
<tr>
<td>Mercuric chloride</td>
<td>(2 \times 10^{-5})</td>
<td>11</td>
</tr>
</tbody>
</table>

Calcium ions have shown an increase in the amylolytic activity, whereas mercuric chloride \((2 \times 10^{-5} M)\) has shown an inhibition \((48\%)\) which is in good agreement with the result of the previous experiment.

**Experiments with Salivary \(\alpha\)-Amylase**

1). **Effect of Enzyme Concentration**

In order to obtain a concentration of \(\alpha\)-amylase which showed a slight hydrolytic activity on glycogen \(\beta\)-dextrin, a freeze-dried preparation \((3.4\) units \((19)\); 1 mg. in 1 ml. water) was diluted with water and combined in digests with glycogen \(\beta\)-dextrin \((3\) mg./ml. in 0.05M sodium chloride; 5 ml.) and 0.2M-acetate buffer \((pH 5.8; 3\) ml.). Samples \((2\) ml.) were removed at intervals for reducing-power determinations.

<table>
<thead>
<tr>
<th>(\alpha)-Amylase</th>
<th>Incubation time 20 hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution ((x 10^3))</td>
<td>Activity/ml. ((x 10^{-4}))</td>
</tr>
<tr>
<td>2.</td>
<td>170</td>
</tr>
<tr>
<td>3.3</td>
<td>103</td>
</tr>
<tr>
<td>5.</td>
<td>68</td>
</tr>
<tr>
<td>10</td>
<td>34</td>
</tr>
<tr>
<td>15</td>
<td>23</td>
</tr>
<tr>
<td>20</td>
<td>17</td>
</tr>
<tr>
<td>50</td>
<td>6.8</td>
</tr>
</tbody>
</table>
2. **Action of Salivary α-Amylase on β-Dextrins of Glycogen, Amylopectin and Amylose.**

A diluted solution of salivary α-amylase \(6.8 \times 10^{-4}\) unit \(/\text{ml.}\) of 0.05M sodium chloride; 1 ml.), was incubated with 0.2M-acetate buffer of pH 5.8 (6 ml.) containing various \(β\)-dextrins (4.8 - 12.0 mg.). Reducing-power or iodine-staining measurements were carried out on samples (1 or 3 ml.) removed after 23 and 42.5 hr. incubation. The following results were obtained:

<table>
<thead>
<tr>
<th>β-Dextrins</th>
<th>Incubation (hr.)</th>
<th>640 m(\mu)</th>
<th>540 m(\mu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylose (4.8 mg.)</td>
<td>23</td>
<td>25</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>42.5</td>
<td>53</td>
<td>-</td>
</tr>
<tr>
<td>Amylopectin</td>
<td>23</td>
<td>-</td>
<td>24</td>
</tr>
<tr>
<td>(12.0 mg.)</td>
<td>42.5</td>
<td>-</td>
<td>39</td>
</tr>
<tr>
<td>Glycogen</td>
<td>23</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>42.5</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fall in A.V. (%)</th>
<th>P_M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylose (4.8 mg.)</td>
<td></td>
</tr>
<tr>
<td>Amylopectin (12.0 mg.)</td>
<td></td>
</tr>
<tr>
<td>Glycogen (12.0 mg.)</td>
<td></td>
</tr>
</tbody>
</table>

In addition, digests containing amylopectin \(β\)-dextrin (17.0 mg.) and glycogen \(β\)-dextrin (17.8 mg.), and either (a) \(6.8 \times 10^{-4}\) unit of α-amylase or (b) \(13.6 \times 10^{-4}\) unit, in a total volume of 16 - 27 ml., the extents of degradation were:

<table>
<thead>
<tr>
<th>Incubation (hr.)</th>
<th>Fall in A.V. (%) at 540 m(\mu)</th>
<th>P_M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylopectin (β)-dextrin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) 10</td>
<td>22</td>
<td>3.0</td>
</tr>
<tr>
<td>(b) 22</td>
<td>42</td>
<td>6.2</td>
</tr>
<tr>
<td>Glycogen (β)-dextrin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) -</td>
<td>0.5</td>
<td>1.4</td>
</tr>
<tr>
<td>(b) -</td>
<td>1.4</td>
<td>3.2</td>
</tr>
</tbody>
</table>
Under similar conditions, the diluted salivary \(\alpha\)-amylase thus hydrolyses more than twice as many bonds in amylopectin \(\beta\)-dextrin as in glycogen \(\beta\)-dextrin.

3. **Effect of Mercuric Chloride on the Action of Salivary \(\alpha\)-Amylase**

This was examined by preparing the following digests: diluted \(\alpha\)-amylase solution (6.8 x 10\(^{-4}\) unit/ml. of 0.05 M sodium chloride; 0.4 ml.) was added to 0.2M-acetate buffer (pH 5.6; 5 ml.), water or mercuric chloride solution (1 ml.), and soluble starch (0.1% in water; 20 ml.). Samples (2 ml.) were removed after 1, 2, and 24 hr. for staining with iodine solution (1 ml.). The A.V.'s measured at 680 \(\mu\) after 1 hr. were:

<table>
<thead>
<tr>
<th>Concentration (M) of HgCl(_2)</th>
<th>10(^{-4})</th>
<th>10(^{-5})</th>
<th>10(^{-6})</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decrease in A.V. (%)</td>
<td>0.9</td>
<td>4.5</td>
<td>13.5</td>
<td>17</td>
</tr>
<tr>
<td>Inhibition (%)</td>
<td>94</td>
<td>74</td>
<td>21</td>
<td>-</td>
</tr>
</tbody>
</table>

The results after 2 and 24 hr. showed the same fall in A.V. for the digests containing mercuric chloride whereas the A.V. of the control digest fell by 26 and 41%, respectively.

Mercuric chloride exerts an inhibitory effect on dilute salivary \(\alpha\)-amylase in a fashion comparable to that of other \(\alpha\)-amylases.

**Discussion**

The action of \(Z\)-enzyme, from the emulsin and
Wallerstein $\beta$-amylase preparations, has been simulated by dilute solutions of barley, malt, and salivary $\alpha$-amylases. For example the iodine-staining power of amyllose is decreased considerably with a much smaller effect on that of amylopectin; and the amyloytic action is increased by calcium ions and partially or totally inhibited by mercuric chloride and EDTA.

It was found that, in general, the $\alpha$-amylases attacked twice as many bonds in amylopectin $\beta$-dextrin as in glycogen $\beta$-dextrin. This may be governed by the fact that the interior chain length of amylopectin is usually twice that of glycogen. The lowered affinity of an $\alpha$-amylase for a branched polysaccharide, may be related to the interior chain length.

In comparable experiments, amylopectin and glycogen $\beta$-dextrins were degraded to a greater extent, by salivary $\alpha$-amylase than by equivalent concentrations of malt and barley $\alpha$-amylases. For example with glycogen $\beta$-dextrin, the $P_M$ values with $7 \times 10^{-4}$ units of salivary $\alpha$-amylase and $3.6 \times 10^{-2}$ units of malt $\alpha$-amylase were 1.4 and 1.1, respectively. This suggests that the $\alpha$-amyloytic action of the salivary $\alpha$-amylase is different from that of the malt and barley $\alpha$-amylases, since it can hydrolyse the interior chains of the branched $\beta$-dextrins at a greater rate than that of the other $\alpha$-amylases. This is probably due to the ability of salivary $\alpha$-amylase to hydrolyse $\alpha$-$1,4$-glucosidic linkages close to the branch point in amylopectin and glycogen (120), whereas malt $\alpha$-amylase can only hydrolyse
linkages when they are 5 to 6 units away from the non-reducing ends or branch points (119,122) (Figure 18). The malt (and barley) \( \alpha \)-amylase would, therefore, only attack the \( \beta \)-dextrins of amylpectin and glycogen with difficulty, the latter least of all. The failure of Banks et al (117) to detect any \( Z \)-enzyme action on glycogen, was probably due to the use of too low a concentration of enzyme. The conclusion that salivary \( \alpha \)-amylase has a greater affinity for branched substrates than plant \( \alpha \)-amylases is supported by the results of a previous investigation (123). The successive action of barley \( \beta \)-amylase, malt \( \alpha \)-amylase and finally barley \( \beta \)-amylase on rabbit liver glycogen, resulted in \( \alpha \)-dextrins, which were resistant to malt \( \alpha \)-amylase, but were partially hydrolysed by salivary \( \alpha \)-amylase.

Emulsin \( \alpha \)-amylase (\( \L 4 \times 10^{-2} \) unit) and barley \( \alpha \)-amylase (\( \L 2 \times 10^{-2} \) unit), in similar experiments, hydrolyse glycogen into maltose to the extent of 2.1 and 5.7% respectively; the former activity appears to be related to the malt and barley \( \alpha \)-amylase activities rather than that of salivary \( \alpha \)-amylase. The use of salivary \( \alpha \)-amylase by Peat et al. (102) to distinguish \( Z \)-enzyme from \( \alpha \)-amylase was not, therefore, an ideal choice since the action of the amylases from plant sources differs from that of the animal amylase.

Pretreatment of amylpectin and glycogen with a concentration of barley \( \alpha \)-amylase, which decreases the limiting viscosity number of an amylose from 204 to 83, caused the \( \beta \)-amylolysis limits to increase by 5 and 2%, respectively.
Assuming that random degradation of the amylose occurred and that there was not more than one anomalous structure per molecule, the expected \( \beta \)-amylolysis limit of the degraded amylose would be 90% (117), compared with 77% for the whole amylose. Thus, a considerable increase in the \( \beta \)-amylolysis limit of an amylose by pretreatment with a trace of \( \alpha \)-amylase, can be compared with the slight increase in \( \beta \)-amylolysis limit of amylopectin and glycogen after similar treatment.

The experiments described in Parts 2 - 4 of this Section clearly show that Z-enzyme action involves a limited random hydrolysis of \( \alpha \)-1,4-glucosidic linkages in contrast to the "debranching" action postulated by previous workers (74); no deductions concerning the structural anomalies can therefore be made. Useful information on these anomalies could, however, be obtained by the successive treatment of large amounts of pure amylose with (a) Z-enzyme (\( \alpha \)-amylase) and (b) \( \beta \)-amylase. This would yield large amounts of maltose and a small amount of residual polysaccharide.

Part 5

Effect of Various Ions on the \( \alpha \)-Amylase Activity of Cladophora rupestris

Introduction

Cladophora rupestris, a green algae of the class
Chlorophyceae and order Cladophorales, was shown by Dr. W. A. M. Duncan to contain a weak \(\alpha\)-amylase (124). This activity was greater in B.D.H. (British Drug Houses) Universal buffer than in acetate buffer. This activation effect was subsequently shown to be due to the borate ions present in the buffer. The present study was undertaken to confirm the above activation effect, and to compare the effect of various ions on the algal \(\alpha\)-amylase and on \(\alpha\)-amylases from other sources.

Methods and Materials

Unless otherwise stated the method of Smith and Roe (69) was used to estimate \(\alpha\)-amylase activity.

(a) Substrates

Commercially prepared soluble starch was used.

(b) Enzyme Preparations

Malt diastase and bacterial \(\alpha\)-amylase as supplied by Wallerstein Company, New York were used as sources of malt and bacterial \(\alpha\)-amylases, respectively. Salivary \(\alpha\)-amylase was obtained by centrifuging fresh human saliva and diluting the supernatant solution to a suitable activity with water.

Algal \(\alpha\)-amylase was obtained by extracting 5.7 Kg. of minced Cladophora rupestris (collected Dunbar 28.10.58) with aqueous sodium carbonate solution (0.25%; 9 litres). The separated extract was then dialysed at room temperature for four days against running tap-water before precipitation.
of the mixed proteins with solid ammonium sulphate to 70% saturation. The precipitate was separated at the centrifuge, dialysed at 0° against running tap-water, and freeze-dried.

Yield of Fraction A: 60 gm. (9.5% protein N). Fraction A (10 g.) was dissolved in water (600 ml.) and solid ammonium sulphate added to 25% saturation, the precipitate was separated and discarded. Further amounts of solid ammonium sulphate were then added to the supernatant solution to 75% saturation; the resulting precipitate was separated, dissolved in water, and dialysed free from inorganic material at 0°. This solution was diluted to 60 ml. with 0.2M-acetate buffer of pH 5.6 and cold isopropanol added. The precipitates which formed at 40 and 65% saturation were removed, dissolved in cold water, dialysed, and freeze-dried.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Saturation with isopropanol (%)</th>
<th>Yield (mg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0 - 40</td>
<td>225</td>
</tr>
<tr>
<td>2</td>
<td>40 - 60</td>
<td>112</td>
</tr>
</tbody>
</table>

Results

1. **Effect of Acetate and B.D.H. Buffers on α-Amylase Activity**

Digests were prepared with buffers of pH 5.6 following the conditions of Smith and Roe (69). Iodine-staining measurements were carried out at 620 mμ (Unicam Spectrophotometer) or using a 608 filter (EEL Colorimeter).
The results were as follows:

<table>
<thead>
<tr>
<th>α-Amylase</th>
<th>Relative Activities</th>
<th>Acetate</th>
<th>B.D.H.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salivary</td>
<td></td>
<td>558</td>
<td>482</td>
</tr>
<tr>
<td>Bacterial</td>
<td></td>
<td>550</td>
<td>537</td>
</tr>
<tr>
<td>Malt</td>
<td></td>
<td>365</td>
<td>204</td>
</tr>
<tr>
<td>Algal</td>
<td></td>
<td>187</td>
<td>224</td>
</tr>
</tbody>
</table>

* Incubation time 0.5 hr.
* * " " " 2 hr.

With the exception of the algal α-amylase, the activity was greatest in the acetate buffer.

2. Effect of Various Ions on the Activity of the Algal α-Amylase

(a) Effect of boric acid

Boric acid, phenylacetic acid, and sodium dihydrogen phosphate are the constituents of B.D.H. Universal buffer. The first two compounds were incorporated with malt diastase and algal α-amylase (0.3% fraction 3; 0.2 ml) into digests with the following results:

<table>
<thead>
<tr>
<th>Added Reagent</th>
<th>Concentration (M)</th>
<th>Relative Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>232</td>
</tr>
<tr>
<td>Phenylacetic acid</td>
<td>$2 \times 10^{-3}$</td>
<td>232</td>
</tr>
<tr>
<td>Boric acid</td>
<td>$7 \times 10^{-3}$</td>
<td>262</td>
</tr>
<tr>
<td></td>
<td>$14 \times 10^{-3}$</td>
<td>232</td>
</tr>
<tr>
<td></td>
<td>$21 \times 10^{-3}$</td>
<td>224</td>
</tr>
</tbody>
</table>

* 4 hr. incubation
* * 0.5 hr. incubation

The Malt α-amylase had been slightly inhibited
by the added reagents, whilst the algal $\alpha$-amylase had been activated by the low concentration of boric acid and not effected by the phenylacetic acid.

The effect of boric acid on the algal $\alpha$-amylase was also examined by determining the reducing-power of a digest after 3 hr. incubation at $37^\circ$.

<table>
<thead>
<tr>
<th>Added Reagent</th>
<th>Concentration (M)</th>
<th>$P_M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>11.8</td>
</tr>
<tr>
<td>Boric acid</td>
<td>$5 \times 10^{-3}$</td>
<td>16.6</td>
</tr>
<tr>
<td>&quot;</td>
<td>$8 \times 10^{-3}$</td>
<td>16.6</td>
</tr>
<tr>
<td>&quot;</td>
<td>$16 \times 10^{-3}$</td>
<td>9.0</td>
</tr>
</tbody>
</table>

Concentrations of boric acid below $14 \times 10^{-3}$ M appear to have an activating effect on the algal $\alpha$-amylase.

(b) **Effect of molybdate ions**

The $\alpha$-amylase activity of fraction A (0.35%; 0.3 ml.) was determined in the presence of ammonium molybdate since like borate, this anion can form complexes with carbohydrates. The results are calculated as a percentage of the activity in the absence of molybdate.

<table>
<thead>
<tr>
<th>Added reagent</th>
<th>Concentration (M)</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Ammonium molybdate</td>
<td>$5 \times 10^{-3}$</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>$8 \times 10^{-3}$</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>$16 \times 10^{-3}$</td>
<td>67</td>
</tr>
</tbody>
</table>

The $\alpha$-amylase activity has been partially inhibited rather than activated by ammonium molybdate.

(c) **Effect of halide ions**

The acetate buffer-starch reagent for the $\alpha$-amylase
activity determination was prepared in the absence of sodium chloride, so that the effect of 0.1M sodium chloride, bromide, iodide, and fluoride (1 ml.) on fraction A (0.4%; 0.2 ml.) could be investigated. The activity was determined by the iodine-staining method and by reducing-power estimations. Results were calculated as for the previous experiment.

<table>
<thead>
<tr>
<th>Added reagent</th>
<th>Activity (%)</th>
<th>Iodine-staining</th>
<th>Reducing-Power</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Sodium chloride*</td>
<td>114</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td>Sodium bromide*</td>
<td>108</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>Sodium iodide*</td>
<td>86</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>Sodium fluoride*</td>
<td>94</td>
<td>86</td>
<td></td>
</tr>
</tbody>
</table>

* final concentration $3 \times 10^{-2}$M.

The results would seemingly indicate that the algal $\alpha$-amylase activity is increased by chloride and bromide ions and decreased by iodide and fluoride ions. It has been observed, however, that the effect of these ions on animal $\alpha$-amylases is pH dependent (29), so the above conclusion is only valid at pH 5.6.

Electrophoresis of Fraction A

This was carried out as described in method 13 using 0.05M phosphate buffer of pH 7.8.

Fraction A (100 mg.) was dissolved in the buffer (3 ml.) and insoluble material centrifuged off. This was applied to the centre of a Whatman No. 54 filter paper curtain and separated continuously at 850 volts (8.5 mA). Development with nigrosine indicated that there was one main band (tubes 1 - 5) and one minor band (tubes 6 - 9) of protein moving towards the anode. The $\alpha$-amylase activity was qualitatively determined by incubating the fractions with soluble starch (0.6%; 0.1 ml.) at 37° for 24 hr.
On addition of iodine-solution, it was observed that there was a visible decrease in iodine-staining power of fractions 6, 7, and 8, the first showing the largest decrease. The protein showing α-amylase activity, in Fraction A, had been therefore separated to some extent from the other proteins.

**Discussion**

In a comparison of the activities of various α-amylases in B.D.H. Universal and acetate buffers of pH 5.6, the *Cladophora ranestris* α-amylase activity was increased in the presence of B.D.H. Universal buffer, whereas the activities of bacterial, animal, and malt α-amylase were partially inhibited.

The different relative activities of bacterial, animal, malt, and algal α-amylases in the two buffers indicates that a comparison of the effect of pH on these enzymes is only valid if the same buffer systems are used.

Boric acid, which is known to inhibit isoamylase (125), was an effective activator of the algal α-amylase at concentrations below $8 \times 10^{-3}M$, and an inhibitor at higher concentrations. The activation effect is in agreement with earlier studies (124). The reason for this effect is not known and investigations should be repeated with preparations of a higher degree of purity, perhaps obtainable from continuous electrophoresis.

In acetate buffer of pH 5.6, the algal α-amylase activity is partially inhibited by molybdate, fluoride, and iodide ions, and activated by chloride and bromide ions.
Summary

1. The presence of a trace of $\alpha$-amylase in barley $\beta$-amylase and emulsin provides an explanation for the observed increase in $\beta$-amylolysis and phosphorolysis of amylose (+), formerly attributed to the presence of Z-enzyme. The random hydrolysis of non terminal linkages will expose sufficient new non-reducing end groups to allow further $\beta$-amylolysis to take place. Amylopectin and Glycogen are also attacked, the rate of hydrolysis being lower than that observed with amylose. There is also limited amylolytic action on their $\beta$-dextrins.

2. The $\alpha$-amylase activity of emulsin is optimum at pH 5.7, stabilised by calcium ions, and partially inhibited by EDTA and mercuric chloride. Further amounts of $\alpha$-amylase can be extracted from the material remaining after the preparation of emulsin. Dilute solutions of barley and malt $\alpha$-amylase can simulate Z-enzyme action. It is suggested that the $\alpha$-amylase of emulsin resembles barley and malt $\alpha$-amylases, rather than salivary $\alpha$-amylase, with regard to detailed mode of action.

Electrophoresis has been used to separate the Z-enzyme activity from the $\beta$-amylase activity in the barley $\beta$-amylase preparation.

Viscometry has provided a rapid and sensitive analytical method for the detection of trace amounts of $\alpha$-amylase, especially, when amylose $\beta$-dextrin is used as substrate.

3. Since Z-enzyme action is $\alpha$-amylolytic in nature it provides no indication of the structural anomalies in amylose. Z-enzyme
is clearly not a debranching enzyme. The structural anomaly in amylose may possibly be 1) a linkage other than the $\alpha$-1,4-type, or 2) a substituted glucose residue, or 3) a combination of 1 and 2. These results agree with the independent findings of other workers (101, 104, 117).

