STRUCTURAL INVESTIGATIONS OF THE POLYSACCHARIDES
OF GREEN SEAWEEDS

by

JOHN P. MCKINNELL

Thesis presented for the degree of Doctor of Philosophy

University of Edinburgh

September 1961.
TO ANN.
ABSTRACT OF THESIS

Name of Candidate: John Philip McKinnell

Address: 14 Braid Hills Road, Edinburgh, 10.

Degree: 

Date: 1st September, 1961.

Title of Thesis: Structural Investigations of the Polysaccharides of Green Seaweeds.

Section 1

1. Alkaline extraction of the green alga Cladophora rupestris after hot water and chlorite treatments produced a heteropolysaccharide \([\alpha_D + 5^\circ]\), consisting of galactose (6.4), glucose (3.4), arabinose (4.3), xylose (4.0) and rhamnose (trace). Acetylation and chloroform extraction of the polysaccharide acetate yielded a polymer which on deacetylation produced a pure Glucan I, \([\alpha_D + 177^\circ]\).

Again, treatment of the alkaline extract from C. rupestris with iodine in potassium iodide precipitated a polysaccharide \([\alpha_P + 170^\circ]\), which hydrolysed to give only glucose. Fractionation of this material with thymol gave Glucan IIa \([\alpha_D + 176^\circ]\) and Glucan IIb \([\alpha_D + 182^\circ]\).

2. The alcohol extracted weed Enteromorpha compressa on hot water treatment yielded a polysaccharide \([\alpha_D - 49^\circ]\), composed of glucose (1.0), xylose (1.34) and rhamnose (6.7) residues, together with sulphate ester (11.45%) and uronic acid groups (17.2%). Fractionation of this polymer by iodine precipitation gave a Glucan III which was separated into Glucan IIIa, \([\alpha_P + 177^\circ]\) and Glucan IIIb, \([\alpha_P + 190^\circ]\), by the thymol method.

3. Examination of the iodine staining properties of the above Glucans indicated that Glucans I—IIIa resembled degraded amylose types and Glucan IIIB was similar to an amylpectin. The "blue-values" were in keeping with these results. Potentiometric iodine titration of Glucans I and IIIa revealed iodine binding values (mg. iodine bound/100 mg. polysaccharide) of 1.05 and 10.47% respectively.

4. Viscosity measurements on these Glucans gave low values indicative of a small molecule.

5. Salivary \(\alpha\)-amyloysis of Glucans I, IIa, IIIa and IIIB gave \(P_M\) (% conversion to maltose) values typical of starch-type polymers. \(\beta\)-Amyloysis showed Glucan IIIB to be a true amylpectin but results obtained for Glucans I—IIIa seem to indicate a polymer with branching though to a lesser extent than amylpectin.

6. Periodate oxidation experiments gave results indicative of 1,4-linked glucose polymers except in the case of Glucan IIa (0.682 moles periodate/C\(_6\) anhydro unit). No unattacked glucose was evident in the hydrolysed exo-polysaccharides.

7. Methylation of Glucans I and IIb supported the evidence obtained from amyloysis and periodate experiments and confirmed the degraded nature of the polymers. The number average molecular weight of methylated Glucan I was found to be 7,200 by the isothermal distillation method. Hydrolysis and chromatographic separation of the methylated sugars yielded 2,3,4,6-tetra-O-methylglucose, 2,3,6-tri-O-methylglucose and a mixture of dimethyl glucoses.

8. It is concluded that the starches of the green algae C. rupestris and E. compressa are similar in many respects to the starches of land plants.
Section II

1. Aqueous extraction of the green alga Ulva lactuca produced a polysaccharide containing 15.3% uronic acid material. Partial acid hydrolysis of this polymer and column chromatographic resolution (cellulose or Amberlite CG-45 resin) of the hydrolysate gave a major acid fraction consisting of an aldobiouronic acid.

2. The acid had \([\alpha]_D - 22.1^\circ\), \(\text{RQal.Acid} 1.06\), equivalent weight 347, and on esterification reduction and hydrolysis produced equimolar proportions of glucose and rhamnose. Periodate oxidation of the disaccharide glycoside resulted in the reduction of 3 moles per mole, indicative of a 1,2'- or 1,4'-linked disaccharide. Methylation, hydrolysis and separation of the methylated sugars resulted in the isolation of 2,3,4,6-tetra-O-methylglucose and 2,3-di-O-methylrhamnose.