4. The $\alpha$-amylase activity of Cladophora runestris has been shown to be increased by low concentrations of borate, chloride, and bromide ions, and decreased by molybdate, fluoride, and iodide ions.
### Table VI.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Principal linkages</th>
<th>Other linkages</th>
<th>Other units</th>
<th>Occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lichenan</td>
<td>$1 \rightarrow 3$ 30%</td>
<td>--</td>
<td></td>
<td>Lichens</td>
</tr>
<tr>
<td></td>
<td>$1 \rightarrow 4$ 70%</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laminarin</td>
<td>$1 \rightarrow 3$</td>
<td>$1 \rightarrow 6$</td>
<td>Mannitol (ca. 2%)</td>
<td>Seaweeds</td>
</tr>
<tr>
<td>Callose</td>
<td>$1 \rightarrow 3$</td>
<td>--</td>
<td>Uronic acid (2%)</td>
<td>Grape vines</td>
</tr>
<tr>
<td>Oat glucan</td>
<td>$1 \rightarrow 3$ 30%</td>
<td>--</td>
<td></td>
<td>Oat grain</td>
</tr>
<tr>
<td></td>
<td>$1 \rightarrow 4$ 70%</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Barley glucan</td>
<td>$1 \rightarrow 3$ 50%</td>
<td>--</td>
<td></td>
<td>Barley grain</td>
</tr>
<tr>
<td></td>
<td>$1 \rightarrow 4$ 50%</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pustulan</td>
<td>$1 \rightarrow 6$</td>
<td>--</td>
<td></td>
<td>Lichens</td>
</tr>
<tr>
<td>Pachyman</td>
<td>$1 \rightarrow 3$</td>
<td>--</td>
<td></td>
<td>Poria sclerotia</td>
</tr>
<tr>
<td>Yeast glucan</td>
<td>$1 \rightarrow 3$</td>
<td>$1 \rightarrow 2$</td>
<td>Glucuronic acid (50%)</td>
<td>Yeast cell walls</td>
</tr>
<tr>
<td>Pneumococci type III polysaccharide</td>
<td>$1 \rightarrow 3$ 50%</td>
<td>--</td>
<td></td>
<td>Pneumococci capsules</td>
</tr>
<tr>
<td></td>
<td>$1 \rightarrow 4$ 50%</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luteic acid</td>
<td>$1 \rightarrow 6$</td>
<td>$1 \rightarrow 3$, $1 \rightarrow 4$</td>
<td>Esterified malonic acid</td>
<td>Fungi (Penicillium)</td>
</tr>
<tr>
<td>Crown gall polysaccharide</td>
<td>$1 \rightarrow 2$</td>
<td>?</td>
<td></td>
<td>Phytomonas tumefaciens</td>
</tr>
<tr>
<td>Mung bean seedlings polysaccharide</td>
<td>$1 \rightarrow 3$</td>
<td>--</td>
<td></td>
<td>Mung beans</td>
</tr>
</tbody>
</table>
Section IV

Studies on the

Preparation of Specific β-Glucosanases.

Introduction

Polysaccharides containing β-glucosidic linkages occur naturally in plants, cereals, seaweeds and microorganisms as cell-wall constituents, reserve materials, capsules, or other extracellular substances (for review see 126). These β-glucosans possess a variety of structures; some appear to be linear polymers whereas others may be branched. The occurrence and main structural features of the most important members of this group are shown in Table VI.
Enzymic Degradation of \( \beta \)-Glucosans.

Cellulose and the related \( \beta \)-glucosans constitute one of the most abundant groups of organic substances in Nature, so that a study of their biological degradation is of industrial and scientific importance. The enzymes responsible for this degradation (127, 128) are found in a variety of living organisms, e.g. as intracellular constituents of the higher plants, in the glands of invertebrates (129), and are produced extracellularly by bacteria and fungi.

Cellulases

The most common sources of cellulases (130) are the digestive gland of the snail, *Helix pomatia*, and the culture media or mycelium extracts of fungi, especially of the genus *Aspergillus*. The latter organisms are responsible for the microbial decay of wood, paper, and cellulosic textiles. Compared with the *invivo* action, the *in vitro* hydrolysis of the natural polymer is limited. This action can be improved considerably by treatment of the cellulose molecule with reagents such as acid or alkali, or the preparation of soluble derivatives (e.g. carboxymethylcellulose). Reese and his coworkers (131) have postulated the existence of two enzymes, one which alters the physical state of the molecule and releases the polymeric chains which can then be degraded by the second enzyme. The former enzyme, \( C_1 \) or "swelling-factor," has not yet been isolated from cell-free culture media.

The hydrolytic action of cellulase may follow one of
The following patterns: a) endwise removal of glucose units; b) endwise removal of groups of glucose units followed by hydrolysis of the oligosaccharides by other enzymes i.e. the existence of a "polyase" and "oligase" or cellobiase as suggested by Grassmann and his coworkers (132); and c) random hydrolysis of the polymeric chains giving initially cellobiose and cellotriose, and eventually glucose, as favoured by the results of Whitaker, and Reese, Samulka, and Perlin (133). The analysis of crude cellulase preparations from various fungi has shown that chromatographically distinct cellulases are present (134) suggesting that there is a complex system of related enzymes involved in the degradation of cellulose.

**Laminarinase**

Recent evidence has suggested that the enzyme which hydrolyses $\beta-1,4$-linked polymers is distinct from that which hydrolyses the $\beta-1,3$-linkages in laminarin. Laminarinase appears to be more heat labile than celldextrinases (135), and is also present in culture filtrates of fungi which do not show cellulolytic activity (136). Murti and Stone have recently distinguished the laminarinase and cellulase activities of *Aspergillus niger* by adsorption chromatography (137). These results are in contrast to those of Ploetz and Pogacar (138) who concluded that the enzymic hydrolysis of $\beta-1,4$-, $\beta-1,6$-, and $\beta-1,3$-linkages was due to a non-specific polysaccharase rather than to specific enzymes.

The enzymic degradation of laminarin may be due to two
distinct enzymes, one which catalyses the hydrolysis of non-terminal \( \beta-1,3 \)-linkages (endo-laminarinase) and has been found in extracts from seaweed (139), fungi (136), and cereals (140), whilst the other enzyme hydrolysates terminal linkages (exo-laminarinase) and occurs in almond emulsin (38), and fungi (136). Malted barley has been shown to contain both enzymes (141).

Lichenase

Lichenase activity is shown by organisms which also possess either laminarinase or cellulase activities or both. Since lichenin contains both \( \beta-1,4 \)- and \( \beta-1,3 \)-linkages, and it is accepted that the enzymes randomly hydrolysing \( \beta-1,4 \) and \( \beta-1,3 \)-linkages are distinct from each other, there is the possibility that either or both of these enzymes will degrade lichenin. Various workers have found that the cellulase and lichenase activities of certain enzyme preparations could not be separated (137, 142), whereas other workers have stated that purified cellulase preparations had no action on lichenin (143). Recent observations by Parrish, Perlin, and Reese (144) indicate that fungal "cellulase" and "laminarinase" can act on polysaccharides containing both \( \beta-1,3 \)- and \( \beta-1,4 \)-linkages.

Information regarding the fine structure of the \( \beta \)-glucosans can be provided by a study of the oligosaccharides resulting from their enzymic degradation. Purified enzyme preparations are required for this purpose, so that it is essential to purify complex mixtures such as those present in ungerminated and malted barley or to selectively inhibit interfering enzymes.
The selective production of enzymes by micro-organisms has recently proved of value and will be discussed later.

This Section is therefore concerned with the attempts to isolate purified enzyme preparations showing hydrolytic activity towards $\beta$-glucosans.

**Nomenclature.**

The following terms will be used subsequently:

- **Cellodextrin** refers to the water-soluble oligosaccharides produced by partial acid hydrolysis of cellulose.
- **Cellodextrinase** activity is equal to the sum of the $\beta$-$1,4$-glucosanase and $\beta$-$1,4$-glucosidase activities. **Laminarinase** activity is similarly equal to the combined action of the $\beta$-$1,3$-glucosanase and $\beta$-$1,3$-glucosidase activities. **Lichenase** activity refers to the enzymic degradation of lichenin to reducing sugars. The term cellulase is used to indicate the enzymic degradation of insoluble natural cellulose. For clarification, it will be assumed that $\beta$-$1,4$-glucosidase and cellobiase are identical, and $\beta$-$1,3$-glucosidase and laminaribiose are also identical.
Part I

Fractional Precipitation by Ammonium Sulphate

The fractional precipitation and subsequent purification of a wide variety of enzymes has been reported using inorganic salts, especially ammonium sulphate, as precipitants. Barley extracts were fractionated in this way by Dr. F. B. Anderson and the fractions so obtained have now been examined for $\beta$-glucosidase and $\beta$-glucosanase activities.

Methods and Materials

a) Substrates

Celllobiose, salicin, and phenyl $\beta$-glucoside were commercial preparations; laminarin was supplied by the Institute of Seaweed Research; lichenin was prepared by Dr. F. B. Anderson; cellodextrin, kindly donated by Professor C.S. Hanes, F.R.S., had a D.P. of 6 - 7 (Method 8b).

b) Enzyme Preparations

Barley flour (1 kg.) was extracted with 0.2M-acetate buffer (3.5L; pH 5). The pH of the extract was then brought to 4.5 and solid ammonium sulphate added to give fractions 0 - 35, 35 - 50, 50 - 65, 65 - 80 (% w/v). This fractionation was carried out by Dr. F. B. Anderson.

c) Analytical Methods

Reducing sugars were estimated by the methods of Somogyi, and Philips and Caldwell.
The activity of the enzyme fractions was determined by preparing the following digests:
Substrate (3 mg./ml.; 5 ml.) and an aliquot (3 ml.) from a solution containing enzyme preparation (20 mg.), 0.2M-acetate buffer (pH 5.0; 5 ml.) and water (5 ml.) were incubated at 35° for 2 hr. The reaction was terminated by the addition of zinc sulphate (5%; 1 ml.) and barium hydroxide (0.3N; 1 ml.) and after centrifugation, samples (2 or 3 ml.) were withdrawn for reducing-power estimations. Relative activities were calculated as the apparent hydrolysis to glucose (%) per mg. N.

Due to the insoluble nature of natural cellulose, a soluble derivative, sodium carboxymethylcellulose (Cellofas B, I.C.I. Limited), was also used to determine the presence of -1,4-glucosanase activity. Enzyme (0.1, 0.2, or 0.25% in 0.2M-acetate buffer of pH 5.0; 5 ml.) was incubated with Cellofas B (0.25%; 10 ml.) in an Ostwald viscometer at 25°. The activity was then calculated as shown on p.23, t, being measured in hours.

1. Enzymic Activities and Protein N Content of the Fractions

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Yield (mg.)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction</td>
<td>% N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 - 35</td>
<td>2.8</td>
<td>2,600</td>
<td>54</td>
<td>19</td>
<td>20</td>
<td>34</td>
<td>60</td>
</tr>
<tr>
<td>35 - 50</td>
<td>5.9</td>
<td>2,300</td>
<td>189</td>
<td>19</td>
<td>15</td>
<td>63</td>
<td>95</td>
</tr>
<tr>
<td>50 - 65</td>
<td>4.5</td>
<td>2,300</td>
<td>341</td>
<td>38</td>
<td>24</td>
<td>83</td>
<td>178</td>
</tr>
<tr>
<td>65 - 80</td>
<td>1.7</td>
<td>2,000</td>
<td>433</td>
<td>47</td>
<td>55</td>
<td>211</td>
<td>177</td>
</tr>
</tbody>
</table>

Substrates
1. Celllobiose
2. Salicin
3. Phenyl β-glucoside
4. Cellodextrin
5. Laminarin
6. Lichenin
The above results have shown that there has been no complete separation of the barley system into specific enzymes by ammonium sulphate fractional precipitation.

2. "Cellulase" activity

The effect of various concentrations of fraction 50 – 65 on the viscosity of Cellofas B solution was determined.

<table>
<thead>
<tr>
<th>Concentration of enzyme (%)</th>
<th>0.10</th>
<th>0.20</th>
<th>0.25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative Activity</td>
<td>0.60</td>
<td>1.22</td>
<td>1.57</td>
</tr>
</tbody>
</table>

Since there is a linear relationship between enzyme concentration and activity, random degradation had taken place i.e. fraction 50 – 65 contains a true \( \beta-1,4\)-glucosanase. Measurements carried out on the digests showed that the fall in viscosity was accompanied by an increase in reducing-power; paper chromatographic examination indicated that glucose and a trace of oligosaccharides were present after 24 hr. incubation.

**Discussion**

Precipitation with ammonium sulphate had not separated the barley extract into fraction which showed higher specific activity towards cellobiose, salicin, phenyl \( \beta\)-glucoside, laminarin, cellodextrin or lichenin. The distribution of the enzymic activities does not follow a constant pattern indicating that different enzymes are responsible for the hydrolysis of the \( \beta\)-glucosides and \( \beta\)-glucosans. Fractions which show enzymic activity towards laminarin and cellodextrin also show activity towards lichenin.
Part 2.

Adsorption Chromatography

It has been shown by various workers that enzymes can be separated by adsorption techniques (145) involving the reversible adsorption of the enzymes on columns of substances such as alumina, calcium phosphate, ion-exchange resins, and more recently, ion-exchange cellulose; the enzymes can then be removed by selective elution with dilute salt or buffer solution. Alumina was therefore used as an adsorbent in an attempt to separate the $\beta$-glucosidases and $\beta$-glucosanases of malted barley. A preliminary fractionation was also carried out using DEAE cellulose (113).

Methods and Materials

(a) Substrates

In addition to the substrates described in Part I of this Section, maltose and soluble starch were commercial preparations, and gentiobiose was prepared by Dr. W.A.M. Duncan.

(b) Enzyme Preparation

Malted Ymer barley flour (200g.; hand ground) was extracted with 1% aqueous sodium chloride (800 ml.) for 29 hr. at 0°. After centrifugation, the clear supernatant solution was saturated with ammonium sulphate. The precipitate which formed overnight, was separated, dissolved in water, and dialysed
against running tap-water at 0° for 48 hr. The resulting solution was then freeze-dried. Yield: 4 g. (Ml).

(c) Chromatographic Separation

(i) Alumina

The separation was carried out as described in Method 12. Spence (activated) alumina, type H was used since Brockmann alumina was not available. In the first separation, 300 mg; fraction Ml were adsorbed on to a column (20 x 2 cm.) and eluted with buffer of increasing pH; forty five fractions (5 ml.) were collected.

(ii) DEAE Cellulose

DEAE cellulose (Reeve Angel and Co., London; 30 gm.) was added with stirring to 1N sodium hydroxide (1 litre), filtered, and washed with water. The pH of the slurry was adjusted to 8.0 by the addition of concentrated phosphoric acid. before equilibrating the cellulose with phosphate-citrate buffer of pH 8.0. The adsorbent was then poured as a slurry into a glass column fitted with a coarse fritted disc and packed under pressure to a constant height. This column (30 x 3 cm.) was washed with the above buffer.

In the second separation fraction Ml (600 mg.) was adsorbed on to the column and eluted with buffer of decreasing pH, phosphate-citrate buffer (pH 4.0) being added to buffer of pH 8.0 (150 ml.) in the mixing chamber at a constant rate (constant gas pressure applied).

(d) Enzymic Activities
The column fractions were analysed by preparing digests containing column fraction (0.25 ml.) and substrate (2% in phosphate-citrate buffer of pH 5.9) and incubating at 37°. Samples were examined by paper chromatography (Solvent 1).

The cellulase activity was determined by incubating the fraction (2 ml.) with Cellofas B (0.25%; 10 ml.) and phosphate-citrate buffer (pH 5.9; 3 ml.) at 25°. The fall in viscosity of the digest was measured and the activity calculated by method 10.

Results

Chromatography on Alumina

The distribution of protein in the fractions can be seen on Figure 22. Table VII records the enzymic activities of the protein eluted from the alumina column after 24 hr. incubation with the substrates. From the results, there has not been a complete separation of specific enzymic activities. The sugars with R_g values of 0.75 and 0.41 corresponded to laminaribiose and gentiobiose respectively. Incubation of fractions 19, 21, and 23 with laminarin produced a series of four oligosaccharides; assuming that these were di-, tri-, tetra-, and penta-saccharides, there was a linear relationship between DP, and the value of \( \log \frac{R_g}{1 - R_g} \).

Chromatography on DEAE Cellulose

Figure 23 illustrates the distribution of protein in the DEAE column fractions. The enzymic activities of the fractions
## Table VII

### Distribution of Enzymic Activities of Malt Protein Eluted from an Alumina Column

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Sugars produced</th>
<th>Enzyme indicated</th>
<th>Fraction Number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1-14 18 19 21 23 25 28 30 31 35 39 43 45</td>
<td></td>
</tr>
<tr>
<td>Cellofas B</td>
<td>Glucose</td>
<td>β-1,4-glucosanase</td>
<td>t t t t t t t t t t t</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Relative Activity</td>
<td>0.34 0.51 0.52 0.56 0.14 0.11</td>
</tr>
<tr>
<td>Cellobiase</td>
<td>Glucose</td>
<td>cellobiase</td>
<td>t + + +++ +++ +++ +++ +++ +++ + +</td>
</tr>
<tr>
<td>Oligosaccharides</td>
<td></td>
<td></td>
<td>t t t + + + + + + + +</td>
</tr>
<tr>
<td>R₉ 0.75</td>
<td>Trans-β-</td>
<td></td>
<td>t t t + + + + + + + +</td>
</tr>
<tr>
<td>R₉ 0.41</td>
<td>glucosylase</td>
<td></td>
<td>+ + + + + + + + + +</td>
</tr>
<tr>
<td>Laminarin</td>
<td>Glucose</td>
<td>Laminarinase</td>
<td>t t + +++ +++ + + + + t</td>
</tr>
<tr>
<td>Laminaribiose</td>
<td>β-1,3-glucos-</td>
<td></td>
<td>t + + t</td>
</tr>
<tr>
<td></td>
<td>anase</td>
<td></td>
<td>t + +</td>
</tr>
<tr>
<td>Oligosaccharides</td>
<td></td>
<td></td>
<td>t + + + + + + + + + + + +</td>
</tr>
<tr>
<td>ϕ R₉ 0.41</td>
<td>Trans-β-glucosylase</td>
<td></td>
<td>+ + + + + + + + + +</td>
</tr>
</tbody>
</table>

Strength of spot on paper chromatogram indicated by:
- t = trace
- + = light
- ++ = medium
- +++ = heavy

ϕ complete hydrolysis of original substrate
Ø after incubation for 72 Hrs.
are recorded in Table VIII after 24 hr. incubation; all activities are recorded as glucose produced.

Table VIII.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Fraction Number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Cellobiase</td>
<td>++</td>
</tr>
<tr>
<td>Maltase</td>
<td>+</td>
</tr>
<tr>
<td>Laminarinase</td>
<td>+</td>
</tr>
</tbody>
</table>

Extent of activity indicated as in Table VII.

There is an indication that some enzymic activities have been separated, i.e. cellobiase activity is present in fractions which do not exhibit laminarinase or maltase activities.

Discussion

The distribution of laminarinase activity recorded in Table VII indicates that degradation of laminarin is due to two enzymes, one (fractions 19 - 23), catalysing random hydrolysis of the \( \beta \)-1,3-linkages with the production of oligosaccharides, and the other (fractions 39 - 43), catalysing the stepwise degradation of the \( \beta \)-1,3-linkages to form glucose. The intermediate fractions probably also possess \( \beta \)-1,3-glucosanase activity, since oligosaccharides would be necessary for trans-\( \beta \)-glucosylation to take place; glucose alone is not a suitable substrate for trans-\( \beta \)-glucosylation (43). Fractions 25 - 35 show trans-\( \beta \)-glucosylase activity, which results in the formation of oligosaccharides from cellobiose and from the degradation products of laminarin. The final product of this enzymic action is a carbohydrate with
Rg of 0.41. This is tentatively identified as gentiobiose since previous workers have observed the preferential formation of $\beta$-1,6-linkages after trans-$\beta$-glucosylation of cellobiose (43, 45). Dillon and O'Cola (140) observed that extracts of wheat, oats, and barley acting on laminarin caused the formation of a series of oligosaccharides which, with one exception, were eventually hydrolysed to glucose; the oligosaccharide remaining had an Rg of 0.51 (ethylacetate: pyridine: water solvent of unspecified composition). This oligosaccharide did not accumulate during the degradation of laminarin by a potato extract. It is now evident that the oligosaccharide is produced by a trans-$\beta$-glucosylase in the cereal extracts which is absent in the potato extract.

There has been no definite separation of the cellobiase and laminarinase activities on the above alumina in contrast to the results of earlier workers (141) who obtained a separation using Brockmann alumina. DEAE cellulose does, however, show a slight separation of activities which would merit further investigation.

$\beta$-1,4-Glucosanase activity appears to be concentrated in alumina column fractions 18 - 28 indicating a slight separation from the cellobiase activity.

Using the above conditions, the malt preparation has not been separated into fractions showing activity towards the specific $\beta$-1,3 or $\beta$-1,4-linkages. Stone and Murti (137) have fractionated the carbohydrases of \textit{Aspergillus niger} by gradient-elution chromatography on calcium phosphate and anion-exchange resins. Although they obtained fractions which showed differences in specificity towards substrates containing
β-1,4-glucosidic linkages, they were unable to completely separate the β-1,4- and β-1,3-glucosanases. This emphasises the difficulty of such separations.

Part 3.

Electrophoresis

Since the β-amylase and Z-enzyme activities of a barley preparation had been successfully separated (Section III) by continuous electrophoresis, it was decided to make use of this method in an attempt to separate the β-glucosanases of a malted barley preparation.

Methods and Materials

(a) Substrates

With the exception of laminaribiose, which was kindly supplied by Dr. W. J. Whelan, the substrates are described in Parts 1 and 2 of this Section.

(b) Enzyme Preparation

This was malted barley fraction M1, the preparation of which has been described in Part 2 of this Section.

(c) Electrophoresis

The electrophoretic separation of fraction M1 (100 mg) was carried out as described in Method 13 (600 volts; 24 mA; 40 hr.) using 6.05M phosphate buffer of pH 7.3 at room temperature. On development with nigrosine, two main bands of protein were
FIGURE 24.
observed, one travelling vertically (fractions 15 - 18), and the other moving to the anode (fractions 12 - 14). The distribution of protein in the fractions was determined by comparing the A.V. of the solutions at 280 \( \mu \) and is shown on Figure 24.

(d) **Enzymic Activities**

The fractions (0.3 ml) were incubated with substrate (4 - 5 mg./ml; 1 ml.) and 0.2M-acetate buffer of pH 5.6 at 37\(^\circ\) for 2 hr. The reducing-power of samples (4 ml.) was measured and the increase (in ml. 0.01N sodium thiosulphate) taken as an estimate of the relative activity.

**Results and Discussion**

Figure 24 records the activity of the fractions towards cellobiose, laminaribiose, lichenin, cellodextrin, and laminarin.

The cellobiase and laminaribiase activities have been separated to some extent, fraction 14 possessing high laminaribiase activity with low cellobiase activity, whilst fraction 17 shows the reverse. The laminarinase activity occurs mainly in fractions 15 and 17, the high activity in the former probably being partly due to the presence of large amounts of laminaribiase. Lichenase activity is at a maximum in fraction 17, but no conclusion can be made as to which \( \beta \)-glucosanase is responsible for the degradation.

The complexity of the enzyme mixture in malted barley is well illustrated by Figure 24; and it can be seen that continuous electrophoresis of malted barley has not separated the enzymes catalysing the hydrolysis of the \( \beta \)-glucosans, laminarin,
lichenin, and cellodextrin; partial separation of the \( \beta \)-glucosidases, cellobiase and laminaribiase has occurred and with further fractionation, laminaribiase free from cellobiase could be obtained. There is, however, a definite indication that laminaribiase, cellobiose, laminarin and cellodextrin are hydrolysed by different enzymes.

Part 4.

Selective Inhibition

One method of studying the action of a specific enzyme in a mixture is to selectively inhibit the interfering enzymes. Conchie and Levvy (146) have shown that glyconolactones are specific inhibitors of the glycosidases, in sheep rumen liquor, the delta and gamma lactone being equally effective. This was confirmed by Reese and Mandels (147) who found that in particular, \( \beta \)-glucosidases in fungal extracts were strongly inhibited by gluconolactone. These extracts also contained polysaccharases which could then be studied without interference from the contaminating oligosaccharases. Festenstein (148) also found that the cellobiase activity of sheep rumen liquor could be completely inhibited by glucono-1,4-lactone at a concentration which inhibited the \( \beta \)-1,4-glucosanase activity to the extent of 60%. In studies by Preece and Hoggan (149), phenylmercuric nitrate was used to completely inhibit the exo-glucosanase activity of a malted barley preparation with a retention of 50% of the endo-glucosanase activity.

This part deals with the selective inhibition of the
enzymic activities of barley and malt preparations by glucono-
1,4-lactone (gamma lactone) phenylacetic acid and phenylmercuric
acetate.

Methods and Materials.

(a) Substrates

The substrates have been described in Part 1 of this
Section, with the exception of the commercial preparations of
glucono-1,4-lactone, phenylmercuric acetate and phenylacetic
acid.

(b) Enzyme Preparations

(i) The barley fraction 50 - 65 described in Part 1
of this Section was used in inhibition studies with phenylacetic
acid (PA) and phenylmercuric acetate (PMA).

(ii) An enzyme preparation from malted barley was
prepared by extracting ground flour (1 Kg.) with 0.2M-acetate
buffer (2.3L; pH5) at room temperature for 2 hr. with constant
stirring. The grain residue was removed and the solution dia-
lysed for 16 hr. against running tap-water. The protein in
the solution (pH 5.7) was completely precipitated at 0° by the
addition of varying amounts of solid ammonium sulphate to 100%
saturation. The resulting precipitates were dissolved in ice-
cold water and dialysed against running distilled water at 0°
for 48 hr. This solution was then freeze-dried.

<table>
<thead>
<tr>
<th>Ammonium Sulphate (% Saturation)</th>
<th>Yield (G.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 28</td>
<td>3.70</td>
</tr>
<tr>
<td>28 - 55</td>
<td>2.50</td>
</tr>
<tr>
<td>55 - 100</td>
<td>Nil.</td>
</tr>
</tbody>
</table>
From qualitative tests, fraction 28 - 55 (M2) was found to be the most active and was, therefore, used in the subsequent experiments.

(iii) Emulsin II described in Section III, Part 3 was also used as a source of \( \beta \)-glucosidases.

**Enzymic Activities in Presence of Inhibitors**

(i) The effect of PA and PMA on laminarinase, celldextrinase and lichenase was determined by preparing the following digests: Substrate (3 mg./ml.; 4 ml.), water or inhibitor (5 mg./ml.; 1 ml.) and an aliquot (3 ml.) from a solution containing fraction 50 - 65 (20 mg.), 0.2M-acetate buffer (pH 5.6; 5 ml.), and water (5 ml.), were incubated at 37\(^\circ\) for 2 hr. Samples (3 ml.) were deproteinised with zinc sulphate - barium hydroxide and the reducing-power determined. The inhibition is calculated as a percentage of the uninhibited reaction.

(ii) The inhibitory effect of 0.02M glucono-\(1,\beta\)-lactone on the \( \beta \)-glucosanases and \( \beta \)-glucosidases was studied by incorporating varying amounts (0 - 3 ml.) with substrate (5 mg./ml.; 1 ml.) water (0. - 3 ml.), and enzyme solution (5 mg./ml. of 0.2M-acetate buffer pH 5.6; 1 ml.), to a total volume of 5 ml. These digests were incubated at 37\(^\circ\) and reducing-power measurements carried out on a sample (2 ml.) after 0 and 1 hr. The inhibition (%) was calculated from a comparison with the reducing-power of a control digest.

Lichenase activity (150) was also measured in the
presence and absence of gluconolactone by determining the fall in viscosity of a digest containing lichenin solution (12 mg./ml. of 4% aqueous urea; 5 ml.), enzyme solution (10 mg./ml. of 0.2M-acetate buffer of pH 5.6; 3 ml.) and water or 0.02M glucono-1,4-lactone (2 ml.) at 37° during a period of 1 hr. The activity was calculated as previously indicated (p. 23).

Results
1. **Effect of Phenylacetic Acid and Phenylmercuric Acetate**

The inhibition of the enzymic activities of fraction 50 - 65 is tabulated below:

<table>
<thead>
<tr>
<th>Substrate</th>
<th>P.A.</th>
<th>PMA.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminarin</td>
<td>6</td>
<td>60</td>
</tr>
<tr>
<td>Cellobextrin</td>
<td>5</td>
<td>61</td>
</tr>
<tr>
<td>Lichenin</td>
<td>13</td>
<td>50</td>
</tr>
</tbody>
</table>

Incubation with phenylacetic acid and phenylmercuric acetate has not, therefore, selectively inhibited the enzyme activities of barley.

2. **Effect of Glucono-1,4-lactone**

(a) **Effect of incubation time on inhibition of \( \beta \)-1,4-glucosidase activity.**

Digests were prepared containing cellobiose (5 mg./ml.; 2 ml.), enzyme solution (5 mg. barley fraction 50 - 65 in 0.2M-acetate buffer of pH 5.6; 2 ml.), 0.02M glucono-1,4-lactone or water (1 ml.) in a total volume of 10 ml. i.e. 2 in M lactone present. Samples (1 - 2 ml.) were removed
FIGURE 25

Lactone absent
Lactone present

FIGURE 26

α-Cellobiase
Celldextrinase
Laminaribiase
Laminarinase

CONCENTRATION of LACTONE (m Moles)
after intervals of incubation at 37° and the total reducing-power determined (Figure 25).

<table>
<thead>
<tr>
<th>Incubation (hr.)</th>
<th>Control</th>
<th>Lactone present</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>10.15</td>
<td>0.25</td>
<td>98</td>
</tr>
<tr>
<td>1.0</td>
<td>12.05</td>
<td>0.35</td>
<td>97</td>
</tr>
<tr>
<td>1.5</td>
<td>12.75</td>
<td>2.25</td>
<td>82</td>
</tr>
<tr>
<td>2.0</td>
<td>13.85</td>
<td>3.65</td>
<td>74</td>
</tr>
<tr>
<td>2.5</td>
<td>14.05</td>
<td>4.95</td>
<td>65</td>
</tr>
</tbody>
</table>

At a concentration of 2mM, glucono-1,4-lactone will competitively inhibit the β-1,4-glucosidase activity of a barley preparation during an incubation period of 1 hr. The decrease in inhibitory action is due to the hydrolysis of the lactone to gluconic acid which does not act as a competitive inhibitor. Subsequent digests were incubated for 1 hr.

(b) **Inhibition of the β-glucosidases and β-glucosanases of barley**

The effect of gluconolactone on the above enzymes in the barley preparation 50 - 65 was as follows:

<table>
<thead>
<tr>
<th>Concentration of gluconolactone (mM)</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-1,3-Glucosidase</td>
<td>80</td>
<td>91</td>
<td>99</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>β-1,4-Glucosidase</td>
<td>97</td>
<td>99</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Laminarinase</td>
<td>67</td>
<td>72</td>
<td>78</td>
<td>79</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>Cellodextrinase</td>
<td>77</td>
<td>88</td>
<td>94</td>
<td>-</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

These results (Figure 26) indicate that at a concentration of 8 mM gluconolactone, the β-glucosidase and cellodextrinase activities of barley are completely inhibited whilst 22% of the laminarinase activity is retained. Since the β-glucosidases
FIGURE 27.
are inhibited, the remaining laminarinase activity must be due completely to the random action of a \( \beta-1,3 \)-glucosanase. This therefore suggests that this enzyme is distinct from the \( \beta-1,4 \)-glucosanase which is completely inhibited.

In similar digests with emulsin II as the source of the \(-\)glucosidases, the gluconolactone inhibited the \( \beta-1,3 \)-glucosidase activity to the extent of 74, 86, and 89% at concentrations of 2, 4, and 6 mM respectively, whereas the \( \beta-1,4 \)-glucosidase activity was suppressed. These results, together with the above results for barley, indicate that two distinct enzymes are responsible for the hydrolysis of laminaribiose and cellobiose.

(c) Inhibition of the \( \beta \)-glucosanases of Malted barley
Reducing-power measurements

The enzymic activity of barley is known to increase during malting (151) so that the effect of gluconolactone on the malted barley fraction, M2, was examined. The results can be seen on Figure 27.

In the presence of 10 mM glucono-1,4-lactone, the laminarinase activity has been reduced by 67%, and the cellodextrinase and \( \beta-1,4 \)-glucosidase activities have been completely inhibited. The enzymes which hydrolyse lichenin and laminarin are inhibited in an identical manner indicating that the same enzyme may be finally hydrolysing both substrates. The results of this inhibition study are similar to those obtained with the barley fraction 50 - 65. It must be noted, however, that several barley preparations, made by fractional precipitation with ammonium sulphate, did not show the preferential
inhibition of the celloextrinase activity by glucono-1,4-lactone with a retention of laminarinase activity. The alteration in enzyme source and, hence, in the proportion of the enzymes present, appears to signify that the inhibition is dependent on the enzyme preparation.

In a digest prepared with laminarin (5 mg./ml.; 1 ml.), enzyme solution (malt fraction M2, 5 mg./ml of 0.2M-acetate buffer of pH 5.6; 1 ml.), and water or 0.02M glucono-1,4-lactone (pH adjusted to 5.6 with sodium hydroxide) to a total volume of 5 ml., it was observed that the inhibition of the laminarinase after 1 hr. incubation at 37° was not the same as the above result.

<table>
<thead>
<tr>
<th>Concentration of lactone (mM)</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition of laminarinase (%)</td>
<td>5</td>
<td>10</td>
<td>24</td>
<td>28</td>
</tr>
</tbody>
</table>

This is due to the increased rate of hydrolysis of the lactone at pH 5.6 (ca. 1 hr.) to produce gluconic acid which does not inhibit the enzyme action to the same extent. For example 24% and 65% inhibition was observed at a concentration of 8 mM of hydrolysed and unhydrolysed gluconolactone, respectively.

When solutions of glucono-1,4-lactone were used more than 2 - 3 days after preparation; it was found that there was a decrease in the inhibitory action similar to the above. This is due to the spontaneous hydrolysis of the lactone in solution (cf. 117b).
Viscometric measurements

The hydrolytic action of the malted barley fraction, M2, on lichenin was shown to be of a random type by observing the fall in viscosity of a digest contained in an Ostwald viscometer at 37°. An incubation period of 1 hr. was used to measure the inhibitory effect of 4 mM gluconolactone-1,4-lactone

<table>
<thead>
<tr>
<th>Incubation (min.)</th>
<th>Control digest</th>
<th>Lactone present</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>1.08</td>
<td>0.94</td>
</tr>
<tr>
<td>15</td>
<td>1.31</td>
<td>1.14</td>
</tr>
<tr>
<td>30</td>
<td>1.55</td>
<td>1.34</td>
</tr>
<tr>
<td>45</td>
<td>1.79</td>
<td>1.50</td>
</tr>
<tr>
<td>60</td>
<td>1.93</td>
<td>1.59</td>
</tr>
</tbody>
</table>

Relative Activities (x 10^-7)

5.8

1/\(\eta_{sp}\)

The random hydrolysis of lichenin had been reduced by 19% in the presence of 4 mM gluconolactone.

Discussion

In the present experiments phenylacetic acid and phenylmercuric acetate did not selectively inhibit the laminarinase, cellodextrinase or lichenase of barley. This result may be compared with the findings of Preece and Hoggan (149) who reported that with barley \(\beta\)-glucosan as substrate, exo-glucosanase activity was inhibited by phenylmercuric nitrate. These observations were based on reducing-power measurements, and since it has not yet been established whether exo-glucosanase action involves the
hydrolysis of a $\beta$-1,4- or $\beta$-1,3-linkage, an examination of the effect of PMA on the cellobiose and laminaribiose activities of barley would be of interest.

The inhibitory effect of glucono-1,4-lactone solution on the carbohydrazes of malted and ungerminated barley is dependent on the time of incubation of the substrate and carbohydrazase, and on the extent of hydrolysis of the lactone to gluconic acid. This hydrolysis is spontaneous, and takes place slowly at 0° and low pH, and is exceedingly rapid at 20 - 37° and a pH of 5.6. (cf. a half life of 10 minutes (pH 6.8) and 180 minutes (pH 4.6) at 4° (147b).

Providing an incubation period of 1 hr. is used, the $\beta$-1,4- and $\beta$-1,3-glucosidases and $\beta$-1,4-glucosanase of barley and malted barley can be completely inhibited in the presence of 10 mM glucono-1,4-lactone whereas the $\beta$-1,3-glucosanase activity is reduced by 67% (malted barley) and 77% (barley). The inhibition studies indicate that the $\beta$-glucosidases acting on cellobiose and laminaribiose and the $\beta$-glucosanases acting on laminarin and cellodextrin are different enzymes. In the presence of gluconolactone, laminarin and lichenin are degraded to the same extent suggesting that the same enzyme may be acting on both substrates viz. the $\beta$-1,3-glucosanase.

The hydrolysis of lichenin by a malted barley extract has been shown to be of a random nature since there is a significant decrease in viscosity of the polysaccharide; this is slightly inhibited by 4 mM glucono-1,4-lactone.
These results confirm previous suggestions that true polysaccharase action of an enzymic extract may be studied by inhibiting the interfering oligosaccharases with glucono-1,4-lactone (147). In this case the $\beta$-1,3-glucosanase action could be studied by selectively inhibiting the $\beta$-glucosidase and $\beta$-1,4-glucosanase activities, and such experiments are described in Section V.

Part 5

Selective Production of Enzymes by Fungi

Reese and Mandels (136) have recently screened a large number of fungi for laminarinase activity and found that the culture medium of the majority showed detectable activity. The enzyme was constitutive and they suggested that it may be responsible for the intracellular hydrolysis and synthesis of reserve materials containing $\beta$-1,3-glucosidic linkages. This suggestion has been verified to a certain extent by the occurrence of $\beta$-1,3-glucosans and $\beta$-1,3-glucosanases in the fungi Porio cocos (152) and Sclerotinia libertiana (153). In some cases, the production of $\beta$-1,3-glucosanase occurred without any $\beta$-1,4-glucosanase activity being detected i.e. there was selective production of a particular $\beta$-glucosanase. The mode of action of the $\beta$-1,3-glucosanases on laminarin was dependent on the organism; culture filtrate from Basidiomycetes and Rhizopus arrhizus hydrolyse laminarin in a stepwise (with the
production of glucose) and random fashion, respectively. Other workers (153b) observed that *Rhizopus chinensis* produced random hydrolysis of the glucosans from yeast and *Sclerotinia*, although there was no proof that the $\beta-1,3$-glucosidic linkages, present in both polysaccharides, had been hydrolysed.

Further studies by Reese and his coworkers (133b) have indicated that culture filtrates of the fungi *Streptomyces* grown on cellulose contained a $\beta-1,4$-glucosanase and no $\beta-1,3$-glucosanase. This filtrate hydrolysed cellulose to cellobiose and cellotriose, i.e. $\beta-1,4$-glucosidase was absent.

Culture filtrates of *Myrothecium verrucaria* have also been used by many workers as a source of "cellulase", of the endo-type (133 a, 154); cellobiose and a tranoglucosidase are also present in varying amounts.

*Rhizopus arrhizus*, *Streptomyces*, and *Myrothecium verrucaria* were, therefore, grown in the laboratory and the culture filtrates examined for $\beta$-glucosanase activity.

**Methods and Materials**

(a) **Substrates**

These have been described in Part 1 of this Section.

(b) **Enzyme Preparations**

Cultures of the fungi were obtained from the Commonwealth Mycological Institute, Kew Gardens, Surrey and grown on a carbon source of cellobiose (0.6%; *Rhizopus arrhizus* IMI 83711) or cellulose (Reeve Angel and Co.; 0.5% *Myrothecium verrucaria* IMI 45541; 1.0% *Streptomyces* IMI 45560)
in a basal medium containing (per litre) KH$_2$PO$_4$, 2.0g; 
(NH$_4$)$_2$SO$_4$, 1.4g; urea 0.3g; MgSO$_4$, 7H$_2$O, 0.3g; CaCl$_2$, 0.3g; yeast extract, 0.1g; proteose peptone, 1.0g. The cultures (100 ml./500 ml. flask) were grown for 7 - 21 days at 27° with shaking.

The cultures were filtered through sintered glass (G3 or 4) and concentrated by evaporation under reduced pressure (Roto vac) at room temperature before freeze-drying; alternatively the enzymes were precipitated by the addition of cold acetone at -15°.

All media were sterilized at 10 lbs/sq. inch pressure for 10 minutes.

(c) **Enzymic Activities**

The laminarinase activity was determined by the method of Reese and Mandels (136) as follows: 0.6% laminarin in 0.05M-citrate buffer of pH 4.8 (0.5 ml.) and enzyme solution to be assayed (0.5 ml.) were incubated at 40°. The reducing-power of the digest was determined after 1 hr.; one unit of activity was shown by 1 ml. of the enzyme solution if 0.5 mg. of reducing sugar, as glucose, was produced in the above digest.

Other enzymic activities were qualitatively demonstrated by paper chromatographic examination of digests containing substrate (10 mg./ml; 1 ml.) and enzyme solution (2.5 mg. in 0.5 ml. 0.05M-citrate buffer of pH 4.8 after incubation at 40°.
Results

1. **Enzymic Activity of Culture Filtrates from Rhizopus arrhizus**

Two enzyme preparations were isolated by acetone precipitation, one (R1) resulting from 500 ml. of the basal medium grown for 7 days at 27°, and the other (R2) from 350 ml. of the basal medium combined with 20 ml% of 1% cellobiose solution grown for 19 days at 27°.

**Laminarinase Activity**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Yield (mg.)</th>
<th>Units/mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>1400</td>
<td>0.4</td>
</tr>
<tr>
<td>R2</td>
<td>970</td>
<td>0.7</td>
</tr>
</tbody>
</table>

The qualitative analysis of R1 gave the following results:

<table>
<thead>
<tr>
<th>Incubation (hr.)</th>
<th>3</th>
<th>28</th>
<th>48</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Sugars produced.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellobiose</td>
<td>Glucose ++</td>
</tr>
<tr>
<td>Laminaribiose</td>
<td>Glucose ++</td>
</tr>
<tr>
<td>Gentibiose</td>
<td>Glucose ++</td>
</tr>
<tr>
<td>Cellodextrin</td>
<td>Glucose ++</td>
</tr>
<tr>
<td>Laminnarin</td>
<td>Glucose ++</td>
</tr>
<tr>
<td>Oligosaccharides</td>
<td>+</td>
</tr>
</tbody>
</table>

The digests containing laminaribiose and cellobiose were heated on a boiling water bath after 28 hr. incubation and reduced in volume. On paper chromatographic examination, an oligosaccharide was produced which was neither cellobiose nor laminaribiose. This suggests that a transglucosidase is present.

Providing the incubation period is restricted to 3 hr., only β-1,3 and β-1,4 glucosidase and laminarinase activities can be detected. With longer incubation, degradation of substrates
by low $\beta-1,6$-glucosidase and cellodextrinase activities can be observed. This fungus appears to selectively produce a laminarinase in quantity, with concomitant production of small amounts of $\beta$-glucosidases and a trace of cellodextrinase.

2. **Enzymic Activity of Culture Filtrates from Myrothecium verrucaria**

The enzyme preparation was isolated by concentrating the culture filtrate from one litre of the basal medium after 1½ days incubation and freeze-drying to a brown powder. Yield 3.05 g. A sample (20 mg.) of this powder was found to contain no laminarinase activity by the method of Reese and Mandels. The preparation was examined for cellobiase and cellodextrinase activity with the following results:

<table>
<thead>
<tr>
<th>Incubation (hrs.)</th>
<th>Substrate</th>
<th>Sugars produced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cellobiose</td>
<td>Glucose ++</td>
</tr>
<tr>
<td></td>
<td>Cellodextrin</td>
<td>Glucose +, Cellobiose +</td>
</tr>
</tbody>
</table>

The preparation contained $\beta-1,4$-glucosanase and $\beta-1,4$-glucosidase.

3. **Enzymic Activity of Culture Filtrates from Streptomyces**

The preparation was isolated by acetone precipitation of culture filtrates from one litre of the basal medium after 21 days incubation; and examined for enzymic activity.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Sugars Produced</th>
<th>Incubation (hr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellobiose</td>
<td>Glucose</td>
<td>16</td>
</tr>
<tr>
<td>Cellodextrin</td>
<td>Glucose</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cellobiose</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cellotriose</td>
<td>+</td>
</tr>
<tr>
<td>Laminarin</td>
<td>Glucose</td>
<td>t</td>
</tr>
<tr>
<td></td>
<td>Oligosaccharides</td>
<td>t</td>
</tr>
</tbody>
</table>

This preparation contained a \( \beta-1,^4 \) glucosanase with trace quantities of \( \beta-1,3 \)-glucosanase.