3. It is concluded that the acid is 4-O-\(\beta\)-D-glucuronosyl-\(\alpha\)-rhamnose.

Section III

1. Hot water extraction of Enteromorpha compressa yielded the polymer described in Section 1.2 of this abstract.

2. Alkaline hydrolysis of this material reduced the sulphate content but rapidly degraded the polymer.

3. Various fractionation experiments indicated that the polymer consisted of a neutral glucan and an acidic polysaccharide. The glucan III separated by iodine precipitation as discussed in Section I of this summary. The residual polysaccharide (I) had \([\alpha]_D - 87^\circ\), ash 1.3%, sulphate 15.0%, and uronic acid 18.3%, and equivalent weight 310. It contained glucose, xylose and rhamnose in the ratios 1.0 : 2.7 : 8.1.

4. All attempts to fractionate this polysaccharide were unsuccessful.

5. The polysaccharide was desulphated using dry methanolic hydrogen chloride to give a material (M), \([\alpha]_D - 36^\circ\), sulphate 0.75%, uronic acid 23.5%, and consisting of glucose, xylose and rhamnose in the ratios 1.0 : 1.4 : 5.0.

6. Periodate oxidation of (I) and (M) indicated a high proportion of 1,3'-linked residues and a branched molecule. The presence of a larger number of free -OH groups in (M) and investigation of the molar proportions of the sugars in the respective oxo-polysaccharides indicated that rhamnose was attacked to a greater extent in polysaccharide (M). Quantitative estimation of the rhamnose in polysaccharides (L) and (M) and their oxo-polysaccharides have confirmed this.

7. These results, together with those of infrared analysis provide evidence that at least some of the sulphate residues in the polymer are sited at position C_6 of rhamnose.
INDEX

GENERAL INTRODUCTION.

General Problems in Structural Studies of Algal Polysaccharides. 3
Polysaccharides of the Phaeophyceae. 9
Polysaccharides of the Rhodophyceae. 20
Polysaccharides of the Chlorophyceae. 32

EXPERIMENTAL.

General methods. 41

SECTION 1. STARCH TYPE POLYSACCHARIDES FROM CLADOPOURA RUPESTRIS AND ENTEROMORPHA COMPRESSA.

Introduction 46

A. C. Rupestris.

Expt.1. Extraction of the weed. 52

2. Preliminary Examination of Extracts (B), (C)and (D). 53

3. Purification of Polysaccharide (C) 54

4. Physical Properties of Polysaccharide (F) 55

5. Quantitative Estimation of the Relative Proportions of the Sugars in the Hydrolysate of the Alkali-Soluble Polysaccharide Mixture (F) 55

6. Acetylation of Polysaccharide (F) 56

7. Iodine Precipitation of a Glucan from Polysaccharide (F) 58

B. E. Compressa.

8. Iodine Precipitation of a Glucan from Polysaccharide (G). 59
C. Investigation of the Structures of Glucans I, II and III.

Expt. 9. Thymol Fractionation of the Glucans. 60
10. Preliminary Examination of the Properties of Glucans I, II and III. 61
11. Properties of the Iodine Complex. 62
12. Potentiometric Iodine Titration. 64
13. Viscosity Measurements on the Glucans. 65
14. Amylolysis Experiments on the Glucans. 68
15. Oxidation of the Glucans with Sodium Metaperiodate. 72
16. Methylation of Glucans I and II(b). 75
17. Determination of the Number Average Molecular Weight of Glucan I by Isothermal Distillation. 76
18. Hydrolysis of the Methylated Glucan I and Quantitative Estimation of the Relative Proportions of the Methylated Sugars. 79
20. Separation and Characterisation of the Methylated Sugars in the Hydrolysate of the Methylated Glucan I. 82

Appendix to C. rupestris Section.

21. Attempted Fractionation of Polysaccharide (F) using Cetavlon. 83
22. Attempted Fractionation of Polysaccharide (F) using Fehling's Solution. 84
23. Preliminary Investigation of Polysaccharide (E). 84
24. Investigation of Extracts (D) and (E). 86

Discussion. 88
SECTION II. THE ACID FRACTION OF ULVA LACTUCA.