**Discussion**

*Rhizopus arrhizus* has been shown to produce a quantity of enzyme which can degrade laminarin to glucose and oligosaccharides having the same \( R_g \) values as laminaribiose and laminaritriose, i.e. \( \beta-1,3 \)-glucosanase activity. By using incubation periods of 3 hr. or less, it was possible to demonstrate no \( \beta-1,^4 \) glucosanase activity and only a trace of \( \beta \)-glucosidase activity. This suggests that there has been almost a selective production of one enzyme.

The other fungal preparations require further study before definite conclusions can be made as to their enzymic content.
Summary

1. The $\beta$-glucosidase and $\beta$-glucanase activities of barley and malted barley have not been completely separated by fractional precipitation with ammonium sulphate, chromatography on alumina, or by continuous electrophoresis. Indications, however, were obtained that $\beta$-1,3 and $\beta$-1,4 glucosidase, laminarinase and cellobextrinase activities were due to distinct enzymes.

2. Inhibition by 10 mM glucono-$\beta$-lactone has verified the above conclusion and shown that 30% of the $\beta$-1,3-glucanase activity can be retained with complete inhibition of the $\beta$-glucosidase and $\beta$-1,4 glucanase activities.

3. The fungi, Rhizopus arrhizus, can selectively produce a $\beta$-1,3-glucanase with only trace amounts of $\beta$-glucosidase and $\beta$-1,4-glucanase.
Section V

The Molecular Structure of Lichenin and Related \( \beta \)-Glucosans

Introduction

The chemistry of a few of the more important \( \beta \)-glucosans appearing on Table VI will now be discussed in greater detail.

Cellulose: The main structural material of the majority of plants (for review see 155), possesses essentially the same chemical structure from whatever source it is isolated. From methylation studies, cellulose was found to consist of polymeric chains of several thousand \( \beta \)-D-glucopyranose units linked through positions 1 and 4. The current conception is that of a polycrystalline structure in which cellulose molecules traverse ordered crystalline and disordered amorphous regions. The latter regions present a greater surface area for the rapid initial chemical and enzymic degradation with a subsequent slower degradation of the crystalline regions. Outside of the plant kingdom, cellulose is also produced by bacteria of the genus Acetobacter (156), certain sea animals, and has recently been found in mammalian tissues (157).

Laminarin, a reserve material of the brown seaweeds, exists in two forms which differ in their solubility in water. The "soluble" and "insoluble" forms were shown in early studies to be \( \beta \)-1,3-glucosans with a CL. of 16–20 glucose residues (158). Recent work by Peat and coworkers (159), and Hirst and his coworkers
The periodate oxidation of lichenin
(160,161), using partial acid hydrolysis, and periodate and methylation techniques, respectively, have indicated that mannitol is a constituent monomer residue (2%) and that a low degree of branching is possible. Gentiobiose was isolated by Peat et al. showing that β-1,6-linkages were present in the molecule, either as branch points, or constituents of the unbranched chains. The periodate and methylation results of Hirst et al. have also shown that ca. 50% of the molecules are terminated by mannitol. The failure to identify ethylene glycol as a product after reduction of periodate-oxidised laminarin, followed by acid hydrolysis, led Smith and coworkers to suggest that the mannitol unit was located in a non-terminal position (162). Smith and Unrau have also isolated mannose from the hydrolysis products of periodate-degraded laminarin (163).

Lichenin: This reserve carbohydrate of various lichens, including Cetraria islandica, is extracted by hot water and settles out on cooling. Early methylation (164,165) and periodate oxidation (164) studies indicated that the molecule was essentially linear with a DP of 62 glucose residues, and contained both β-1,3- and β-1,4-glucosidic linkages in the ratio of 3:7, respectively. Recent periodate oxidation studies (161,166) have confirmed and extended these results. Treatment of periodate-oxidised lichenin with isonicotinhydrazide (Figure 27a) gave a product containing 11.6% N which indicated that these were ca. 66% 1,4-linked glucose residues, and, therefore, 34% 1,3-linked units in the original molecule. The formic acid produced during
controlled periodate oxidation (Figure 27b) gave a CL. of 63 in excellent agreement with the earlier results. Formaldehyde production (1.0 mole per glucose residue) on periodate-overoxidation of lichenin was consistent with the suggested linear structure; this was confirmed by the absence of branched oligosaccharides in the partial acid hydrolysates of lichenin (167). The presence of glucose and absence of laminaribiose in the partial acid hydrolysate of the polyalcohol obtained from the reduction of periodate-oxidised lichenin, suggested that there was a random distribution of the 1,3-linkages (161). This was also shown by the presence of laminaribiose but the significant absence of laminaritriose in partial acid hydrolysates of the polysaccharide (167).

From a study of the tri- and tetrasaccharides produced by the partial acid hydrolysis of lichenin, the molecule was suggested to be made up of cellotriose units joined together by single 1,3-linkages (167).

**Barley β-Glucosan:** This was isolated by the fractionation of aqueous extracts of barley flour with ammonium sulphate (3), and shown by methylation studies (168) to be essentially linear with a DP, of 100, and to contain approximately equal proportions of β-1,3- and β-1,4-glucosidic linkages.

**Oat Lichenin:** Treatment of oat flour with methanol and acetone to destroy hydrolytic enzymes, allowed the isolation of oat lichenin in an undegraded form (167,169). This polysaccharide also contains β-1,4- and β-1,3-glucosidic linkages; from periodate oxidation data,
the ratio of 1,4- to 1,3-linkages has been reported as 3.17:1 (167) 1.77:1 (170), and 1.86:1 (171).

The isolation of 2-0-β-D-laminaritetaosyl erythritol from the partial acid hydrolysate of the polyalcohol obtained by reduction of periodate-oxidised oat lichenin, indicated at least four of the 1,3-linkages occurred in sequence (170). In contrast, Peat et al. (167) had previously considered that there were no adjacent 1,3-linkages in the molecule, and that oat lichenin was similar in structure to lichenin.

A DF. of 1000 has been calculated (170), and methylation studies (170,171) suggest a low degree of branching.

Other β-Glucosans: From periodate oxidation and methylation studies, and analyses of partial acid hydrolysates, the β-glucosans callose (172), pachyman (152), and yeast glucosan have been shown to be mainly β-1,3-linked polymers. Bell and Northcote (173) concluded from their methylation and periodate oxidation results that yeast glucosan had a highly branched structure containing β-1,2-inter-chain linkages. Peat and coworkers (174), however, suggested that the polysaccharide was unbranched and contained ca. 10% of β-1,6-glucosidic linkages; this was proved by examination of partial acid hydrolysates, and esterification of the polysaccharide by toluene-p-sulphonyl chloride.

Pustulan, a polysaccharide isolated from a hot aqueous extract of the lichen, Umbilicaria pustulata, appears to be a
linear β-glucosan containing linkages of the 1,6-type (175).

From the above account, it is seen that the general structural features of many β-glucosans have been elucidated but that many details of their fine structure are still not known. This Section is concerned with some aspects of this subject.

Part 1

Studies on Lichenin

An attempt has been made to elucidate the molecular structure of lichenin by examination of the oligo saccharides resulting from the degradation of the polysaccharide with enzyme preparations showing β-1,3-glucosanase activity.

Materials

(a) Substrate

A sample of lichenin which had been used in previous chemical studies (161) was prepared and purified by Dr. F.B. Anderson. The polysaccharide was linear, had a D.P. of 63, and contained ca. 70% β-1,4- and 30% β-1,3-glucosidic linkages; there was a random distribution of the -1,3-linkages. A solution of lichenin was prepared by dissolution in 0.2N sodium hydroxide followed by neutralisation with sulphuric acid and addition of water to the correct volume. Concentration of the
polysaccharide was determined by acid hydrolysis.

(b) Enzyme Preparations

(i) The malted barley fraction, M2 (p.112), contained a mixture of carbohydrases, but in the presence of 10 mM glucono-1,4-lactone, only β-1,3-glucosanase and lichenase activity could be detected. These conditions were, therefore, applied to a study of the degradation of lichenin.

(ii) The enzyme preparation, R2, from the culture filtrates of *Rhizopus arrhizus* has been shown to contain β-1,3-glucosanase with trace amounts of other enzymes. The hydrolysis of lichenin by this preparation should provide information regarding the mode of action of the enzyme together with details of the molecular structure of the polysaccharide.

(iii) A commercial preparation of glucose oxidase (Takamine Deoxygenase; 1g) was dialysed against distilled water at 0° for 24 hr. This solution (100 ml.) was stored at 0° in the presence of chloroform (0.5 ml.)

Results

1. Enzymic Hydrolysis of Lichenin with a Malted Barley Preparation in Presence of Glucono-1,4-lactone

(a) Small-scale hydrolysis of lichenin

Lichenin (20 mg. in 6 ml. solution), enzyme solution (20 mg. malted barley fraction M2 in 4 ml. 0.2M-acetate buffer of pH 5.6) and 0.02M glucono-1,4-lactone or water (10 ml.) were incubated at 37° for 1 hr. The digestes were heated in a boiling-
water bath for 5 min., filtered through sintered glass (G-2),
deonised with Biodeminrolit (CO₃) ion-exchange resin, concentrated
in vacuo and examined by paper chromatography (Solvent 3).

<table>
<thead>
<tr>
<th>Sugars produced</th>
<th>Rs</th>
<th>Control</th>
<th>Lactone present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gluconic acid</td>
<td>1.25</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.0</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Laminaribiose</td>
<td>0.41</td>
<td>t</td>
<td>+</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>0.32</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>M</td>
<td>0.14</td>
<td>t</td>
<td>++</td>
</tr>
</tbody>
</table>

Sugar $X_M$ was isolated by preparative chromatography on
3 MM paper (Solvent 3). Paper chromatographic analysis showed
that (a) partial acid hydrolysis gave glucose, laminaribiose and
cellobiose and (b) hydrolysis with further amounts of malted barley
fraction, M2, gave only glucose. The DP. by Method Sa was 3.2 and
the electrophoretic mobility was similar to that of laminaribiose.
These results suggest that sugar $X_M$ is $4\beta$-glucosyl laminaribiose.

(b) Large-scale hydrolysis of lichenin

Lichenin (1.9g. in 190 ml. solution) was incubated at 37°
with enzyme solution (0.5g. malted barley fraction M2 in 50 ml.
0.2M-acetate buffer of pH 5.6) and 0.25M glucono-1,4-lactone (10 ml.)
The reducing-power of samples (2 ml.) was measured after incubation
for 0 and 1 hr. The increase corresponded to 14% conversion into
glucose. The digest was then deproteinised (zinc sulphate-
barium hydroxide) and centrifuged. An equal volume of ethanol was
added to the supernatant solution and the precipitate which formed
was removed. The solution was deionised with Biodeminrolit \( (\text{CO}_3) \) ion-exchange resin and concentrated \textit{in vacuo} to 50 ml. The amount of the individual sugars in a portion of this solution was estimated by Method 4b; the results calculated for the whole solution were as follows:

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Rg (Solvent 1)</th>
<th>Yield (mg.)</th>
<th>Yield (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Glucose</td>
<td>1.0</td>
<td>54</td>
<td>0.21</td>
</tr>
<tr>
<td>2. Laminaribiose</td>
<td>0.80</td>
<td>20</td>
<td>0.06</td>
</tr>
<tr>
<td>3. Trisaccharide ( X_M )</td>
<td>0.41</td>
<td>236</td>
<td>0.47</td>
</tr>
<tr>
<td>4. ( Y_M )</td>
<td>0.22, 0.15</td>
<td>29</td>
<td>0.04</td>
</tr>
<tr>
<td>5. Higher oligosaccharides</td>
<td>858</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The trisaccharide, \( X_M \), was the major low molecular weight product together with oligosaccharides higher than sugar \( Y_M \).

Using Method 8a, the DP, of the whole solution was 3.7 indicating that there was a large proportion of sugars of relatively high DP. Electrophoretic analysis of the solution showed the presence of glucose and one other zone with the same mobility as laminaribiose suggesting that the higher oligosaccharides all possessed a terminal \( \beta-1,3 \)-glucosidic linkage at the reducing end of the molecule.

On completion of the above preliminary analyses, the remaining solution was freeze-dried to yield 1.1 gm. of amorphous material.

A portion of this material (400 mg.) was adsorbed onto a charcoal-Celite column (26 x 1.5 cm.) containing Ultrasorb charcoal (British N.U. Ltd.; 10g) and Celite (John Manville & Co.; 10g). The
gluconic acid and glucose which were present in the solution were removed by washing with water and 1% aqueous ethanol, respectively before removal of the remaining sugars with 50% aqueous ethanol to give fraction D. The gluconic acid and glucose content of the remaining amorphous material was quantitatively removed by chromatography on 3MM paper (Solvent 3) and the higher oligosaccharides eluted from the paper with water, combined with the above Fraction D, and separated on 3MM paper (Solvent 1).

(c) Analysis of the hydrolysis products

(i) Glucose

The sugar tentatively identified as glucose was incubated with glucose oxidase solution (10 ml.), McIlvaine buffer of pH 5.8 (2 ml.), and 30% hydrogen peroxide (0.2 ml.) at 37°C for 24 hr. (176). Paper chromatographic analysis showed the complete removal of the original substrate and the appearance of a sugar with the same $R_g$ value as authentic gluconic acid (Solvents 1 and 3). The tentative identification was, therefore, correct.

(ii) Laminaribiose

This was shown to have the same $R_g$ value as authentic laminaribiose in Solvents 1 and 2.

(iii) The analysis of the remaining oligosaccharide material has yet to be undertaken since it was decided, at this stage, to investigate the hydrolysis of lichenin using the fungal preparation, R2. It was hoped that a larger quantity
of oligosaccharide material could be isolated from the hydrolysate for analytical purposes.

2. Enzymic Hydrolysis of Lichenin with the *Rhizopus arrhizus* Preparation

(a) Effect of time of incubation on the hydrolysis of Lichenin

Lichenin (20 mg. in 2 ml.) was incubated with the enzyme (4 mg. in 1 ml. 0.05M-citrate buffer of pH 4.8) at 40°. Reducing-power measurements were carried out on samples (0.5 ml.) after various intervals, and the hydrolysis (%) to glucose was then calculated.

<table>
<thead>
<tr>
<th>Incubation (min.)</th>
<th>Hydrolysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>22</td>
</tr>
<tr>
<td>125</td>
<td>26</td>
</tr>
<tr>
<td>160</td>
<td>28</td>
</tr>
<tr>
<td>360</td>
<td>30</td>
</tr>
</tbody>
</table>

These results indicate that the \(\beta\)-1,3-glucosanase produced by *Rhizopus arrhizus* is capable of slowly and incompletely hydrolysing lichenin; this hydrolysis was of the random type since paper chromatographic examination of the above digest showed the presence of oligosaccharides with \(R_g\)'s of 0.80 and 0.45. The hydrolysis of lichenin by the enzyme preparation is virtually completed after ca. 3 hr. incubation; subsequent action probably involves the degradation of the above oligosaccharides by the trace amounts of \(\beta\)-glucosidase.

(b) Large-scale hydrolysis of lichenin
Lichenin (2.7g in 300 ml. solution of pH 4.8) and enzyme solution (500 mg. R2 in 50 ml. 0.05M-citrate buffer of pH 4.8) were incubated at 40° for 3.25 hr. The digest was heated on a boiling water bath for 10 min. before cooling, filtering through sintered glass (G3 and 4), deionising with Biodeminrolit (CO₃) resin and concentrating in vacuo.

(c) Separation of the hydrolysis products

The concentrated sugar mixture was separated by preparative paper chromatography on Whatman No. 3MM (Solvent 1) as described in Method 4C.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Yield (mg.)</th>
<th>Yield (mL)</th>
<th>Rg values (Solvent 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Glucose</td>
<td>201</td>
<td>1.24</td>
<td>1.0</td>
</tr>
<tr>
<td>2. Laminaribiose</td>
<td>194</td>
<td>0.57</td>
<td>0.80</td>
</tr>
<tr>
<td>3. X₉</td>
<td>924</td>
<td>1.83</td>
<td>0.41</td>
</tr>
<tr>
<td>4. Y₉</td>
<td>137</td>
<td>0.20</td>
<td>0.12</td>
</tr>
<tr>
<td>5. Higher</td>
<td>505</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The major product, X₉, has the Rg of a trisaccharide; small amounts of glucose, laminaribiose, and a sugar with the Rg of a tetrasaccharide were also present.

(d) Analysis of the hydrolysis products

(i) Glucose

This was identified as described previously (p.135).

(ii) Laminaribiose

The sugar isolated from the enzymic hydrolysate was identified as laminaribiose since it (a) had the same Rg value as a sample of authentic laminaribiose (Solvent 1 and 2); (b) was
Tetramethyl-
Glucose

Cellobiose

Laminaribiose

Glucose

FIGURE 28c.
hydrolysed completely to glucose by emulsin; (c) had $[\alpha]_D^{20} = 20.2$ (c.1.14). in water c.f. $[\alpha]_D^{20} 20.3$ in water (167); (d) possessed almost the same reducing-power as cellobiose; and (e) travelled at the same rate as authentic laminaribiose on electrophoresis. This data distinguishes laminaribiose from sophorose, gentiobiose, and cellobiose.

(iii) Sugar $X_R$

Determination of the DP, by Methods 8a and 8b gave values of 3.1 and 3.3, respectively, i.e. $X_R$ was a trisaccharide. Partial acid hydrolysis gave sugars with the same $R_g$ values as laminaribiose, cellobiose, and glucose. In a digest containing the trisaccharide $X_R$ (20 mg. in 1 ml.) and emulsin II (10 mg. in 1 ml. 0.05M-citrate buffer of pH 4.8), paper chromatographic examination after 1 and 4 hr. incubation at $37^\circ$ showed that glucose, laminaribiose and unhydrolysed trisaccharide $X_R$ were present. Since emulsin is known to degrade oligosaccharide from the non-reducing end (38), the latter result suggested that there was a $\beta$-$1,3$-glucosidic linkage at the reducing end of trisaccharide $X_R$.

Previous workers (167,178), observed that on electrophoresis, oligosaccharides with $1,3$-linkages at the reducing end would migrate as a fast-moving zone to the anode, whilst oligosaccharides with $1,4$-linkages in the same position would travel relatively slowly. Comparison of the electrophoretic mobility of $X_R$ with laminaribiose and cellobiose (Figure 28) verified the above suggestion that there was a $1,3$-linkage at the reducing end of the trisaccharide molecule. In the estimation of the DP. of $X_R$ by the
Trisaccharide X

Emulsin

Glucose + Laminaribiose

Hypoiodite oxidation and H⁺

Gluconic acid + Cellobiose

Glucose + Cellobiose + Laminaribiose

∴ X is G.β.4.G.β.3.G.

Site of action of laminarinase on lichenin

(b)

FIGURE 29
periodate oxidation method, it was observed that the yields of formaldehyde were low and variable, if the time of reduction with potassium borohydride was limited to 2 hr. Higher constant results were obtained if $X_R$ was reduced for a period of 30-40 hr; this is a further confirmation of the above structure since a 1,3-linkage at the reducing end of an oligosaccharide stabilises the reducing group to reduction with borohydride (57).

The reducing group of $X_R$ (20 mg. in 5 ml.) was oxidised with alkaline hypoiodite (30 mg, iodine and 0.3 ml. 1N sodium hydroxide) for 24 hr. at room temperature. After deionising the solution with Amberlite IR-4B(OH) ion-exchange resin, the product was partially hydrolysed with acid. Paper chromatographic examination (Solvents 1 and 3) showed the presence of cellobiose and gluconic acid in the hydrolysate; hence, a $\beta$-1,4-linkage was present at the non-reducing end of $X_R$.

The above results (Figure 29a) prove that the trisaccharide $X_R$ is the linear $4^2-\beta$-glucosyllaminaribiose (alternative name: $3-O-\beta$-cellobiosyl-$\alpha$-glucose). The branched trisaccharide $3,4-O-\beta$-diglucosyl-$\alpha$-glucose would have yielded laminaribiose and cellobiose on degradation with emulsin, and after alkaline hypoiodite treatment followed by partial hydrolysis with acid, only glucose would be obtained.

Confirmatory evidence of the structure was obtained from a measurement of the formic acid produced on periodate oxidation. Trisaccharide $X_R$, cellobiose or laminaribiose (18-21 mg. in 30 ml. water) was oxidised with 0.1M sodium metaperiodate (6 ml.) at $0^\circ$.
for 100 hr. Ethylene glycol (1 ml.) was added to samples (3 ml.) to destroy excess periodate and the formic acid content then measured. This initial primary oxidation, carried out at 0° to minimise the hydrolysis of formyl ester intermediates, was virtually complete after 40-50 hr. An aliquot (15 ml.) of the remaining reaction mixture was added to ethylene glycol (5 ml.) and the temperature raised to 20°. The formic acid released by the hydrolysis of the above intermediates could then be measured directly. This hydrolysis was completed in 20-40 hr. The experimental results are tabulated and theoretical values are shown in parentheses, below:

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Initial (0°)</th>
<th>Final (20°)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellobiose</td>
<td>1.6 (2.0)</td>
<td>0.7 (1.0)</td>
<td>2.3 (3.0)</td>
</tr>
<tr>
<td>Laminaribiose</td>
<td>1.0 (1.0)</td>
<td>0.8 (1.0)</td>
<td>1.8 (2.0)</td>
</tr>
<tr>
<td>4²-β-glucosyllaminaribiose</td>
<td>1.0 (1.0)</td>
<td>0.8 (1.0)</td>
<td>1.8 (2.0)</td>
</tr>
<tr>
<td>3²-β-glucosylcellobiose</td>
<td>- (2.0)</td>
<td>- (1.0)</td>
<td>- (3.0)</td>
</tr>
<tr>
<td>3¹,4¹,β-diglucosylglucose</td>
<td>- (2.0)</td>
<td>- (1.0)</td>
<td>- (3.0)</td>
</tr>
</tbody>
</table>

The above results together with a measurement of $^{12} \beta_D + 12.3°(c,2.1\text{ in water})$ in good agreement with that ($^{12} \beta_D + 12.7°$) reported by Peat et al (167), and by Parrish, Perlin, and Reese (144) who isolated the same trisaccharide ($^{12} \beta_D + 12.1°$) from a digest of Rhizopus arrhizus "laminarinase" and oat lichenin, definitely confirm the structure of the trisaccharide $X_R$ as $4²-\beta$-glucosyllaminaribiose.

(iv) Sugar $X_R$. 

Partial acid hydrolysis of this sugar on paper chromatographic examination gave glucose, laminaribiose, celllobiose, cellotriose, and $4^2-\beta$-glucosyllaminaribiose. Since the electrophoretic mobility of sugar $Y_R$ was comparable to laminaribiose and the above $4^2-\beta$-glucosyllaminaribiose (Figure 28c), the structure is most probably $4^2-\beta$-celllobiosyllaminaribiose. Further evidence of the structure would be obtained by methods used in the analysis of sugar $X_R$.

The enzymic hydrolysis of lichenin by a laminarinase preparation from Rhizopus arrhizus has, therefore, yielded a number of oligosaccharides, all of which possess a terminal $\beta$-1,3-glycosidic linkage at the reducing end of the molecule and the major product being $4^2-\beta$-glucosyllaminaribiose.

3. Mode of Action of the Rhizopus arrhizus Preparation on Oligosaccharides containing $\beta$-1,3-Glycosidic Linkages.

This analysis was undertaken to provide information regarding the specificity requirements of this preparation. The substrates examined were 1) laminaribiose, 2) $1-\alpha-\beta$-laminaribiosyl mannitol, 3) $1-\alpha-\beta$-laminaritetraosyl mannitol, 4) $4^2-\beta$-glucosyllaminaribiose, and 5) the tetrasaccharide fraction from the above digest. The mannitol containing oligosaccharides were kindly provided by Dr. J.R. Turvey.

Substrate (5-10 mg. in 0.5 ml. water) and enzyme preparation (2.5 mg. in 0.5 ml. of 0.05M-citrate buffer of pH 4.8) were incubated at 40°. Paper chromatographic examination was carried out during a period of 3 hr. incubation using Solvent 1 and
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Sugars Produced</th>
<th>Incubation (hr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glucose</td>
<td>t</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1-O-β-Glucosyl Mannitol</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Laminaribiose</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Glucose</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1-O-β-Glucosyl Mannitol</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Laminaribiose</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Glucose</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1-O-β-Glucosyl Mannitol</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1-O-β-Laminaribiosyl Mannitol</td>
<td>t</td>
</tr>
<tr>
<td></td>
<td>Laminaribiose</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Laminaritriose</td>
<td>++</td>
</tr>
<tr>
<td>4</td>
<td>Glucose</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Laminaribiose</td>
<td>t</td>
</tr>
<tr>
<td></td>
<td>Cellobiose</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Glucose</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Laminaribiose</td>
<td>t</td>
</tr>
<tr>
<td></td>
<td>Cellobiose</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4-O-β-glucosyl laminaribiose</td>
<td>+</td>
</tr>
</tbody>
</table>

* Tentative based on Rg values.
isopropanol: acetic acid: water (3:1:1 v/v). The results are recorded in Table X.

From a consideration of the results (i) the enzyme preparation rapidly hydrolyses a $\beta$-1,3-linkage which is already attached to a 3-substituted glucose residue; (ii) the linkage between mannitol and a 3-substituted glucose residue is not hydrolysed; (iii) the two oligosaccharides containing the $\beta$-1,3- and $\beta$-1,4-linkages are hydrolysed from the non-reducing end, probably by the $\beta$-glucosidase contaminant. This may account for the hydrolysis of laminaribiose.

4. Enzymic Hydrolysis of Lichenin by Preparations from Myrothecium Verrucaria and Streptomyces.

A digest was prepared containing enzyme preparation (20 mg, in 1 ml, 0.05M-citrate buffer of pH 4.8) and lichenin (20 mg, in 2 ml, solution), and examined chromatographically (Solvent 1) after 16 hr, incubation at 40°.

<table>
<thead>
<tr>
<th>Sugars Produced</th>
<th>Myrothecium verrucaria</th>
<th>Streptomyces</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Laminaribiose</td>
<td>t</td>
<td>+</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Oligosaccharide Rg of 0.36</td>
<td>t</td>
<td>+</td>
</tr>
</tbody>
</table>

After 27 hr. incubation, the Streptomyces preparation had hydrolysed lichenin to an extent of 25% (calculated in glucose equivalents).

Since the Myrothecium verrucaria preparation contains $\beta$-1,4-glucosanase and $\beta$-1,4-glucosidase activities, and the
\( \beta-1,4\)-glucosanase activity of the Streptomyces preparation is contaminated with a \( \beta-1,3\)-glucosanase, no definite conclusions can be derived from the above results.

**Discussion**

Lichenin has been incubated with an extract of malted barley in the presence of 10 mM glucono-1,4-lactone, and with an enzyme preparation from *Rhizopus arrhizus*; both preparations have previously been found to exhibit \( \beta-1,3\)-glucosanase activity (Section IV). Since the hydrolysis products of the former digest were more difficult to separate in the presence of lactone and since there was a lower degree of hydrolysis (14\%) of the lichenin, only a preliminary identification of the oligosaccharides was attempted. However, the greater extent of hydrolysis of lichenin in the second digest, has enabled the products to be separated in quantity and an analysis to be carried out.

The major oligosaccharide component in both digests was a trisaccharide, \( 4^2-\beta \)-glucosyllaminaribiose, named "cellamiose" by Ono and Dazai (178); small amounts of glucose, laminaribiose, and a tetrasaccharide, were also formed. Considering the basic structure proposed by Peat et al (167) as shown in Figure 29b, the absence of cellotriose in the hydrolysate indicates that enzyme action must involve the rupture of a \( \beta-1,4\)-glucosidic linkage to yield \( 4^2-\beta \)-glucosyl laminaribiose i.e. \( \beta-1,3\)-glucosanase shows a preference for a glucosidic linkage attached to a 3-substituted \( \beta-D\)-glucose unit rather than a \( \beta-1,3\)-linkage. This conclusion is in exact agreement
with the results of Parrish et al. (144).

The presence of the trisaccharide as a major product confirms that the major portion of the lichenin molecule must consist of the sequence shown in Figure 29b. However, the isolation of a tetrasaccharide in the hydrolysate, and the previous evidence that lichenin does not contain a sequence of $\beta$-1,3-glucosidic linkages (167), indicates that there must be some sequences of three $\beta$-1,4-gluicosidic bonds joined by a $\beta$-1,3-glucosidic bond. The ratio of tetrasaccharide to trisaccharide in both digests was ca. 1:10 suggesting that the above sequence was only a minor constituent. In order to account for the ratio of linkages obtained by chemical analysis, there must be minor sequences of alternating $\beta$-1,3- and $\beta$-1,4-linkages.

Laminaribiose is present in both digests, the molar ratio to trisaccharide being 1:6 with the malted barley enzyme and 1:3 with the fungal enzyme. In the presence of the lactone, the barley preparation does not show $\beta$-glucosidase activity, and the laminaribiose is probably a primary product of polysaccharase action. This is in accord with minor sequences of alternating $\beta$-1,3- and $\beta$-1,4-linkages. With the Rhizopus arrhizus preparation, part of the laminaribiose may arise from the lichenin and part from the $\beta$-hydrolysis of 4-$\beta$-Glucosyl laminaribiose and the tetrasaccharide $\gamma_2$ by the contaminating $\beta$-glucosidase.

From the present work, it is sugested that lichenin, consists of a major sequence of cellotriosyl units joined together
by $\beta-1,3$-glucosidic linkages with minor sequences of cellotetraosyl and cellobiosyl units joined by the same linkage.

A complete outline of the fine structure of lichenin could also be obtained from the above results together with those from the enzymic degradation of the polysaccharide with a pure $\beta-1,4$-glucosanase preparation. This was not attempted since the "cellulase" preparation of Streptomyces, in contrast to that reported by Reese et al. (133b), showed some $\beta-1,3$-glucosanase activity.

In a study of the hydrolysis of oligosaccharides, containing more than one type of $\beta$-glucosidic linkage, by the Rhizopus arrhizus preparation, evidence was obtained that linkages containing the sequence $-3\text{G}1\beta\text{G}$ were rapidly hydrolysed, whilst linkages of the $-3\text{G}1\beta$ Mannitol and $-4\text{G}1\beta\text{G}$ were not attacked. The hydrolysis of $4^2-\beta$-glucosyl laminaribiose and laminaribiose occurred slowly, the former being degraded from the non-reducing end of the molecule probably by the $\beta$-glucosidase contaminant.

These results suggest that the specificity of the $\beta-1,3$-glucosanase from Rhizopus arrhizus is mainly controlled by the linkage (a) in the annexed diagram.

\[
\begin{array}{cccc}
(a) & (b) & X & \text{Activity of enzyme} \\
1,3 & 1,3 & \text{Glucose} & + \\
1,3 & 1,4 & " & + \\
1,4 & 1,3 & " & - \\
1,3 & 1,6 & " & ? \\
1,3 & 1,1 & \text{Mannitol} & - \\
\end{array}
\]
Since the β-1,3-glucosanase preparation can hydrolyse both β-1,3- and β-1,4-linkages, then in studying the action on laminarin, the absence of gentiobiose from the enzymic hydrolysates would not necessarily prove the absence of β-1,6-linkages in the polysaccharide. Extreme caution will, therefore, be required in future studies on the enzymic degradation of polysaccharides.

Part 2

The Molecular Structure of the Reserve Polysaccharide from Peranema.

Introduction

The protozoon, Peranema (180), is a colourless member of the Eugleninaeae and is one of the largest zootrophic flagellates. It is found in stagnant waters which contain plant residues. The dimensions of the irregularly sack-shaped body (Figure 30) vary with environmental conditions, newly collected individuals having an average size of 42 x 16 μ which increased to ca. 65 x 25 μ on culturing in milk. There are two flagella, one directed in a forward direction, and the other in a backward direction.

Peranema readily ingest a great variety of food particles and organisms such as Euglena gracilis and Saccharomyces converting them to the reserve food material, paramylon. This occurs as disk-shaped granules which can be observed scattered randomly in the cytoplasm. "Paramylon" also occurs in other members of the family
Euglenineae, and was originally named by Gottlieb (181) who observed that the paramylon granules were similar in form to starch granules but did not give a blue stain with iodine solution. Until the recent investigation of the paramylon from the organism, *Euglena gracilis*, by Clarke and Stone (182), no complete chemical characterisation of the granules had taken place, apart from their designation as glucose containing polysaccharides. Paramylon granules were therefore isolated from *Peranema* and a preliminary chemical investigation carried out.

**Materials**

(a) **Substrate**

Cultures of *Peranema* were grown by Dr. J.F. Ryley on an inorganic salt-milk medium and the cells separated by centrifugation, washed with water and ethanol. The cells were disrupted by passing ultrasonic vibrations (M.S.E. Ultrasonic Disintegrator) through an aqueous suspension and the fatty material discarded. The cell debris was washed with alcohol followed by ether and air dried. Yield of dried cells material: 1.8g. This material was incubated with trypsin (Nutritional Biochemical Company; 95 mg. in 50 ml. 0.1M-phosphate buffer of pH 7.6) at 40° for 40 hr. After centrifugation, the residue was extracted twice with saturated urea solution and washed with water. The remaining denatured protein was then removed by shaking the off-white material for 60 min. with chloroform (50 ml.), water (130 ml.) and amyl alcohol (20 ml.)
(183), and separating the chloroform–protein gel at the centrifuge; the white particles of paramylon in the aqueous phase were precipitated by the addition of 3 vol. ethanol and the precipitate washed with ethanol followed by ether and air-dried. Yield: 1.06g. representing 59% of the weight of dried cell material.

(b) **Enzyme preparations**

The preparation, R2, isolated from the culture filtrates of *Rhizopus arrhizus* (p.123) and a solution of glucose oxidase (p.132) were used.

**Results**

(a) **Acidhydrolyses**

The paramylon was insoluble in 2N sulphuric acid and hence complete hydrolysis was carried out by adding 20 mg. to 2ml. 90% formic acid and heating the solution in a boiling water bath for 2 hr. followed by the addition of 4 ml. 2N sulphuric acid and heating for a further 3 hr. On paper chromatographic examination of the neutralised hydrolysate (Solvents 1,2,3) only a monosaccharide having the same *Rf* value as glucose was observed. The glucose content of the paramylon was 97%.

A partial acid hydrolysate was prepared by heating 15 mg. of paramylon with 2 ml. 90% formic acid on a boiling-water bath for 3 hr. The formic acid was then removed by evaporating the solution to dryness; the residue was dissolved in water and examined by paper chromatography (Solvent 1) Whatmann 3MM paper). Glucose and
FIGURE 31.

Laminarin

700  900  1100  1300

Polysaccharide from Peranema

700  900  1100  1300

Starch

$\text{cm}^{-1}$
a series of four oligosaccharides having \( R_g \) values of 0.75, 0.44, 0.27, 0.16 were produced, the lowest member corresponding to laminaribiose; a plot of \( \log \frac{R_g}{1-R_g} \) against the expected DP. was linear suggesting that the oligosaccharides formed a homologous series. This hydrolysate was incubated with glucose oxidase solution (10 ml.) at 37° for 24 hr. and on chromatographic examination of the digest (Solvent 3) the glucose content was observed to decrease with the production of gluconic acid.

(b) Specific rotation and infra-red spectrum

Paramylon had \( [\alpha]_{16}^0 + 16^0 \) (0.12; 5N sodium hydroxide). A dry sample of the polysaccharide formed a mull with nujol and the infra-red spectrum examined as a pressed disc in a Perkin-Elmer Infracord Spectrophotometer. The spectrum was almost identical to one obtained with laminarin (Figure 31), both giving a band at 890 cm\(^{-1}\) which was not shown by a starch sample i.e. evidence that paramylon is a \( \beta \)-glucosan (14).

(c) Periodate oxidation

Paramylon (59 mg.) was treated with 0.015M sodium periodate (10 ml.) at 27°, and the reduction of periodate determined spectrophotometrically (Method 7c); 0.03 mole were reduced / mole anhydroglucose after 72 hr. reaction, in contrast to a value of 0.3 mole / mole anhydroglucose for a control experiment containing laminarin. This low consumption of periodate by the paramylon can only be explained if the polysaccharide is a linear \( \beta-1,3 \)-glucosan. This latter conclusion is consistent with the partial acid hydrolysis.
results which indicate the presence of oligosaccharides containing \( \beta-1,3 \)-glucosidic linkages.

Sodium meta-periodate will oxidise the reducing glucose unit of a glucosan and liberate one mole of formaldehyde; the DP. of the molecule may thus be estimated. Paramylon (20 mg. in 5 ml. water) was, therefore, oxidised with 0.3M sodium periodate (0.5 ml.) at 27° and the formaldehyde content determined (Method 7a). In this estimation, performed by Mr W.D. Annan, the paramylon in the sample (1 ml.) was removed by ethanol precipitation prior to determining the formaldehyde content. It was found that, after 72 hr., 0.009 mole formaldehyde was liberated per mole of anhydroglucose i.e. equivalent to a DP. of ca. 100 glucose units. This result indicates that the paramylon is not a low molecular weight polysaccharide.

(d) **Enzymic degradation**

A solution of paramylon was prepared by dissolving the polysaccharide in 5N sodium hydroxide and dialysing this against several changes of distilled water. An aliquot (10 ml. containing 5 mg. polysaccharide) was incubated with enzyme solution (100 mg. fraction R2 in 2 ml. 0.05M-citrate buffer of pH 4.8). On chromatographic examination of the digest after 24 hr. and 48 hr. incubation at 40°, only glucose was detected.

**Discussion**

The infra-red spectrum, acid hydrolysis, and optical rotation data characterise the paramylon from *Peranema* as a
\( \beta \)-glucosan. The low reduction of sodium periodate, the slow hydrolysis by the *Rhizopus arrhizus* preparation, and the preliminary identification of the products of partial acid hydrolysis by paper chromatography indicate that the linkages in the \( \beta \)-glucosan are mainly of the 1,3-type.

This preliminary characterisation of the reserve carbohydrate, paramylon, from *Peranema* as a \( \beta \)-1,3-glucosan is in agreement with the results of Clarke and Stone (182) and Archibald et al. (32) in their analyses of the reserve materials from *Euglena gracilis* (paramylon) and *Ochromonas malhamensis* (leucosin), respectively.

In future work, the isolation and chemical identification of laminaribiose and the homologous series of higher oligosaccharides produced by partial acid and enzymic hydrolysis of the paramylon from *Peranema* would enable the characterisation to be completed.

**Part 3**

**Studies on Laminarin**

No reducing monosaccharide other than glucose had ever been detected in acid or enzymic hydrolysates of laminarin by previous workers in this Department. The recent report by Smith and Unrau (163) that D-mannose is a constituent of the polysaccharide led to a re-examination of an acid hydrolysate of laminarin in an effort to
detect trace amounts of this sugar.

Materials

(a) **Substrates**

The laminarin sample, BB2, was that described by Anderson et al. (160). Mannose was a commercial sample.

(b) **Enzyme preparation**

The glucose oxidase solution has been described previously (p.132).

Results

(a) **Acid hydrolysis**

Laminarin (1g.) was refluxed for 3 hr. on a boiling-water bath with 1.5N sulphuric acid (100 ml.). This was neutralised with Amberlite IR-4B/OH and 120(H) ion-exchange resins and the resulting solution freeze-dried. The white amorphous powder was examined by preparing ascending and descending paper chromatograms and developing them in a variety of solvent systems. It was found that control glucose solutions containing 3, 4, and 6% mannose could be satisfactorily separated into the component monosaccharides by ascending chromatography (Solvent 2) on Whatman 3MM paper; under similar conditions the laminarin hydrolysate gave only glucose. A control glucose solution containing mannitol (13%) and mannose (2.5%) (monomer concentrations of a laminarin sample quoted by Professor F. Smith in a personal communication to Dr. D.J. Manners) was separated into the component sugars by ascending chromatography on Whatman 3MM paper using a boric acid saturated solvent containing methylethyl Ketone: acetic acid: water (9:1:1 v/v) and developed with periodate-rosaniline reagent (184). Using these conditions,
the laminarin acid hydrolysate separated into glucose and a trace amount of mannitol.

In a second, acid hydrolysis, aliquots were neutralised with i) sodium hydroxide, evaporated to dryness, extracted with ethanol and concentrated in volume; and (ii) barium acetate, centrifuged, and concentrated *in vacuo*. Paper chromatographic examination (Solvent 2; ascending) showed that the sodium hydroxide-neutralised hydrolysate contained a trace amount of mannose; this may have arisen from the alkaline transformation of glucose. Fructose, another possible transformation product was not observed with the aniline oxalate spray reagent.

(b) **Glucose oxidase treatment**

Substrate (100 mg. glucose or 5 mg. mannose), glucose oxidase solution (5 ml.), McIlvaine buffer (pH 5.8; 10 ml.), hydrogen peroxide (0.1 ml.) and water to a total volume of 50 ml. were incubated at 37°. Reducing-power measurement were carried out on samples (5 ml.) after 24 hr. incubation and the monosaccharide content estimated and found to be glucose, 0.4 mg., and mannose, 4.9 mg.

A digest was prepared containing glucose (100 mg.), mannose (1 mg.), glucose oxidase solution (7 ml.), McIlvaine buffer (pH 5.8; 10 ml.), hydrogen peroxide (0.1 ml.), and water to a total volume of 50 ml. A sample (5 ml.) was removed after 17 hr. incubation at 37° and the total reducing-power estimated. This corresponded to a value of 1.2 mg. monosaccharide, and from the previous
results this was considered to be due to the mannose originally present. The digest was concentrated and examined by paper chromatography; only a trace of mannose was observed.

In a similar digest containing the freeze-dried laminarin acid hydrolysate (100 mg.), no monosaccharides were observed.

Discussion

Analysis of acid hydrolysates of laminarin by paper chromatography and enzymic studies have failed to demonstrate the presence of mannose although control experiments were carried out in which 1-3% of mannose could be detected in the presence of glucose. It has been shown, however, that care must be taken in neutralising the acid hydrolysates since a trace quantity of mannose may be formed by alkaline transformation from glucose, or may be lost by adsorption on the ion-exchange resins used for deionisation. It is concluded that mannose is not a significant constituent of laminarin sample BB2.

Part 4

Studies on Yeast Glucan

Yeast glucan was incubated with an enzymic preparation showing β-1,3-glucosanase activity in an attempt to identify possible oligosaccharide products and hence provide information of the fine structure of the molecule.
Materials

(a) Substrate
A small sample of yeast glucan was kindly provided by Dr. D.H. Northcote.

(b) Enzyme preparations
A preparation, R2, from the culture filtrate of Rhizopus arrhizus has been described previously (p.123).

Results
Yeast glucan (10 mg.) was incubated with enzyme (10 mg. R2 in 2 ml. 0.05M-citrate buffer of pH 4.8) at 40° and examined chromatographically (Solvent 1) at intervals.

<table>
<thead>
<tr>
<th>Incubation (hr.)</th>
<th>3</th>
<th>8</th>
<th>20</th>
<th>42</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugars produced</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>t</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Laminaribiose</td>
<td>t</td>
<td>t</td>
<td>t</td>
<td>+</td>
</tr>
<tr>
<td>Oligosaccharide (Rg 0.39)</td>
<td>t</td>
<td>t</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

An oligosaccharide has been produced by the enzymic hydrolysis of yeast glucan which is not celllobiose (Rg 0.55), sophorose (Rg 0.52), laminaritriose (Rg 0.46), but which may be gentiobiose (Rg 0.40).

Discussion
There is evidence to suggest that the enzymic hydrolysis of yeast glucan by a β-1,3-glucosanase yields glucose, laminaribiose, and an oligosaccharide which may be gentiobiose or a trisaccharide with β-1,2- and β-1,3-linkages but is definitely not sophorose. Bell
and Northcote (173) suggested that the polysaccharide was highly branched and contained $\beta-1,3$- and $\beta-1,2$-linkages i.e. laminaribiose and sophorose might be formed on hydrolysis. In contrast, Peat et al. have isolated gentiobiose and laminaribiose from a partial acid hydrolysate of yeast glucan, and concluded that $\beta-1,6$- and $\beta-1,3$-linkages were present.