Introduction

Experimental
Expt. 25. Extraction of the Acid Polysaccharide.
Expt. 27. Equivalent Weight Determinations.
Expt. 28. Small Scale Hydrolysis of the Polysaccharide.
Expt. 29. Large Scale Separation of an Aldobiuronic Acid from the Water-Soluble Polysaccharide.
Expt. 31. Conversion of the Aldobiuronic Acid to the Ester Glycoside and Reduction to the Neutral Disaccharide.
Expt. 32. Hydrolysis of the Disaccharide Glycoside and Characterisation of the Neutral Sugars.
Expt. 34. Methylation of the Disaccharide Glycoside.
Expt. 35. Hydrolysis of the Methylated Disaccharide and Separation of the Methylated Sugars.
Expt. 36. Investigation of Higher Acid Fraction.

Acid Fraction of E. compressa.
Expt. 37. Separation of an Acid Fraction of E. compressa Water-Soluble Polysaccharide and Preliminary Investigation.

Discussion

SECTION III. THE WATER-SOLUBLE POLYSACCHARIDE OF ENTEROMORPHA COMPRESSA.

Introduction.

Experimental
Expt. 38. Preliminary Alcoholic Extraction.
Expt. 39. Examination of the Alcoholic Extract.
SECTION III (Contd.).

Expt. 40. Extraction of the Polysaccharide Materials. 134

41. Preliminary Examination of the Extracts. 136

42. Removal of Protein from the Water-Soluble Polysaccharide. 136

43. Properties of the Purified Water-Soluble Polysaccharide. 137

44. Quantitative Estimation of the Relative Proportions of the Sugars in the Hydrolysate of the Water-Soluble Polysaccharide. 137

45. Alkaline Hydrolysis of the Water-Soluble Polysaccharide. 138

46. Attempted Fractionation of the Water-Soluble Polysaccharide with a Xylanase. 139

47. Treatment of the Water-soluble Polysaccharide with Salivary $\alpha$-Amylase. 139

48. Attempted Cetavlon Fractionation of the Water-Soluble Polysaccharide. 140

49. Fractionation of a Glucan from the Water-Soluble Polysaccharide by Iodine Precipitation. 142

50. Properties of Polysaccharide (L), the Residue after Removal of Glucan III. 142

51. Quantitative Estimation of the Relative Proportions of Sugars in the Hydrolysate of Polysaccharide (L). 143

52. Separation and Characterisation of the component Sugars of Polysaccharide (L). 143

53. Attempted Separation of a Sulphate-Rich Fraction from Polysaccharide (L) by Chromatography. 144

54. Treatment of Polysaccharide (L) with Salivary $\alpha$-Amylase. 145

55. Attempted Fractionation of Polysaccharide (L) with Fehling's Solution. 145

56. Attempted Barium Hydroxide Fractionation of Polysaccharide (L). 146
SECTION III. (Contd.).

Expt. 57. Attempted Potassium Chloride Fractionation. 146

58. Hydrolysis of Polysaccharide (L) with Taka-Diastase and Hemicellulase. 147

59. Sodium Periodate Oxidation of Polysaccharide (L). 148

60. Desulphation of Polysaccharide (L) using Dry Methanolic Hydrogen Chloride. 149

61. Preliminary Examination of Polysaccharide (M). 151


63. Periodate Oxidation of Polysaccharides (L) and (M). 151

64. Quantitative Estimation of Rhamnose in Polysaccharides (L) and (M) and their Respective Oxo-polysaccharides. 154

65. Carbazole/Sulphuric Acid Estimation of Uronic Acid Content of Polysaccharides (L) and (M) and their Respective Oxo-polysaccharides. 158

Discussion 160

Bibliography 181
INTRODUCTION
The algae belong to the Thallophyta, a group of plants devoid of the physical characteristics of higher land plants. Their form has not changed since their first appearance, and it is probable that an investigation of the products of metabolism of such plants will provide a link between marine life and terrestrial vegetation. It is to be expected that the green seaweeds, members of this group of plants, will resemble the land plants most closely since chlorophyll plays a major role in metabolic processes.

A layman’s interest in seaweed usually stems from the knowledge that it was at one time a source of iodine. However, practical use was made of many algal species centuries prior to their discovery as an iodine source, especially in China, Japan and Polonesia. The cultivation of seaweeds plays a considerable part in the economy of Japan even today. A casual inspection of any seashore is enough to show that seaweeds are of varying colour and in fact they fall largely into four well-known groups depending on the major pigment in the plant.

**SEAWEEDS**

- Phaeophyceae (Brown)
- Rhodophyceae (Red)
- Cyanophyceae (Blue-Green)
- Chlorophyceae (Green)

- Fucoxanthin
- Xanthophyll
- Phycoxanthin
- Chlorophyll
From the first two groups come the major commercial products in use today and for this reason they have received the most attention during the past few decades. However, the others are no less important and it is probable that, in these times of increasing population and food shortage all four groups may assume much greater importance. Through the centuries the fortunes of the seaweed industry have varied considerably. In early times it was used mainly as a food for humans and animals and also as a manure, some of the Chlorophyceae being particularly rich in nitrogen. Some varieties are still consumed, as for example, Porphyra in South Wales. With the introduction of the Kelp industry in the 18th Century the harvesting of seaweeds increased considerably and at the turn of the last century the discovery of alginic acid as a constituent of the brown algae by Stanford (1883) led to a revival of interest in seaweeds, and indeed, modern uses of alginic acid and its derivatives are very numerous in the textile and paper industries, in emulsions, plastics, linoleum, imitation leather, paints, ice-cream and confectionery.

Like the polysaccharides of the land plants algal polysaccharides can be divided into food reserve materials and those with structural significance. In the former category are starches similar to those of higher plants for example floridean starch, cyanophycean starch and other glucans such as laminarin. Examples of structural polysaccharides are to be found in the many seaweeds which contain cellulose in the inner walls of the plant and this has been shown
to be the case in *Cladophora rupestris* and *Chaetomorpha melagonium* (Preston 1948). The galactan sulphates of the Rhodophyceae and alginic acid in the Rhaeophyceae are also considered to be structural polymers.

2. General Problems in Structural Studies of Algal Polysaccharides

The general methods in polysaccharide analysis such as hydrolysis, methylation and periodate oxidation have been applied in the structural investigation of algal polysaccharides with varying success. Many difficulties are encountered in the examination of such polysaccharides and these vary in degree depending on the algal source (i.e. brown, green, etc.).

In the first case, identification of the alga is not always a simple matter and botanists still disagree over many points in this field. It is of interest to note that botanically similar species have been found to contain rather similar polysaccharides e.g. *Ulva lactuca* and *Enteromorpha compressa*, while other cases have been reported in which the polysaccharides of two similar species bear no resemblance to each other, e.g. *Cladophora rupestris* and *Acrosiphonia centralis* (*Spongomorpha arcta*).

Again, the algae are known to undergo wide seasonal variations in the proportions of their major components (Black 1948) and for this reason care must be taken in obtaining a comparative sample.

One of the major problems encountered in the analysis of polysaccharides from the Chlorophyceae is their purification from colouring matter and protein contaminants, an obstacle not always surmountable. This is illustrated by investigations on the
mucilage of *Gladophora rupestris* which contained up to 25% protein which defied complete separation by the conventional methods (Fisher 1956). This cannot apparently be completely removed without extensive degradation of the polysaccharide.

Further major obstacles which confront the investigator in structural determinations on the polysaccharides of the Chlorophyceae is their heterogeneity and the presence of sulphate and uronic acid residues. It is no criterion that morphologically simple plants like algae have simple metabolic processes and this is shown by the variety of components found in water-soluble extracts of the green weeds that have so far been investigated. (see Table 1).

**Table 1**

Molar proportions of:

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactose</td>
<td>2.8</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
<td>2.3</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.2</td>
<td>1.0</td>
<td>1.0</td>
<td>7.5</td>
<td>8.4</td>
</tr>
<tr>
<td>Mannose</td>
<td>-</td>
<td>-</td>
<td>0.2</td>
<td>-</td>
<td>1.1</td>
</tr>
<tr>
<td>Arabinose</td>
<td>3.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>Xylose</td>
<td>1.0</td>
<td>1.3</td>
<td>1.6</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>0.4</td>
<td>4.4</td>
<td>1.4</td>
<td>9.8</td>
<td>0.2</td>
</tr>
<tr>
<td>Uronic acid %</td>
<td>3.0 (?)</td>
<td>20.3</td>
<td>19.3</td>
<td>16.4</td>
<td>22.7 (?)</td>
</tr>
<tr>
<td>Sulphate %</td>
<td>19.6</td>
<td>17.5</td>
<td>7.3</td>
<td>20.0</td>
<td>7.5</td>
</tr>
<tr>
<td>Ash %</td>
<td>13.7</td>
<td>19.0</td>
<td>10.0</td>
<td>30.8</td>
<td>13.5</td>
</tr>
<tr>
<td>$\left[\alpha\right]_D$</td>
<td>+69°</td>
<td>-47°</td>
<td>-31°</td>
<td>-25°</td>
<td>+120°</td>
</tr>
</tbody>
</table>
Whereas the brown and many of the red weeds appear to consist of homopolysaccharides which are relatively easily fractionated, this is not so in the Chlorophyceae, few instances of the separation of homopolysaccharides from the latter having been reported. (Mackie 1959 and 1960). Many fractionation procedures applicable to polysaccharides have failed.