The above preliminary results are not conclusive and a full investigation is clearly required. The insoluble nature of yeast glucan hinders enzymic hydrolysis and prevents the isolation and identification of the oligosaccharides under the above conditions; continuous dialysis of the enzymic digest (185) may prove of value in isolating the oligosaccharides.

**Summary**

1. The $\beta-1,3$-glucosanases from the culture filtrates of *Rhizopus arrhizus* and malt extracts have been shown to be specific for $1,3$- or $1,4$-glucosidic linkages attached to a glucosyl unit substituted in position 3.

2. This $\beta$-glucosanase can degrade lichenin to oligosaccharides, the major product being $4^2-\beta$-glucosyl laminaribiose; glucose laminaribiose and a tetrasaccharide are also formed. These results together with previous chemical data indicate that the polysaccharide consists mainly of cellotriose units joined by $\beta-1,3$-linkages; cellotenraosyl and cellobiosyl units may also be present.

3. The reserve polysaccharide from *Paranema*, paramyton, has been characterised as a $\beta$-glucosan probably containing glucose units
joined by 1,3-linkages.

4. A critical examination of acid hydrolysates from laminarin have failed to show the presence of \( \beta \)-mannose.

5. Paper chromatographic examination of an enzymic hydrolysate of yeast glucan have failed to provide conclusive evidence on the nature of the minor \( \beta \)-glucosidic linkages.
Section VI

Studies on Mannanases

Introduction

Polysaccharides containing D-mannose as the major constituent are found as reserve or structural substances in woods, tubers and seeds of many plants, yeast, and certain algal and micro-organisms. The mannans from plant tissues have a basic linear structure of β-D-mannose units linked through positions 1- and 4-; β-D-glucose (glucomannans) and β-D-galactose (galactomannans) may also be present, either in the main chain, or as side-chains.

Mannans

Ivory nut mannans: Two mannans, A and B, have been isolated from vegetable ivory (Phytelephas macrocarpa), A being extracted with alkali (186), and B being separated from the cellulose by precipitation with cuprammonium solution (187). Both polysaccharides contain β-1,4-linked D-mannose residues with a small proportion of β-1,6-linkages; they differ, however, in CL., mannan A having a CL. of 10-13, and mannan B one of 39-40 (187).

Salep mannan: This water soluble reserve polysaccharide is obtained from the dried tubers of the orchids, Orchis and Eulophia. The mannan was first prepared by Pringsheim and Genin (188) but subsequent work by Husemann (189) showed that this had been enzymically
degraded during extraction. Methylation studies (190) indicated that the structure was similar to ivory nut mannan.

Yeast mannan: This has been shown by methylation (191) and periodate oxidation (192) studies to have a highly branched structure containing 1,6-, 1,2- and 1,3-linkages in the proportions 2:3:1. Recently Peat and his coworkers (193) have examined a partial acid hydrolysate of yeast mannan and confirmed the presence of 1,6-linkages; they suggest that the backbone of the molecule contains sequences of α-1,6-linkages with side chains containing other linkages attached to this backbone.

Galactomannans

With the exception of lucerne seed galactomannan (194), mannose is the major constituent of these polysaccharides. It has been suggested (195) that most galactomannans can be represented by linear polymeric chains of 1,4-linked β-D-mannopyranose residues with single galactose units attached to this chain by α-1,6-linkages. The more important members of this group have been discussed by Anderson (196), Jones and coworkers (194), and more recently by Smith and Montgomery (2).

Glucomannans

These account for ca. 50% of the hemicellulose fractions of coniferous woods, and have also been isolated from the seeds of various land plants. The basic feature appears to be a chain of 1,4-linked β-D-mannopyranose and β-D-glucopyranose residues (197).
Mannose-containing polysaccharides have also been isolated from marine algae e.g. the \( \beta-1,4 \)-mannan from *Porphyra umbilicalis* (198), and from micro-organisms e.g. the glucomannan from *Penicillium charlesii* G. Smith (199).

**Enzymic Degradation of Mannose-containing Polysaccharides**

Enzymes catalysing the hydrolysis of mannose-containing polysaccharides have been demonstrated in malt, bacterial and fungal extracts, digestive juices of invertebrates, marine algae, and preparations from the germinating seeds of locust bean, fenugreek, lucerne and guar (for reviews see 2, 127, 128).

There is evidence to suggest that two enzymes may be involved in the hydrolysis of mannans, one (endo-mannanase), causing random hydrolysis of the polysaccharide has been demonstrated in algal (37) and aged malt preparations (188) to yield a homologous series of oligosaccharides, the second (exo-mannanase or mannnobiase) which hydrolyses the oligosaccharides formed by endo-mannanase action to mannose is present in normal malt extracts (188).

Galactomannans isolated from plant seeds are hydrolysed by enzyme preparations from the germ of the same seed. For example, guaran, a reserve polysaccharide occurring in the endosperm of guar seeds, is hydrolysed by an enzyme preparation from the germinated seeds. Characterisation of the resulting oligosaccharides has confirmed the chemical evidence that guaran is composed of a linear chain of \( \beta-1,4 \)-linked D-mannose units with single \( \alpha-D \)-galactose units linked to \( C_6 \) of some of the mannose residues (200). Structural comparisons
may be possible using this or similar preparations to degrade other galactomannans.

Recently, Perila and Bishop (201) examined the enzymic hydrolysis of a glucomannan from jack pine with a commercial "hemicellulase" preparation. The results agreed closely with previous chemical studies.

This Section is concerned with a preliminary investigation of the enzymic hydrolysis of mannose-containing polysaccharides by extracts of malt, together with an attempted purification of the "mannanase".

**Materials and Methods**

(a) **Substrates**

Soluble oligosaccharides were isolated by Dr. R.B. Rashbrook from a partial acid hydrolysate of ivory nut mannan A, and used to test the mannanase activity of the enzyme fractions; the oligosaccharides were free from mannose, mannobiose, mannotriose and mannotetraose. Other samples of mannan were kindly provided by Dr. D.H. Northcote (yeast mannan), Dr. W.J. Whelan (salep mannan), and Mr J. Love (mannan from Codium fragile). Galactomannans from Honey locust bean (Gleditsia tricanthos) and flame tree (Delonix regia) and a glucomannan from Lilium candidum were provided by Dr. L. Hough. Mr R. Begbie isolated a glucomannan from Lilium henryii.

(b) **Enzyme preparations**

(1) Malt flour (1 Kg.) was extracted with 0.2M-acetate
buffer (2.5L; pH 5.0) at 20° for 2.5 hr. The grain residue was removed and the resulting solution dialysed at 0° against running tap water for 3 days. After separation of the precipitate which had formed, the protein was then fractionally precipitated with ammonium sulphate. These precipitates were dissolved in ice-cold water and dialysed against running distilled water at 0° for 2 days. The solutions were then freeze-dried.

(ii) The *Rhizopus arrhizus* preparation, RL, has been described on p.123.

(c) Activity measurements

These were estimated by incubating mannan-oligosaccharides (2.5 mg. in 0.5 ml.) with enzyme fraction (2.5 mg. in 0.5 ml. 0.2M-acetate buffer of pH 5.5) at 37° and determining the increase in reducing-power after 1 or 4 hr. The activity is expressed as mannose (mg.) produced /mg. fraction, and is assumed to represent the combined activity of an endo-and exo-mannanase. Ivory nut mannan itself is extremely insoluble, and is not, therefore, a satisfactory substrate for quantitative measurements.

Results

1. Fractional precipitation with ammonium sulphate

The following yields were obtained
No distinct separation of mannanase activity has occurred.

(a) Effect of pH

Enzyme fraction, MN3, 2.5 mg. in 0.5 ml. water) was incubated with mannan-oligosaccharides (2.5 mg. in 0.5 ml. water) and 0.2M-phosphate-citrate buffer (1 ml.; pH 3.5-7.0) at 37°, and the increase in reducing-power determined after 4 hr.

<table>
<thead>
<tr>
<th>pH</th>
<th>3.5</th>
<th>4.0</th>
<th>4.4</th>
<th>4.8</th>
<th>5.2</th>
<th>5.6</th>
<th>6.0</th>
<th>6.5</th>
<th>7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increase in reducing-power (mg. mannose)</td>
<td>0.30</td>
<td>0.68</td>
<td>0.71</td>
<td>0.75</td>
<td>0.86</td>
<td>0.81</td>
<td>0.72</td>
<td>0.60</td>
<td>0.42</td>
</tr>
</tbody>
</table>

The preparation has optimum activity in phosphate-citrate buffer of pH 5.2.

(b) Effect of temperature on activity

A digest was prepared containing mannan-oligosaccharides (2.5 mg. in 0.5 ml. water) and enzyme fraction MN3 (2.5 mg. in 0.5 ml. 0.2M phosphate-citrate buffer of pH 5.2). After 4 hr. incubation at various temperatures, the increase in reducing-power was estimated.

<table>
<thead>
<tr>
<th>Temperature (°)</th>
<th>20</th>
<th>25</th>
<th>30</th>
<th>37</th>
<th>45</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increase in reducing-power (mg. mannose)</td>
<td>0.64</td>
<td>0.74</td>
<td>0.78</td>
<td>0.82</td>
<td>0.89</td>
</tr>
</tbody>
</table>
These results indicate that the enzyme has not been inactivated by incubation for 4 hr. at temperatures up to 45°.

2. Attempted purification by fractional precipitation with acetone

Portions of fractions MN2 and MN3 were combined to give a total weight of 3.0g. This was dissolved in 0.2M-acetate buffer (250 ml.; pH 5.0) and fractionally precipitated by the addition of acetone at -15°. The fractions obtained were dissolved in cold water and dialysed against distilled water at 0° for 9 hr. The resulting precipitates were separated and the solutions then freeze-dried.

<table>
<thead>
<tr>
<th>Acetone added (%)</th>
<th>Yield (mg.)</th>
<th>Activity 1 mg. (X10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-20</td>
<td>90</td>
<td>2.6</td>
</tr>
<tr>
<td>20-30</td>
<td>304</td>
<td>3.2</td>
</tr>
<tr>
<td>30-40</td>
<td>470</td>
<td>3.6</td>
</tr>
<tr>
<td>40-53</td>
<td>760</td>
<td>3.0</td>
</tr>
<tr>
<td>53-68</td>
<td>870</td>
<td>2.6</td>
</tr>
<tr>
<td>68-75</td>
<td>32</td>
<td>1.6</td>
</tr>
</tbody>
</table>

A complete separation of mannanase activity has not, therefore, been obtained by acetone fractionation.

(a) Hydrolysis of mannose-containing polysaccharides by fraction 40-53

The substrates were dissolved in water or 0.2N sodium hydroxide and the pH of the solution adjusted to 5.5. A portion (0.5 ml.) of the solution was then incubated with enzyme (10 mg. fraction 40-53 in 0.5 ml. 0.2M-acetate buffer of pH 5.5) at 37°
and samples examined by paper chromatography after 12, 22, and 60 hr.

Sugars produced after 22 hr.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Source</th>
<th>Galactose</th>
<th>Glucose</th>
<th>Mannose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannan</td>
<td>Yeast</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucosamine</td>
<td><em>Codium fragile</em></td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucosamine</td>
<td>Salep</td>
<td>+ +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucosamine</td>
<td><em>Lilium henryii</em></td>
<td>+ +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucosamine</td>
<td><em>Lilium candidum</em></td>
<td>+ t</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Galactomannan</td>
<td>Honey Locust</td>
<td>t</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Galactomannan</td>
<td>Flame tree</td>
<td>t</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

Examination after prolonged incubation did not reveal oligosaccharide material in any of the digests.

4. Hydrolysis of mannose-containing polysaccharides by the Rhizopus arrhizus preparation

A digest was prepared containing enzyme (10 mg. in 0.5 ml. 0.05M-citrate buffer of pH 4.8) and substrate (10 mg. in 1 ml. water). Samples were removed after 20 hr. incubation at 40°C for chromatographic examination (Solvent 1).

Sugars produced

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Source</th>
<th>Glucose</th>
<th>Mannose</th>
<th>Oligosaccharides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannan-Oligosaccharide</td>
<td><em>Ivory nut</em></td>
<td>+++</td>
<td>+Rm 0.51, 0.16</td>
<td></td>
</tr>
<tr>
<td>Mannan</td>
<td><em>Codium fragile</em></td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucosamine</td>
<td><em>Lilium henryii</em></td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

No oligosaccharide material was observed in the digests containing the polysaccharide material after a further 60 hr. incubation.
Discussion

Although extracts of malted barley show appreciable "mannanase" activity, fractional precipitation with ammonium sulphate and acetone did not separate the activity into specific fractions. The activity of a malt fraction in phosphate-citrate buffer was found to be optimum at pH 5.2 and to be relatively heat stable since at a temperature of ca. 45°C, inactivation did not occur.

With the exception of yeast mannan, an active malt fraction hydrolysed various mannose-containing polysaccharides to the constituent monomers; no oligosaccharide material was detected. This suggests that the malt extract possesses exo-mannanase activity, or both exo- and endo-mannanase activity. The failure of the preparation to hydrolyse yeast mannan containing α-1,6-linkages (and perhaps α-1,2- and α-1,3-linkages) would suggest that the malt enzymes are specific for β-1,4-mannosidic linkages. The hydrolysis of the galactomannans to mannose and galactose indicates that an α-galactosidase is present in addition to the β-1,4-mannanase. The relation of the mannanase activity to the cellobextrinase activity has not been established.

The fungal preparation also exhibits hydrolytic activity towards ivory nut mannan oligosaccharides; since mannose and oligosaccharides of low DP. were produced, it follows that the ratio of endo-mannanase to exo-mannanase (mannobiose) activity is different
to that present in the malted barley preparation.

The present preliminary investigation suggests that both malted barley and *Rhizopus arrhizus* extracts merit further study as sources of mannanases. By the application of chromatographic and electrophoretic techniques, it should be possible to obtain homogeneous enzyme preparations which are essential for determination of their action pattern. These purified enzyme preparations could then be applied to the determination of the fine structure of mannose-containing polysaccharides.

**Summary**

1. Malted barley and *Rhizopus arrhizus* preparations contain enzymes capable of hydrolysing mannose-containing polysaccharides.

2. No complete separation of the "mannanase" activity of malted barley was obtained by fractional precipitation with ammonium sulphate or acetone.

3. With the exception of yeast mannan, the mannose-containing polysaccharides were hydrolysed to their constituents monomers; no oligosaccharide material was detected.
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The Z-enzyme contaminant in a barley β-amylase preparation has no action on the anomalous linkages in amylose, but catalyses random hydrolysis of a small number of α-1,4-glucosidic linkages. Amylopectin and amylose dextrin are also slowly attacked, but under similar conditions, the rate of hydrolysis of glycogen and glycogen β-dextrin is not measurable. The activity, which is optimum at pH 5.6, is stabilised by calcium ions, and partly inhibited by EDTA and mercuric chloride, is attributed to a minute trace of α-amylase.

The Z-enzyme activity of soya-bean β-amylase preparations, and of almond emulsin, is also due to the presence of very small traces of α-amylase.

The methods available for the detection of α-amylase contaminants in carbohydrate preparations are discussed. The most sensitive assay is that using amylose β-limit dextrin as substrate and viscometry as the method of analysis.

The action of purified β-amylase on most samples of amylose is incomplete, since only 65—80% conversion into maltose is observed. This indicates the presence of a small number of enzymically resistant or anomalous structures in the substrate. Further, amylose is heterogeneous with respect to both degree of polymerisation (DP) and behaviour on β-amylolysis; with potato amylose of DP 3200, 40% (by weight) of DP ca. 2000 is completely hydrolysed by β-amylase, and the anomalous structures occur only in the remaining material of DP ca. 6000. For complete amylolysis a second enzyme, named Z-enzyme, is required; this occurs together with β-amylase in soya-beans and barley, is inactivated at pH 3.6, and has no action on α-1,3- or α-1,6-glucosidic linkages or on β-glucosidic linkages. We now report evidence that the action of Z-enzyme involves hydrolysis of a small number of non-terminal α-1,4-glucosidic linkages in amylose rather than selective hydrolysis of anomalous linkages and is due to the presence of a trace of an α-amylase in the β-amylase preparation. Z-Enzyme (α-amylase) also slowly degrades amylopectin and its β-dextrin.

In our earlier studies on α-1,4-glucans, a highly active preparation of barley β-amylase (Wallerstein Analytical reagent) was used. By conventional tests, α-amylase could not be detected: (a) the iodine-staining power of amylopectin β-dextrin measured at 680 mg did not decrease within 24 hr.; (b) the β-amylolysis limit of glycogen was independent of enzyme concentration; (c) the molecular weight (13 x 10^6) of the β-limit dextrin of fetcal sheep liver glycogen was in good agreement with that calculated from the molecular weight (29 x 10^6) and β-amylolysis limit (40%) of the original glycogen; (d) during enzyme action on amylose, the intermediate 50% conversion dextrin had the same molecular size as the original substrate. The same enzyme preparation was considered to contain Z-enzyme since complete degradation of amylose at pH 4.6 but not at 3.6 was observed.

The first indication that the apparent Z-enzyme activity might be due to traces of α-amylase was obtained during studies of the β-amylolysis of amylopectin, when an apparent relation between enzyme concentration and β-amylolysis limit was found. With digests containing 33, 66, 99, and 128 units of β-amylase per mg. of polysaccharide, the apparent β-amylolysis limits were 64, 65, 68, and 71 after 92 hours' incubation at pH 4.9 and 37°. Such a relation is characteristic of the α-amylases. Since this finding was at variance with the previous results, the enzymic homogeneity of the β-amylase preparation was investigated.

Degradation of Amylopectin and β-Dextrin by Barley Z-Enzyme.—The above possibility has been examined in detail with amylopectin β-dextrin as substrate, and (a) iodine staining, (b) viscosity, and (c) reducing-power measurements to follow enzyme action.

Peat, Pirt, and Whelan carried out iodine-staining by measuring the decrease in absorption value (A.V.) of the polysaccharide–iodine complex at 680 μν, the wavelength used for “blue-value” (B.V.) determinations. In our experiments, the wavelength of maximum absorption (λmax) has been used, namely, 530—540 μν. Under these conditions and with an increase in the relative enzyme concentration and time of incubation, a marked decrease in iodine-staining power was observed. For example, with 0-1% of substrate and 0-2% of barley preparation (equivalent to ca. 250 β-amylase units per mg. of polysaccharide) at pH 4-6, an 87% decrease in A.V. occurred in 70 hr., and the residual polysaccharide–iodine complex then had λmax at 420 μν. This indicates random hydrolysis of non-terminal α-1,4-glucosidic linkages. Under similar conditions the A.V. of glycogen β-dextrin at 470, 430, 420, and 410 μν was unchanged.

The decrease in A.V. of amylopectin β-dextrin could be detected with only 0-05% barley preparations; at 480, 520, and 560 μν, the A.V. fell by 17, 30, and 40%, respectively, after 70 hr. Further experiments (Table 1) showed that this activity was increased by pre-incubation of the enzyme with 5 × 10^{-3} M-calcium for 30 min., and was decreased by similar treatment with 5 × 10^{-3} M-ethylenediaminetetra-acetic acid (EDTA). The function of the calcium appears to be that of an enzyme-stabiliser rather than a specific activator. The presence of this ion decreased the rate of inactivation of the enzyme at pH 4-6 and 37° during incubation for 67 hr. in the absence of substrate, and did not restore the activity of a partly inactivated enzyme preparation. EDTA appears to lower the activity by partial removal of the calcium (cf. ref. 15). The protection from inactivation of other α-amylases, including malt α-amylase, by calcium ions has been noted by several workers.

The activity towards β-dextrin was greater in sodium acetate buffer of pH 5-6 than at pH 4-8 or 6-5 (see Figs. 1 and 2); none was detected at pH 3-6. At pH 5-6, the activities in acetate and B.D.H. Universal buffer were identical, in contrast to the behaviour of Cladophora rupes tris amylocase which is more active in the latter buffer. Addition of 5 × 10^{-3}M borate, which inhibits isoamylase and activates Cladophora amylocase, to the acetate buffer had no effect; phenylacetate (7 × 10^{-3} M) and phosphate (7 × 10^{-2} M) likewise did not alter the rate of decrease of A.V. (at 540 μν).

### Table 1. The effect of the barley β-amylase preparation on the A.V. of amylopectin β-dextrin.

<table>
<thead>
<tr>
<th>Barley prep.</th>
<th>Pre-treated with Ca⁺⁺</th>
<th>Pre-treated with EDTA</th>
<th>Pre-treated with Ca⁺⁺</th>
<th>Pre-treated with EDTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fall in A.V. (%)</td>
<td>Normal</td>
<td>480</td>
<td>75</td>
<td>85</td>
</tr>
<tr>
<td>λ (μν)</td>
<td>530</td>
<td>0-67</td>
<td>0-66</td>
<td>530</td>
</tr>
<tr>
<td>Original λmax, (μν)</td>
<td>530</td>
<td>430</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final λmax, (μν)</td>
<td>430</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final A.V.</td>
<td>0-22</td>
<td>0-23</td>
<td>0-49</td>
<td></td>
</tr>
<tr>
<td>Pre-treated</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fall in A.V. (%)</td>
<td>0-039</td>
<td>0-36</td>
<td>0-037</td>
<td>0-033</td>
</tr>
<tr>
<td>A.V. (680 μν)</td>
<td>0-132</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A.V. (540 μν)</td>
<td>0-147</td>
<td>0-136</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Digests contained 0-1% of substrate and 0-2% of enzyme preparation and were incubated at pH 4-6 and 35° for 72 hr.

### Table 2. Action of normal concentrations of β-amylase on amylopectin.

<table>
<thead>
<tr>
<th>Time of incubation (hr.)</th>
<th>Time of incubation (hr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>27</td>
<td>49</td>
</tr>
<tr>
<td>β-Amylase (units)</td>
<td>Amylopectin limit (%)</td>
</tr>
<tr>
<td>β-Amylase (units)</td>
<td>A.V. (680 μν)</td>
</tr>
<tr>
<td>β-Amylase (units)</td>
<td>A.V. (540 μν)</td>
</tr>
</tbody>
</table>

* Digests contained ca. 40 units of β-amylase per mg. of amylopectin.
In contrast to the above results (with ca. 250 units of β-amylase per mg. of substrate), the enzyme preparation caused only a slight decrease in the iodine-staining power of amylopectin when experimental conditions similar to those of Peat, Pirt, and Whelan were used (ca. 40 units per mg. of substrate; A.V. (680 mp)) as shown in Table 2. This result illustrates the importance of varying the enzyme concentration when testing for contaminating enzymes.