Another feature of fundamental importance in algal polysaccharides is the occurrence of sulphate esters R.\(\text{OSO}_3\text{H}\). Sulphated polysaccharides have been isolated from all three major groups of seaweeds yet their occurrence is rare in all non-algal plant species although well known in animal polysaccharides such as the Chondroitin sulphates and the blood anti-coagulant heparin. Their presence in polysaccharides gives rise to many problems in structural investigations. Unequivocal proof of their location in the individual sugar units is difficult. They are exceptionally stable towards alkali and in fact under alkaline conditions the polysaccharide degrades more rapidly than sulphate is liberated. Furthermore, the strength of acid required for their cleavage also hydrolyses the glycosidic links. If the sulphate groups were easily removable without alteration of the polysaccharide structure then it would be relatively easy to determine the point of linkage since a comparison of the methylated sugars present in the hydrolysates of the methylated sulphated and desulphated polymers would give the required information.
Percival (1949) was able to show that sulphate esters having a free trans hydroxyl on an adjacent carbon atom hydrolyses in alkaline solution with epoxide ring formation and simultaneous Walden inversion. (fig.(i))

![Diagram](image)

3,6-Anhydrogalactose residues are of common occurrence in the galactans isolated from red seaweeds. (Mori 1953). Recently Rees (1961), working on the enzymes of the red seaweed *Porphyra*, has been able to show that galactose-6-sulphate units present in the water-soluble polysaccharide extracted from this seaweed are enzymatically converted into 3,6-anhydro-L-galactose residues without cleavage of the molecule, thus providing for the first time an explanation of the origin of these anhydro units and evidence that they are not artefacts produced during the extraction of the polysaccharide. Hence the sulphate group appears to be directly involved in the biosynthesis of one of the major polysaccharides of the Rhodophyceae.

In those sulphate-containing polysaccharides of the green and brown algae which are devoid of galactose no evidence of anhydro-sugar units has been obtained and the functions of the sulphate groups is completely unknown. It will be seen later that in these polysaccharides the major sugar residues in many
of the green algae are rhamnose and glucuronic acid, and in
the brown algae fucose mannanuronic and guluronic acids and it
is possible that L-rhamnose and L-fucose have the same metabolic
function as the anhydro-sugars in the galactans. At the same
time glucose-containing polysaccharides are to be found in the
majority of seaweeds. However, other than conferring upon the
polysaccharides those properties of a hydrophilic group, little
can be said of the sulphate residues in the green seaweed
polysaccharides since no indication of their position has been
given. It has been suggested that the hydrophilic nature of the
O.SO$_3$H. group acts as an ionic sieve excluding any material
harmful to the plant's growth. Furthermore, it seems likely
that the 3,6- anhydro-galactose confers upon the polysaccharides
the ability to gel, while it may be that sulphated polysaccharides
play an important part in forming the protective slime found on
the surfaces of seaweeds.

The presence of uronic acids in algal polysaccharides is
widespread for example in _Laminaria digitata_ , _L. cloustoni_ etc.
However, most of the green weeds so far investigated also contain
uronic acids, although there are notable exceptions (e.g. _C. rupestris_).

The major difficulty in uronic acid containing materials is
due to the high resistance of the uronosyl linkage to acid
hydrolysis, and degradation of the mono-uronide occurs rapidly
under conditions necessary for its cleavage. This has been of
use however, in analysing acid fragments of polysaccharides
containing both uronic acid and neutral sugar residues. Controlled
acid hydrolysis of such substances produces aldobiuronic, triuronic, etc. acids and elucidation of the structure of these compounds by reduction and methylation yields valuable information as to the structure of the main polysaccharide.
3. POLYSACCHARIDES OF THE PHAEOPHYCEAE

The brown seaweeds (e.g. Laminaria and Macrocystis spp.), are among the largest to be found, some growing to 150-200 feet in length and weighing almost one hundred pounds. In physical characteristics they can broadly be compared to land plants having an obvious stem or stipe which bears a leaf-like lamina at its upper end.