A slow and limited degradation of 0.5% amylopectin β-dextrin solution by 0.5% barley preparation at pH 5.6 and 25° was also shown by viscosity measurements. After 5, 40, and 70 min., the specific viscosity ($\eta_s$) values were 0.194, 0.186, and 0.179 respectively. At pH 3.6, the viscosity was unchanged.

**Fig. 1.** Effect of barley Z-enzyme on the iodine-staining power of amylopectin β-dextrin. Enzymic reactions carried out at pH 3.6 (A), 4.6 (B), 5.6 (C), 6.5 (D), and at 5.6 in presence of $5 \times 10^{-3}$M-borate (E).

**Fig. 2.** Effect of pH on barley Z-enzyme activity. Substrate: amylopectin β-dextrin; (A, B) acetate buffer analysed after 6 and 24 hr.; (C, D) phosphate-citrate buffer analysed after 8-75 and 27 hr.

Despite the limited degradation of β-dextrin in the above experiments, the effect of this initial α-amylolysis can be magnified by the presence of an excess of β-amylase. Hydrolysis of only a small number of interior α,1,4-linkages liberates non-reducing end-groups which are susceptible to this enzyme. The resultant increase in reducing power is then appreciable, whereas neither β-amylase nor low concentrations of α-amylase, acting separately on β-dextrin, yield measurable amounts of reducing sugars. The results summarised in Table 3 show the apparent percentage conversion into maltose ($P_M$) during degradation of β-dextrin by the barley preparation. In a further experiment, the relative $P_M$ values at pH 3.6, 5.6, and 6.5 were 4, 45, and 33 respectively.

**Table 3. Action of barley preparation on amylopectin β-dextrin.**

<table>
<thead>
<tr>
<th>Age of digest (hr.)</th>
<th>Apparent conversion (%) into maltose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Digest conditions:</td>
<td></td>
</tr>
<tr>
<td>(a) Acetate buffer pH 4.6</td>
<td>25 33 37</td>
</tr>
<tr>
<td>(b) pH 5.6</td>
<td>35 54 55</td>
</tr>
<tr>
<td>(c) pH 5.6 (with $5 \times 10^{-3}$M-borate)</td>
<td>35 54 56</td>
</tr>
<tr>
<td>(d) B.D.H. Universal buffer pH 5.6</td>
<td>31 49 52</td>
</tr>
</tbody>
</table>

* For composition of digests, see p.
Degradation of Amylose and β-Dextrin by Barley Z-Enzyme.—Although the highly branched amylopectin β-dextrin may be used to detect relatively high concentrations of Z-enzyme, the use of a linear substrate is preferable since the hydrolysis of only a small number of linkages will produce a more marked change in physical properties. The effect of certain inhibitors on barley Z-enzyme was therefore investigated by using amylose β-limit dextrin (prepared by the prolonged action of β-amylase at pH 3.6 on potato amylose): the results are summarised in Table 4.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibn. (%) †</th>
<th>Inhibitor</th>
<th>Inhibn. (%) †</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁻²M-Iodoacetate</td>
<td>100</td>
<td>10⁻²M-p-Chloromercuribenzoate</td>
<td>7</td>
</tr>
<tr>
<td>1.5 × 10⁻³M-Mercuric chloride</td>
<td>80</td>
<td>10⁻³M-Silver nitrate</td>
<td>86</td>
</tr>
<tr>
<td>10⁻⁴M-Phenymercuric acetate</td>
<td>79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10⁻⁵M-Phenymercuric acetate</td>
<td>73</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* All inhibitors caused 100% inhibition of β-amylase.
† Based on A.V. (600 m/x) measurements; see p.

Peat, Thomas, and Whelan³ reported that Z-enzyme was not inhibited by ca. 1.5 × 10⁻⁶M-mercuric chloride and concluded that this distinguished Z-enzyme from α- and β-amylase. Although the extreme sensitivity of β-amylase towards mercury compounds and related thiol-reactants is well known,¹⁹ there is evidence that thiol groups are not essential for the activity of α-amylases.²⁰ We have examined the effect of various concentrations of mercuric chloride on the activity of a number of α-amylases, and the results (Table 5) show that only partial inactivation occurs with concentrations of 10⁻⁵ to 10⁻⁴M. Further, the action of barley Z-enzyme is only partially inhibited by mercuric chloride (Table 4 and 5). Notwithstanding the qualitative nature of these studies (the concentration of α-amylase was not identical in the various assays), it is concluded that the use of mercuric chloride shows, in fact, a similarity between Z-enzyme and α-amylase.

<table>
<thead>
<tr>
<th>Concentration (m) of HgCl₂</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁻³</td>
<td>100</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>66</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>67</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>67</td>
</tr>
</tbody>
</table>

* Assay under Hobson, Whelan, and Peat’s conditions.¹⁵
† Assay under Bernfeld’s conditions (Methods in Enzymology, 1955, 1, 149).
‡ See p.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley β-amylase *</td>
<td>100</td>
</tr>
<tr>
<td>Salivary α-amylase †</td>
<td>84</td>
</tr>
<tr>
<td>Bacterial α-amylase †</td>
<td>87</td>
</tr>
<tr>
<td>Malt α-amylase ‡</td>
<td>96</td>
</tr>
<tr>
<td>Barley Z-enzyme ‡</td>
<td>86</td>
</tr>
</tbody>
</table>

* Assay under Hobson, Whelan, and Peat’s conditions.¹⁵
† Assay under Bernfeld’s conditions (Methods in Enzymology, 1955, 1, 149).
‡ See p.

10⁻³ to 10⁻⁴M. Further, the action of barley Z-enzyme is only partially inhibited by mercuric chloride (Table 4 and 5). Notwithstanding the qualitative nature of these studies (the concentration of α-amylase was not identical in the various assays), it is concluded that the use of mercuric chloride shows, in fact, a similarity between Z-enzyme and α-amylase.

p-Chloromercuribenzoate (1 × 10⁻⁵M) completely inhibits β-amylase¹⁹ and yet has less effect on barley Z-enzyme than have similar concentrations of mercuric chloride (Table 4). This reagent has therefore been used for the selective inactivation of β-amylase. On incubation of amylose (0.2%) with barley preparation (0.07%, corresponding to ca. 45 β-amylase units/mg) and p-chloromercuribenzoate, a marked decrease in specific viscosity was observed, but without a concomitant decrease in iodine-staining power or increase in reducing power. This is attributed to the random hydrolysis of a small number of non-terminal linkages. Since the iodine-staining power of amylose as obtained by “blue-value” or λ_{max} measurements is approximately the same for samples of DP 500 or 2000,²² this result emphasises the caution required in following limited α-amylolysis by iodine-staining. Measurements of the change in reducing power or of the sedimentation constant of the residual amylose are also unsatisfactory when the concentration of α-amylase is extremely low. It is clear that viscometry provides the only sensitive method when concentrations of β-amylase contaminated by Z-enzyme similar to those used in our previous studies⁶-⁸ and by Peat and his co-workers³,⁴ are employed.
The presence of traces of α-amylase in unpurified barley β-amylase preparations has been noted by earlier workers including Hopkins, Murray, and Lockwood. The amount of α-amylase appears to depend upon the condition of the grain, and the variety of the barley. Part of the α-amylase may represent precursors of the enzyme which develops in quantity during germination, and part may arise from contamination of the barley husk by amylase-secreting bacteria. The α-amylase constituents of ungerminated and germinated barley are undoubtedly closely related, and we have found that the initial action of malt α-amylase on amylose β-dextrin is also not activated by calcium ions, and is partially inhibited by mercuric chloride (1.5 × 10⁻⁵ and 1.5 × 10⁻⁶M). Further, the optimum pH of unpurified malt α-amylase is ca. 5.4, a value similar to that shown in Fig. 2 and different from that of bacterial α-amylase (ca. 6.5).

Z-Enzyme Activity of Soya-bean β-Amylase and Almond Emulsin.—Since Z-enzyme was originally detected in unpurified ("stock") preparations of soya-bean β-amylase and in almond emulsin (a complex mixture of carbohydrases including β-glucosidases), samples of these have been examined for contamination with α-amylases.

Neither preparation had a significant effect on the production of maltose from amylpectin-β-amylase or Floridean starch-β-amylase systems (cf. Table 2 and ref. 6); by this criterion gross contamination with α-amylase could be ruled out. However, when amylose, amylose β-dextrin, or amylpectin β-dextrin was used as substrate, and assay was by iodine-staining and reducing power or viscosity, the presence of a trace of α-amylase was established. Slight random degradation of the substrates occurred; for example, incubation of amylose (0.1%) with emulsin (0.5%) at pH 4.6 for 24 hr. reduced νsp by 88% and increased the β-amylolysis limit from 75 to 95%. This α-amylolytic activity was increased by calcium ions (which again act as a stabiliser rather than activator), and partly inhibited by EDTA and mercuric chloride (10⁻⁴—10⁻⁶M), and was optimum in the region pH 5.8–6.1 (cf. Peat, Thomas, and Whelan who reported the action of soya-bean Z-enzyme on amylose β-dextrin as maximum at pH 6). Typical results are shown in Table 6 and Figs. 3 and 4. In similar conditions, the extent of hydrolysis of glycogen β-dextrin by emulsin was approximately one-half that of amylpectin β-dextrin (P₉₃ 3.7 and 7.8 respectively). The properties of the α-amylase present in soya-beans and almond emulsin are therefore generally similar to, although not necessarily identical with, those of barley Z-enzyme.

**Discussion and Conclusions.**—The recognition of the presence of a trace of α-amylase in the barley and soya-bean β-amylase preparations, and in emulsin, provides an explanation for the observed increase in the β-amylolysis of amylose. The slight random hydrolysis will expose sufficient new non-reducing end-groups to enable further β-amylolysis to take place. If the presence of only one anomalous structure per amylose molecule is assumed, the random hydrolysis of only one α-1,4-glucosidic linkage will cause a 10—15% increase in β-amylolysis limit, e.g., from ca. 75 to ca. 87%. The ability of Z-enzyme to increase the phosphorylase limit of amylose, from 70 to 95%, conversion into glucose 1-phosphate, can now also be explained in terms of slight α-amylolytic activity.

**Table 6. Effect of emulsin on the iodine-staining power of amylose β-dextrin.**

<table>
<thead>
<tr>
<th>Wavelength (μm)</th>
<th>580</th>
<th>600</th>
<th>640</th>
<th>680</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digest conditions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Control</td>
<td>56</td>
<td>60</td>
<td>65</td>
<td>69</td>
</tr>
<tr>
<td>(b) Mercuric chloride: 1.5 × 10⁻⁶M</td>
<td>33</td>
<td>35</td>
<td>38</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>1.5 × 10⁻⁸M</td>
<td>44</td>
<td>47</td>
<td>55</td>
</tr>
<tr>
<td>(c) Pre-treated with CaSO₄ †</td>
<td>72</td>
<td>74</td>
<td>79</td>
<td>82</td>
</tr>
<tr>
<td>(d) Pre-treated with EDTA: (i) alone †</td>
<td>23</td>
<td>24</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>(ii) diluted with water</td>
<td>27</td>
<td>28</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>(iii) diluted with CaSO₄ †</td>
<td>47</td>
<td>50</td>
<td>51</td>
</tr>
</tbody>
</table>

* Incubated for 25 hr. at 35°. † Final concentration 2 × 10⁻⁶M.
The nature of the structural anomalies in amylose is not yet known.* These may include one or more of the following possibilities: (a) an anomalous linkage (i.e., a glucosidic linkage other than the α-1,4-type) in the amylose chain or as a branch point; (b) an anomalous residue, i.e., an α-1,4-linked hexose residue derived from D-glucopyranose by substitution with a phosphate group, probably at position 6, or by acylation or oxidation at position 2, 3, or 6; (c) both a residue and its linkage may be anomalous. Recent evidence 28 suggests that a small number of glucose residues in amylose may become modified by oxidation during isolation of the polysaccharide, becoming resistant to β-amylase and phosphorylase. Since Z-enzyme is an α-amylase, its action will involve the “by-passing” of such structural anomalies rather than their removal by selective hydrolysis. Similarly, any anomalous linkage which is present as a branch point will not be hydrolysed by Z-enzyme (or any other α-amylase), i.e., Z-enzyme does not act as a “debranching” enzyme.

Fig. 3. Effect of pH on the Z-enzyme activity of emulsin and “stock” soya-bean β-amylase preparation.

Curve A represents action of soya-bean preparation on amylopectin β-dextrin (reducing-power measurements expressed as P₃₈ values); curve B shows the effect of emulsin on the A.V. (840 μg) of amylose β-dextrin expressed as percentage decrease.

Some anomalous structures are present in unfractionated starch since the β-amylolysis limits with purified and “stock” soya-bean β-amylase are 53 and 61% respectively. 4 This difference was attributed to the action of Z-enzyme on the amylose component. However, Hopkins and his co-workers 23 had previously shown that barley β-amylase prepared by a method involving pretreatment at pH 3·4 caused 56% conversion of soluble starch into maltose, and that if this treatment was omitted, or if a trace of bacterial α-amylase was added to the purified preparation, the β-amylolysis limit was 63%.

These findings are in accord with our observations, and the view that “Z-enzyme” is a trace of α-amylase adequately explains the effect of pH on the β-amylolysis limit of unfractionated starch (cf. ref. 13).

The amount of α-amylase present in the barley preparation is too small to be assessed accurately but, in comparative experiments, a salivary α-amylase solution containing 34 units 26 was diluted 50,000 times and found to cause a decrease in the iodine-staining power of both amylose and amylopectin β-dextrin and to have only a limited action on glycogen β-dextrin. This suggests that the α-amylase activity of the barley preparation

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Fig. 4. Effect of soya-bean β-amylase on the A.V. (540 μg; O; 680 μg, •) of amylopectin β-dextrin.

Concn. of mercuric chloride in the digests was 0 (A), 1·5 × 10⁻⁶ M (B), and 1·5 × 10⁻⁵ M (C).

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* Since the proportion of these is extremely low (probably less than 0·1%), their presence in enzymic hydrolysates of amylose cannot be revealed by available methods of analysis, and is inferred from the known specificity requirements of α- and β-amylase.
is of the order of $10^{-3}$ unit/mg. A further indication of the minute degree of contamination is shown by a comparison of the turn-over number of the related malt $\alpha$-amylase, equivalent to the hydrolysis of 10,000 bonds per min. per mole, with the limited degradation of amylose $\beta$-dextrin observed by viscometry in our experiments during incubation for some hours, and the previous failure to detect degradation of glycogen $\beta$-dextrin by light-scattering, or of amylose by sedimentation measurements. It is suggested that the inability of barley Z-enzyme to cause appreciable degradation of glycogen is a consequence of (a) the low concentration of enzyme and (b) the lowered affinity of $\alpha$-amylases in general for glycogen, rather than to an absolute specificity requirement (cf. $\beta$-enzyme which hydrolyses 1,6-linkages in amylpectin but not in normal 12-unit glycogen). The hydrolysis of glycogen or its $\beta$-dextrin with normal concentrations of other $\alpha$-amylases, including preparations from ungerminated barley and soya-beans, can readily be detected.

Our general conclusions are in accord with the findings of Hopkins and Bird, who have emphasised the difficulty in detecting traces of $\alpha$-amylase when using amylpectin rather than amylose as a substrate, and with the recent results of Baba and Kojima and of Banks, Greenwood, and Jones. Baba and Kojima also showed the presence of $\alpha$-amylase in emulsin, and Banks et al. independently proved random hydrolysis of starch components, using light-scattering and viscosity measurements, by the Z-enzyme contaminant of several unpurified $\beta$-amylase preparations.

It must be noted that the present results do not alter our earlier conclusions on the molecular structure of starch and glycogen-type polysaccharides, or on the mechanism of $\beta$-amylase action, which are derived, in part, from results obtained with the Wallerstein barley $\beta$-amylase preparation.

**Experimental**

**Analytical Methods.**—The general methods used were those described in earlier papers.

For viscometry, digests were prepared in modified Ubbelohde or Ostwald viscometers, and the viscosity at 25° was measured at intervals. Since the activity of an $\alpha$-amylase is related to $d(1/\eta)/dt$, graphs of $1/\eta$ against $t$ were prepared. With identical enzyme and substrate concentrations, the effect of added reagents could then be observed by a comparison of the slopes. The polysaccharide solutions were filtered through sintered glass (G4) before analysis.

In the iodine-staining experiments with amylpectin $\beta$-dextrin, measurements at 540 mp increased the A.V. to ca. 0.5 in the most sensitive region of the spectrophotometer (Unicam S.P. 500) (cf. A.V. of ca. 0.1 at 680 mp).

**Enzyme Preparations.**—The properties of the barley $\alpha$-amylase are reported in ref. 7.

"Stock" and purified soya-bean $\beta$-amylase were prepared by the methods of Bourne, Macey, and Peat and Peat, Pirt, and Whelan. Sweet-almond emulsin was isolated by Tauber's method; a weighed amount was centrifuged in the stated volume of water, and insoluble material was discarded. Wallerstein malt diastase was used as a source of malt $\alpha$-amylase, with short incubation periods to minimise the effect of $\beta$-amylase.

**Substrates.**—(a) Amylose. Various samples made by the fractionation of starch from potatoes (var. Kerr's pink) with thymol and butanol were used, together with amyllose VI, VII, and VIII. Amylose $\beta$-dextrin was prepared by incubating amyllose VIII (500 mg.) with barley $\beta$-amylase (100 units/mg.) at pH 3.6 for 24 hr. The $\beta$-amyloysis limit was 72%. The digest was heated for 10 min., cooled, and filtered (G4 sinter), and the pH was adjusted to 5.6 with aqueous sodium hydroxide. The dextrin was stored under toluene at room temperature; the maltose present did not interfere with subsequent measurements.

(b) Amylopectin. Fractionation of potato starch with thymol or pyridine gave samples I and II respectively. Waxy-maize and sorghum starch were commercial samples. Amylopectin $\beta$-dextrin was prepared from waxy-maize starch I (5 g.) treated with purified $\beta$-amylase (6000 units) in a total volume of 250 ml. at 35° for 48 hr. The $\beta$-amyloysis limit was 53%. After dialysis, the dextrin was isolated by freeze-drying. Samples of amylpectin $\beta$-dextrin were also prepared from waxy-sorghum starch and potato amylopectin by similar methods.

(c) Glycogen $\beta$-dextrin. This was isolated from a digest of Ascaris lumbricoides glycogen and $\beta$-amylase.
Action of Barley Z-Enzyme on Amylopectin β-dextrin.—(a) Iodine-staining measurements. Polysaccharide (25 mg.), barley preparation (52 mg.), 0.2M-sodium acetate buffer (pH 4.6; 3 ml.), and water to 20 ml. were incubated at 35° for 70 hr. Samples (2 ml.) were removed, heated to inactivate the enzyme, and stained with iodine solution (0.2% in 2% potassium iodide solution; 2-5 ml.) in a total volume of 25 ml. The A.V. (540 μl) of amylopectin β-dextrin decreased from 0-740 to 0-097 and the product showed λ_{max} 420 mp and A.V._{max} 0-210. With glyccogen β-dextrin, the initial and final A.V.'s were: at 470 mp, 0-044 and 0-042; at 430 mp, 0-073 and 0-074; at 420 mp, 0-075 and 0-078; at 410 mp, 0-087 and 0-089. When only 6-25 mg. of barley preparation were used, the following results were obtained:

<table>
<thead>
<tr>
<th>λ (μl)</th>
<th>480</th>
<th>500</th>
<th>520</th>
<th>540</th>
<th>560</th>
<th>580</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial A.V.</td>
<td>0-545</td>
<td>0-622</td>
<td>0-674</td>
<td>0-680</td>
<td>0-630</td>
<td>0-532</td>
</tr>
<tr>
<td>Final A.V.</td>
<td>0-446</td>
<td>0-469</td>
<td>0-472</td>
<td>0-440</td>
<td>0-377</td>
<td>0-319</td>
</tr>
</tbody>
</table>

Barley preparation (ca. 50 mg.), pretreated with 5 × 10^{-3}m-calcium sulphate or 5 × 10^{-2}m-EDTA (pH 4-7) for 30 min. at 37°, was incorporated into similar digests. Samples (3 ml.) were removed after 72 hr.; the results are reported in Table 1. In a further experiment with 0-67 mg. of EDTA-treated enzyme, only a slight decrease in iodine-staining power was noted:

<table>
<thead>
<tr>
<th>λ (μl)</th>
<th>480</th>
<th>500</th>
<th>520</th>
<th>540</th>
<th>560</th>
<th>580</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial A.V.</td>
<td>0-529</td>
<td>0-507</td>
<td>0-675</td>
<td>0-655</td>
<td>0-510</td>
<td>0-531</td>
</tr>
<tr>
<td>Final A.V.</td>
<td>0-529</td>
<td>0-573</td>
<td>0-600</td>
<td>0-575</td>
<td>0-517</td>
<td>0-436</td>
</tr>
</tbody>
</table>

The optimum pH for EDTA-calcium complex formation is 7-5; hence, in the above experiments, the calcium ions may not have been completely removed.

(b) Measurement of reducing power. Digests (20 ml.) were prepared containing amylopectin β-dextrin (19-2 mg.), barley α-amylase (3800 units), buffer solution (3 ml.), and water. At intervals samples (5 ml.) were deproteinised, and the apparent maltose contents were determined. The results are shown in Table 3.

(c) Viscometry. Amylopectin β-dextrin (1%, filtered solution; 10 ml.), 0.2M-acetate buffer (5 ml.) of pH 3-6 or 5-6, and barley α-amylase (100 mg. in 5 ml. of water) were mixed in a viscometer. \( \tau_{sp} \) was determined during 2 hr. At pH 5-6, \( d/(\tau_{sp})/dt \) indicated 38 a relative activity of 7-8 × 10^{-3} unit, and in presence of mercuric chloride (1-5 × 10^{-5} at 7-2 × 10^{-3} unit. At pH 3-6, there was no change in viscosity.