Polysaccharides typical of this group are (1) laminarin, (2) fucoidin, (3) alginic acid.

(1) Laminarin is the major carbohydrate constituent of the sublittoral brown algae, particularly the Laminaria spp. As a food reserve the amount of laminarin in seaweeds shows considerable seasonal variation (Black 1948) and may constitute more than 45% of the dry weight of the weed. (Colin 1930).

Laminarin exists in two forms which differ in solubility. L. digitata contains a soluble form whilst other species exhibit an insoluble laminarin which precipitates on standing in cold water. Methylation studies carried out by Barry (1939) showed that insoluble laminarin consisted of 1,3\(^1\) linked \(\beta-D\)-glucopyranose units and this was substantiated by the isolation of the disaccharide 3- (\(\beta-D\)-glucopyranosyl) -D- glucose or laminaribiose, from laminarin treated with a laminarase from Helix pomatia. (Barry 1941).
Laminaribiose (fig.ii).

The yield of tetra-O-methyl-\(\text{D}\)-glucose liberated on hydrolysis of methylated laminarin corresponded to a chain length of 20 \(\text{D}\)-glucose units (Connell 1950) although earlier oxidation studies by Barry (1942) using periodate and bromine indicated an apparent chain length of 16 \(\text{D}\)-glucose residues. Identical methylation results were obtained from "soluble" laminarin (Percival 1951) indicating the essential similarity between the two forms.

Recent investigations have indicated that the structure of laminarin is more complicated than previously supposed. Hence Peat (1955) and his colleagues isolated 6- (\(\alpha\) -\(\text{D}\)-glucopyranosyl) -\(\text{D}\)-glucose, (gentiobiose)(fig.iv), mannitol, 1-O-\(\alpha\) -\(\text{D}\)-glucosyl-mannitol (fig.iii) 1-O-laminaribiosyl-mannitol and other glucose oligosaccharides from a partial acid hydrolysate of laminarin.
The isolation of a small quantity of gentiobiose together with two isomeric trisaccharides indicated the presence of \( \beta -1,6' \) linkages in the polymer (Peat 1955).

The isolation of the above oligosaccharides was considered to be evidence that a proportion of laminarin molecules was terminated by mannitol residues, and a measure of the amount of mannitol present was obtained by Anderson et al. (1957).

Reduction of laminarin with sodium borohydride converts the glucose units at the reducing ends of the chains to sorbitol producing laminaritol. The increase in the production of formaldehyde during periodate oxidation of laminaritol in comparison with unreduced laminarin (2 moles formaldehyde/mole sorbitol) gives a measure of the proportion of glucose-ended chains and hence of mannitol present. (ca. 2%)
Smith and his co-workers (1959) demonstrated that the reducing fraction, laminarose, after oxidation to the acid, "laminarin acid", could be separated from the non-reducing mannitol-terminated entity by anion exchange resin. Mannitol was then found only in the non-reducing fraction of the molecule and they suggested that it functioned as an aglycone residue for two glucose chains:

\[
\begin{align*}
R_1 & \quad \text{o} \quad \text{CH}_2 \\
R_2 & \quad \text{o} \quad \text{CH} \\
& \quad \text{(CHOH)}_3 \\
& \quad \text{CH}_2\text{OH}
\end{align*}
\]

(fig. v)

In addition they have forwarded further evidence for the presence of $\beta-1,6'$-linkages in laminarin (Smith 1959a). By subjecting the non-reducing mannitol-ended fraction to periodate oxidation followed by reduction with borohydride and mild acid hydrolysis ethylene glycol was obtained. Laminarose, the glucose-ended fraction, likewise afforded ethylene glycol. The proposed reaction was:

\[
\begin{align*}
\text{G}_1 & \quad \text{3G}_1 \quad \text{o} \quad \text{CH}_2
\end{align*}
\]

(1) Oxidation
(2) Reduction
(3) Mild hydrolysis
The amount of ethylene glycol measured by chromotropic acid showed that the non-reducing laminaritol contained two $\beta$-1,6'-linkages whilst the reducing laminarose only contained one such link per molecule.
The recent report by Smith (1959b) that mannose is present in the laminarin molecule to the extent of 1 in 7 glucose residues does not appear to be true for all samples of laminarin since other workers have been unable to confirm this.

(2) Fucoidin

Fucoidin, a mucilaginous sulphated polysaccharide, is present in the intercellular tissues of all brown seaweeds. It is most abundant in the Fucaeeae where it possibly acts as a food reserve. Kylin (1913) demonstrated that the polysaccharide contained L-fucose (6-deoxy -L-galactose) by isolation of L-fucose phenylosazone.

Pure fucoidin preparations are difficult to achieve due to retention of solvents, whilst small amounts of galactose, xylose, and uronic acid residues also contaminate extracts, possibly from closely associated polysaccharides (Percival 1950). The high sulphate content (38.3%) of fucoidin indicated that the main residue was L-fucose monosulphate and it has been shown that 90% of these residues are alkali stable. (Conchie 1950).

Methylation followed by methanalysis of fucoidin gave rise to methyl 3-O- methyl -L-fucoside (58%), methyl L-fucoside (ca.2%) and methyl 2,3-di-O-methyl -L-fucoside (ca.20%). This indicated that C₂ and C₄ were involved in linkage with adjacent residues or with sulphate groups and it is considered that the main repeating unit is a 1,2'- linked L-fucose sulphated on C₄ although 1,4' linked units may also occur. The presence of free L-fucose was accounted for either by the existence of a branching point with linkages at C₁,
C₂ and C₃ or by a residue containing sulphate groups at C₃ and C₄. The presence of 1,2'-linked residues in fucoidin was ratified by the isolation, on mild acetolysis and reduction of 2-O-α-L-fucopyranosyl-L-fucitol (fig. vii), the structure of which was proved by periodate oxidation. (O'Neill 1954).

2-O-α-L-fucopyranosyl-L-fucitol (fig. vii)

Gótte (1959) verified the presence of 1,4'-linked entities in a similar experiment which yielded 4-O-α-L-fucopyranosyl-L-fucose and he suggested that 1,2'- and 1,4'-linked units might arise from different polysaccharides present in different proportions. Indeed it is worth noting that two peaks were obtained on electrophoresis of a purified fucoidin sample. (O'Neill 1954).

(3) Alginic acid

Alginic acid is the most commercially important algal polysaccharide and as such has received a great deal of attention both to chemical and physical properties. It plays an important part in the constitution of the cell wall of the Phaeophyceae along with cellulose and other polymers and like laminarin shows a marked
seasonal variation, reaching a minimum (12%) in the late summer. (Black 1943).

Alginic acid was first identified by Stanford (1883) although he was unable to obtain a pure sample. Initially much difficulty was encountered in identifying the uronic acid unit and it was not until 1930 that D-mannuronic acid was isolated, in lactone form (Nelson 1930). The drastic conditions required for complete hydrolysis of the polymer caused decomposition of the uronic acid and theoretical yields of D-mannurono-lactone have never been achieved, for example the highest yield, by formic acid hydrolysis produced 45% of the lactone. (Spoehr 1947).

The postulation of \(\beta-1,4\) linkages by Lunde et al (1933) based on chemical and X-ray data was supported by the methylation experiments of Hirst and co-workers (1939). Experiencing difficulty in complete methylation of alginic acid, they obtained a partially degraded acid by subjecting it to hydrolysis with methanolic hydrogen chloride, which they were then able to methylate (OMe 43%). Although hydrolysis of the methyl ether was also accompanied by degradation they were able to isolate 2,3-di-O-methyl D-mannuronic acid (50% yield based on the degraded material) which they characterised by oxidation to meso-dimethoxysuccinic acid (fig.viii) via 2,3-di-O-methyl D-mannosaccharic acid.

\[
\text{CO}_2\text{H} \quad \text{O} \quad \text{OH} \quad \text{OMe} \quad \text{OMe} \quad \text{OMe} \quad \text{OMe}
\]

\[
\text{H}_2\text{O} \quad \text{Ba}\text{O}_2 \quad \text{H}_2\text{O}
\]
meso-dimethoxysuccinic acid (fig. viii)