Effect of pH and Various Ions on Activity.—Amylopectin β-dextrin (ca. 30 mg.), barley preparation (6000 units), buffer (3 ml.), and water (to 25 ml.) were incubated at 35°. The buffers used were 0.2M-acetate of (a) pH 3-6, (b) pH 4-8, (c) pH 5-6, (d) pH 6-5, and (e) pH 5-6 containing borate to give a final concentration of 5 × 10^{-3}m. The A.V. of samples (2 ml.) was measured at intervals. The results obtained at 540 μl are shown in Fig. 1. Similar results were obtained over the range 460—680 μl.

For the pH-activity curves, β-dextrin (10 mg.) was incubated with β-amylase (1250 units) and 0.2M-acetate buffer (pH 4-6—7-6; 5 ml.) in a total volume of 15 ml. Samples (3 ml.) were removed after 6 and 24 hr. and the A.V.'s at both 540 and 680 μl were determined. After 6 hr., at 540 μl., the maximum decrease was at pH 5-5; after 24 hr., over the range pH 6-1—6-4 (see Fig. 2). The small change in pH is attributed to the decreased stability of the enzyme in acetate buffer at pH 4—6. The same results were obtained from A.V. determinations at 680 μl. The experiment was repeated with phosphate-citrate buffer (pH 5-2—7-3; 0.1M-citric acid and 0.2M-disodium hydrogen phosphate; 3 ml.) in a 10 ml. digest. The maximum fall in A.V. (540 μl) occurred at pH 5-6 after 8-75 hr. and at pH 5-8 after 27 hr.

Digests containing β-dextrin (10 mg.), barley preparation (2000 units), and buffer (5 ml.) in a total volume of 15 ml. were incubated at 35°. The following results were obtained.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Acetate (pH 5-6)</th>
<th>B.D.H. Universal (pH 5-6)</th>
<th>Phenyl-acetate *</th>
<th>Borate *</th>
<th>Phosphate *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fall (%) in A.V. (540 μl):</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>after (a) 6 hr.</td>
<td>34</td>
<td>33</td>
<td>34</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>(b) 27 hr.</td>
<td>70</td>
<td>69</td>
<td>70</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>F_{2}, after 99 hr.</td>
<td>12-6</td>
<td>12-9</td>
<td>13-1</td>
<td>12-5</td>
<td>12-6</td>
</tr>
</tbody>
</table>

* These digests contained 5 ml. of sodium acetate buffer (pH 5-6) and 5 ml. of 2 × 10^{-2}m-anion.

The function of the calcium ion was examined by incubating enzyme solution (2 ml.) with β-dextrin (10 mg.) and acetate buffer (pH 5-8; 3 ml.) in a total volume of 10 ml. Digest no. 1
contained barley preparation pre-incubated at 37° and pH 5-8 for 67 hr.; digest no. 2 contained enzyme solution as above, but also 5 x 10^{-3}m-calcium acetate; digest no. 3 was as digest no. 1 except that the enzyme was added to a mixture of β-dextrin and calcium acetate.

**Fall (%) in A.V. (540 μL)**

<table>
<thead>
<tr>
<th>Incubation (hr.)</th>
<th>Digest no. 1</th>
<th>Digest no. 2</th>
<th>Digest no. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>13</td>
<td>31</td>
<td>13</td>
</tr>
<tr>
<td>7</td>
<td>18</td>
<td>45</td>
<td>19</td>
</tr>
<tr>
<td>24</td>
<td>49</td>
<td>83</td>
<td>51</td>
</tr>
</tbody>
</table>

*Action of Barley β-Enzyme on Amylose β-Dextrin.—(a) Iodine-staining measurements.* Digests containing amylose β-dextrin solution (0-46 mg./ml. by acid hydrolysis; 14 ml.) and β-amylase (14 mg. in 0-5 ml. of water; pre-incubated at 20° for 20 min. with 0-5 ml. of inhibitor solution) were incubated at 35°. Samples (4 ml.) were withdrawn after 2-5 and 4-5 hr., stained with iodine solution (1 ml.), and diluted with water to 25 ml. A.V.'s were measured at 560, 580, 600, and 640 μm. The trend of results was the same at all wavelengths; the results at 600 μm are given in Table 4.

The effect of mercuric chloride was examined in digests containing amylose β-dextrin (10 mg.), β-amylase (15 mg.), 0-2m-acetate buffer (pH 5-5, 3 ml.), mercuric chloride solution (1 ml.), and water (6 ml.). Samples (3 ml.), removed after 2-5 hr., gave the following results:

<table>
<thead>
<tr>
<th>Conc. of HgCl₂ (m)</th>
<th>Decrease (%) in A.V. at (600 μm)</th>
<th>10^{-4}</th>
<th>10^{-3}</th>
<th>10^{-6}</th>
<th>Nil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7</td>
<td>15</td>
<td>46</td>
<td>49</td>
<td></td>
</tr>
</tbody>
</table>

(b) **Viscometry.** A digest containing amylose solution (70 mg.; 20 ml.; with p-chloromercuribenzoate, 10^{-3}m) and β-amylase (25 mg. in 15 ml. of 0-2m-acetate buffer of pH 4-6; with p-chloromercuribenzoate, 10^{-5}m) was prepared in a viscometer. The following results were obtained:

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>97</th>
<th>120</th>
<th>155</th>
<th>205</th>
<th>20 hr.</th>
<th>45 hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/ν₀</td>
<td>313</td>
<td>332</td>
<td>373</td>
<td>394</td>
<td>420</td>
<td>448</td>
<td>465</td>
<td>625</td>
<td>676</td>
</tr>
</tbody>
</table>

Samples were also removed for the measurement of A.V. at both 540 and 680 μm, and of the reducing power. No change was detected within 24 hr. A control experiment showed that 10^{-6}m-p-chloromercuribenzoate had no effect on the reaction of maltose with the Somogyi reagent; in the absence of this material the viscosity change is accompanied by a marked decrease in iodine-staining power and rapid production of reducing sugars.

*Action of Normal Concentrations of β-Amylase on Amylopectin.—* Digests were prepared containing waxy-maize starch (30 mg.), 0-2m-acetate buffer (pH 4-6; 10 ml.), barley β-amylase or stock 'soya-bean β-amylase (1300 units), and water to a final volume of 50 ml. Samples (2 ml. for iodine-staining; 3 ml. for reducing-power measurements) were removed at intervals. The results are in Table 2.

*Action of 'Stock' Soya-bean β-Amylase on Amylopectin β-Dextrin.—* Polysaccharide (23-9 mg.), 0-2m-acetate buffer (pH 4-6; 3 ml.), enzyme solution (3 ml.), and water (10 ml.) were incubated at 35°. [The enzyme solution was prepared by dissolving 50 mg. of powder (activity ca. 100 units/ml) in 5 ml. of buffer and centrifuging the mixture.] Samples (2 ml.) were removed after 27 and 72 hr.: the results after 27 hr. were:

<table>
<thead>
<tr>
<th>A (μm)</th>
<th>480</th>
<th>500</th>
<th>520</th>
<th>540</th>
<th>560</th>
<th>580</th>
<th>680</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial A.V.</td>
<td>0.528</td>
<td>0.612</td>
<td>0.687</td>
<td>0.700</td>
<td>0.649</td>
<td>0.574</td>
<td>0.198</td>
</tr>
<tr>
<td>Final A.V.</td>
<td>0.034</td>
<td>0.039</td>
<td>0.039</td>
<td>0.038</td>
<td>0.038</td>
<td>0.038</td>
<td>0.020</td>
</tr>
</tbody>
</table>

Similar results were obtained after 72 hr.

The effect of pH was examined in digests containing β-dextrin (5 mg.), phosphate-citrate buffer (pH 4-6—7-6; 5 ml.), 1% β-amylase solution (2 ml.) and water (3 ml.). The P₉₅ values of 3 ml. portions were determined after 25 hr. at 35° (see Fig. 3).

Amylopectin β-dextrin (10 mg. in 5 ml. of water) was added to 0-3% β-amylase solution (4 ml.) pre-incubated as follows: (a) with 0-2m-acetate buffer (pH 6-1; 10 ml. containing 5 x 10^{-8}m-calcium acetate); (b) with buffer containing 5 x 10^{-8}m-EDTA; (c) with buffer alone. The total volume was 24 ml. The decreases (%) in A.V. (540 μm) of samples (2 ml.) measured after 1, 13, and 42 hr. were: (a) 7, 66, and 92; (b) 0, 6, and 13; (c) 1, 24, and 50 respectively. The P₉₅ values after 42 hr. were 19, 7, and 14 respectively.

Digests containing β-dextrin (10 mg.), 0-2m-acetate buffer (pH 6-0; 5 ml.), 0-25% β-amylase
solution (2 ml.), water, and mercuric chloride (to give final concentrations of $1\times10^{-3}$ and $1\times10^{-4}\text{M}$ severally) in a total volume of 15 ml. were prepared. The change in A.V. (540 and 680 mg.) is shown in Fig. 4. The $P_M$ values determined after 8-3 and 27-5 hr. were: (a) without mercuric chloride, 15 and 18; (b) $1\times10^{-3}\text{M}$, 14 and 18; (c) $1\times10^{-4}\text{M}$, 5 and 9.

*Action of "Storch" Soya-bean $\beta$-Amylase on Other Polysaccharides.*—Potato amylpectin II (40 mg.), 0-2M-acetate buffer (pH 4-6; 9 ml.), 0-2$\%$ $\beta$-amylase solution (1 ml.), and water to 30 ml. were incubated at $35^\circ$. The $\beta$-amylolysis limit was 49 (0-5 hr.), 50 (1 hr.), 53 (4 hr.), and 53 (24 hr.). In similar conditions soluble starch had a $\beta$-amylolysis limit of 62$\%$ but with purified soya-bean $\beta$-amylase the value was 57$\%$. When potato amylase VI was used, $\beta$-amylolysis limits of 77$\%$ were found after 4 and 22 hours' incubation with 0.5 units per ml. of polysaccharide at pH 4-6; with higher enzyme concentrations, complete degradation occurred.

*Action of Emulsin on Amylopectin.*—Waxy-maize starch (20 mg.), barley $\beta$-amylase (50 units/mg.), 0-2M-acetate buffer (pH 4-6; 1 ml.), and water (to 25 ml.) were incubated at $35^\circ$ for 48 hr. The $\beta$-amylolysis limit was 57$\%$. Emulsin (20 mg.) was added; after a further 24 hr., the $\beta$-amylolysis limit was 58$\%$. In a second digest in which $\beta$-amylase and emulsin acted together on waxy-maize starch, the $\beta$-amylolysis limit was 56 and 56$\%$ after 24 and 48 hr.

*Action of Emulsin on Amylose.*—Amylose VIII (30 mg.) was incubated at pH 3-6 with barley $\beta$-amylase (100 units/mg.) in a total volume of 50 ml. for 24 hr. The $\beta$-amylolysis limit was 75$\%$. The enzyme concentration was then doubled, and after 24 hr. the $\beta$-amylolysis limit was 76$\%$. The pH of the digest was then adjusted to 4-8, and to a 15 ml. portion 1$\%$ emulsin solution (5 ml.) was added. After 1 and 24 hr., the $\beta$-amylolysis limits were 88 and 95$\%$, and the A.V.'s (680 mg.) (measured on a 3 ml. sample stained with 1 ml. of iodine solution and diluted to 25 ml.) were 0-005 and 0-002 respectively compared with an original A.V. of 0-200.

A second 15 ml. portion of the digest was incubated with emulsin and 0-01M-mercuric chloride (0-5 ml.) in a total volume of 25 ml. (final concentration $2\times10^{-3}\text{M}$). The A.V.'s (680 mg.) were 0-202, 0-195, and 0-185 after 0, 1, and 24 hr. respectively.

For amylase VI, $\beta$-amylolysis limits of 73$\%$ before, and 101$\%$ after, addition of emulsin were obtained; the A.V. (680 mg.) of a sample fell from 0-21 to 0-08.

Amylose VII solution (2 mg./ml.: 25 ml.) was then incubated at pH 4-6 with 2$\%$ emulsin solution (12-5 ml.) in a total volume of 50 ml. After 24 hr. the digest was heated, then cooled, and denatured protein was removed at the centrifuge. The residual polysaccharide was precipitated with ethanol, washed, and dried. The specific viscosity at 25$^\circ$ of 25 ml. of polysaccharide dissolved in 20 ml. of 0-2N-potassium hydroxide was 0-025, and the $\beta$-amylolysis limit at pH 3-6 was 93$\%$. Under similar conditions, amylase VII has a $\beta$-amylolysis limit of 75$\%$. In a control experiment with heat-denatured emulsin, the residual polysaccharide had a specific viscosity of 0-212.

*Effect of pH on Activity.*—Digests containing amylose $\beta$-dextrin (2-4 mg. in 2 ml. of water), phosphate-citrate buffer (pH 4-6 to 7-6; 2 ml.), and emulsin solution (15 mg. in 1 ml. of water) were incubated at 37$^\circ$. Control digests (a) without enzyme and (b) without $\beta$-dextrin were also prepared. After 24 hr. samples (2-5 ml.) were withdrawn, heated, and centrifuged. Iodine solution (1 ml.) was added to 2 ml. of solution and the A.V.'s at 640 mg. were measured after dilution to 25 ml. The results are shown in Fig. 3. No correction was required for the enzyme control.

*Effect of Calcium Ions and Inhibitors on Emulsin.*—(a) Iodine-staining measurements. Digests were prepared containing 0-4$\%$ amylose solution (5 ml.), emulsin (0-5$\%$ in 0-2M-acetate buffer of pH 5-6; 5 ml.), and calcium acetate ($5\times10^{-3}\text{M}$; 0-5 ml.) or water (0-5 ml.). Digest (a) contained newly prepared enzyme and substrate and water; digest (b) contained enzyme pre-incubated at 37$^\circ$ for 40 hr.; digest (c) contained enzyme pre-incubated with calcium, and digest (d) contained pre-incubated enzyme added to calcium. Samples (2 ml.) were withdrawn at intervals, heated, and coagulated, protein was removed, and 1 ml. portions were used for

<table>
<thead>
<tr>
<th>Incubation (hr.)</th>
<th>(a)</th>
<th>(b)</th>
<th>(c)</th>
<th>(d)</th>
<th>Incubation (hr.)</th>
<th>(a)</th>
<th>(b)</th>
<th>(c)</th>
<th>(d)</th>
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<tbody>
<tr>
<td>5</td>
<td>11</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>21-5</td>
<td>9</td>
<td>29</td>
<td>12</td>
<td>—</td>
</tr>
<tr>
<td>6-5</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>—</td>
<td>40</td>
<td>52</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>16</td>
<td>35</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>47-3</td>
<td>12</td>
<td>47</td>
<td>15</td>
<td>—</td>
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</table>
A.V. (680 mp) measurements. Results, tabulated, show that the calcium ions have a stabilising rather than an activating action.

(b) Viscosity measurements. Digests containing amylose β-dextrin (13.5 mg.), ~2% emulsin solution (4.5 ml.; pre-incubated for 20 min. with reagent), and water (total volume 15 ml.) were incubated in a viscometer. The relative activities in two series of experiments were (a) 6.5 and 5.7 × 10⁻⁴ unit with water and mercuric chloride (1.5 × 10⁻⁴m); respectively, (b) 9.3, 8.2, and 6.5 × 10⁻³ unit with water, calcium sulphate (2 × 10⁻⁴m), and EDTA (2 × 10⁻⁴m) respectively.

Action of Emulsin on Amylopectin and Glycogen β-Dextrin.—Digests were prepared containing either amylopectin β-dextrin (30 mg. in 15 ml. of 0.2m-acetate buffer of pH 5-8) or glycogen β-dextrin (20 mg. in 5 ml. of buffer) and 0.5% emulsin (5 ml.). The annexed results were obtained.

<table>
<thead>
<tr>
<th>Time of incubation (hr.)</th>
<th>Amylopectin β-dextrin</th>
<th>Glycogen β-dextrin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Decrease (%) in A.V. (640 mp)</td>
<td>Pₘ</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>—</td>
</tr>
<tr>
<td>24</td>
<td>26</td>
<td>4.2</td>
</tr>
<tr>
<td>30</td>
<td>33</td>
<td>7.8</td>
</tr>
</tbody>
</table>

Action of Malt α-Amylase on Amylose β-Dextrin.—β-Dextrin (5 mg.), 0.2-m-acetate buffer (pH 5-6; 9 ml.), 0.01% diastase solution (0.5 ml.), and water or reagent (0.5 ml.) were incubated at 37°C for 30 min. The A.V. (640 mp) of a sample (3 ml.) was then determined. The following results were obtained (expressed as % fall in A.V.); control, 68; calcium acetate (2.5 × 10⁻⁴m), 67; EDTA (2.5 × 10⁻³m), 66; mercuric chloride (1.5 × 10⁻³m), 25; (1.5 × 10⁻⁴m), 2; (1.5 × 10⁻⁸m), 51. The last observation shows the effect caused solely by the α-amylase, and the results in Table 5 are calculated on this basis.

Action of Salivary α-Amylase on β-Dextrins.—Freeze-dried salivary α-amylase (34 units; 26 mg. in 1 ml. of water) was diluted 50,000 times, and sodium chloride was added to a final concentration of 0.05m. Digests were prepared containing various β-dextrins (4.8—12.0 mg.) dissolved in 0.2m-acetate buffer of pH 5-8 (6 ml.) and diluted salivary amylase (1 ml.). Samples (1 or 3 ml.) were removed for analysis by iodine-staining or reducing-power measurements. After incubation for 23 and 42.5 hr., the following results were obtained: with amylose β-dextrin (4.8 mg.), the A.V. (640 mp) fell by 25 and 53%; with amylopectin β-dextrin (12.0 mg.), the A.V. (540 mp) fell by 24 and 39%; with glycogen β-dextrin (12.0 mg.), the Pₘ values were 0.9 and 1.8 respectively.

In additional digests containing amylopectin β-dextrin (17.0 mg.) and glycogen β-dextrin (17.8 mg.), and either (a) 6.8 × 10⁻³ unit of α-amylase or (b) 13.6 × 10⁻⁴ unit, in a total volume of 16—27 ml., the extents of degradation were:

<table>
<thead>
<tr>
<th>Incubation (hr.)</th>
<th>Amylopectin β-dextrin</th>
<th>Glycogen β-dextrin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24.5</td>
<td>48.5</td>
</tr>
<tr>
<td>(a) Fall (%) in A.V. (540 mp)</td>
<td>10</td>
<td>22</td>
</tr>
<tr>
<td>Pₘ</td>
<td>3.0</td>
<td>3.2</td>
</tr>
<tr>
<td>(b) Fall (%) in A.V. (540 mp)</td>
<td>22</td>
<td>42</td>
</tr>
<tr>
<td>Pₘ</td>
<td>6.2</td>
<td>7.0</td>
</tr>
</tbody>
</table>

Under similar conditions, the diluted salivary α-amylase thus hydrolyses more than twice as many bonds in amylopectin β-dextrin as in glycogen β-dextrin.

The authors are grateful to Professor E. L. Hirst, C.B.E., F.R.S., for his interest, to Drs. D. J. Bell and W. J. Whelan for helpful comments on the manuscript, to the Rockefeller Foundation for a grant, and to the Department of Scientific and Industrial Research for maintenance allowances (to W. L. C., I. D. F., and A. W.).

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Enzymic Degradation of Lichenin

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Lichenin, a reserve polysaccharide from Iceland moss, was shown by methylation and periodate oxidation studies to be a linear polymer composed of 70% \( \beta-1:4 \)- and 30% \( \beta-1:3 \)-linked D-glucopyranose residues (Chanda, Hirst & Manners, 1957). Partial acid hydrolysis studies (Peat, Whelan & Roberts, 1957) then showed that the major repeating sequence consisted of \( \beta \)-cellotriose units joined through 1:3-linkages. We now report the degradation of this polysaccharide by two different laminarinase preparations.

Extracts of malted barley, like those of ungerminated barley (Manners, 1955), contain several carbohydrases, including cellobiase, laminaribiase, celloextrinase and laminarinase. In the presence of 10 mM-glucono-1:4-lactone (cf. Festenstein, 1959) the latter activity is reduced by 77% but the other activities are completely inhibited. Under these conditions, slow random hydrolysis of lichenin occurred (paper chromatography), the major product having the \( R_g \) value of a trisaccharide; small amounts of glucose, laminaribiose and a sugar with the \( R_g \) value of a tetrasaccharide were also produced.
The same oligosaccharides were formed when lichenin was incubated with an enzyme preparation from the culture medium of *Rhizopus arrhizus*, known to produce a laminarinase (Reese & Mandels, 1959).

The latter trisaccharide has been isolated by chromatography on Whatman no. 3MM paper and characterized as 3-0-β-cellobiosyl-D-glucose; paper chromatographic analysis showed that (a) partial acid hydrolysis gave glucose, laminaribiose and cellobiose, (b) after hypoiodite oxidation, partial acid hydrolysis gave glucose, gluconic acid and cellobiose and (c) hydrolysis with emulsin gave glucose and laminaribiose. The specific rotation ([α]_D^+ 12.3° in water) is in good agreement with that ([α]_D^+ 12.7°) reported by Peat et al. (1957) and by Parrish, Perlin & Reese (1960) who isolated the same trisaccharide ([α]_D^+ 12.1°) from a digest of *Rhizopus arrhizus* laminarinase and oat glucan (a polysaccharide closely related to lichenin in molecular structure).

Since lichenin does not contain a sequence of two adjacent 1:3-linkages (Chanda et al. 1957; Peat et al. 1957) enzyme action must involve the rupture of a β-1:4-glucosidic linkage. The specificity of laminarinase may therefore be defined by

(a) - G - G - G -

(b) where G represents a -D-glucopyranose residue, (a) a 1:3 linkage and (b) the linkage hydrolysed, which may be a 1:3 or a 1:4-linkage.
These conclusions are in exact agreement with those of Parrish et al. (1960).

We are indebted to Professor E. L. Hirst, C.B.E., F.R.S., for his interest and encouragement.