Viscosity measurements by Chanda, Hirst and Percival (1952) on a less degraded methylated alginic acid indicated a chain length of ca. 100 units. Formic acid hydrolysis of this material followed by reduction and hydrolysis of the methyl mannosides produced, 2,3-di-O-methyl-D-mannose as the main component thus indicating that a large proportion of the alginic acid molecule consisted of 1,4'-linked mannnuronic acid residues. The high negative rotation of alginic acid (\([\alpha]_D -139^\circ\)) and its salts suggests a \(\beta\)-link, hence :-
The presence of L-guluronic acid in the alginic acid molecule first reported by Fischer and Dorfel (1955) upset the generally accepted conception of the structure of alginic acid. They examined twenty-two species of Phaeophyceae and determined the ratio of the two acids in each, examining under varying conditions the possibility that guluronic acid was an artefact produced by C5 epimerisation of mannanuronic acid. Hirst (1958) suggested that an alternative in vivo transformation was the transfer of H and OH between position C6 and C1 in the D-glucose molecule followed by enzymic transformation of the glucose moiety to gulose.

The presence of L- (fig.x) guluronic acid was confirmed by
Drummond & Percival (1958) who have shown it to be 1,4'-linked by subjecting alginic acid to oxidation by periodate then bromine oxidation, separating both erythraric (meso-) and \( \text{L- threaric} \) (\( \text{L} (+) \) - tartaric) acids on hydrolysis of the oxopolysaccharide. It is considered that the \( \text{L} (+) \) - tartaric acid could only have arisen from 1,4'-linked guluronic acid, the optically inactive isomer deriving from the 1,4'-D - mannuronic acid units.

![Chemical structure of L-thearic acid](image1)

(L-thearic (L (+) - tartaric) acid)

![Chemical structure of Erythraric (meso-tartaric) acid](image2)

(Fig xi) Erythraric (meso-tartaric) acid
More recently partial hydrolysates of alginic acid have yielded oligosaccharides (Vincent (1960) which on reduction contained mannose and gulose in varying ratios indicating that mannuronic acid and guluronic acid are chemically combined in alginic acid. The fractionation of alginic acid by Haug (1959) does not conflict with these results. Although two distinct fractions of alginic acid were obtained both contained mannuronic and guluronic acids in varying proportions.

4. POLYSACCHARIDES OF THE RHODOPHYCEAE

A number of polysaccharides characteristic of the Rhodophyceae have been isolated and they have been shown to be useful in the food industry as stabilizing, thickening and gelling agents; in the cosmetics industry for "beauty" preparations etc., in photography, pharmacy, medicine (e.g. agar plates) and many other commercial undertakings. These polysaccharides are characterised by the presence of galactose sulphates and 3,6-anhydrogalactose, and include agar, carrageenin and the mucilage from Dilsea edulis. In addition to these galactan sulphates there are other polysaccharides devoid of sulphate, including the glucan from D. edulis mucilage (Floridean starch), the mannan from Porphyra sp. and the xylan from Rhodymenia palmata.

(a) Agar

Agar is a gel-forming water-soluble extract of several varieties of red seaweeds including Gelidium and Gracilaria species. Such agar bearing weeds are called agarophytes.
Although more extensive work has been applied to this than to practically any other red algal polysaccharide the constitution still evades complete solution. Agar has been reported to contain D-galactose (40%) (Bauer 1884), 3,6- anhydro-L-galactose (Hands 1938; Araki 1938), pyruvic acid, and small amounts of L-galactose (Firse 1936) and D-glucuronic acid (Cotterell 1942). The presence of ester sulphate was first reported by Neuberg and Ohle (1921) since when the sulphur content of several agarophytes has been reported to vary between 0.3 and 2.2% (Percival 1944). It is the opinion of some workers that the sulphated material is a contaminant.

By methanolysis of methylated agar Percival (1937) and Peat (1942) isolated 2,4,6-tri-O-methyl-α-D-galactose (fig. xii) (9 moles) together with 2,4-di-O-methyl-3,6-anhydro-L-galactose (fig. xiii) (1 mole) and hence suggested that the main part of the molecule consisted of 1,3′-linked D-galactose residues.

\[ \text{2,4,6-tri-O-methyl-}^\alpha\text{-D-galactose.} \quad \text{(fig. xii)} \]

\[ \text{2,4-di-O-methyl-3,6-anhydro-}^\text{L-}\text{galactose.} \quad \text{(fig. xiii)} \]
The isolation of 2,4-di-O-methyl-3,6-anhydro-L-galactose by these workers, and later by Araki and Arai (1940) showed that the free anhydro-sugar was an integral part of the agar molecule and much speculation occurred as to its origin. Peat (1942) and his colleagues were of the opinion that it was an artefact produced during alkaline methylation of agar by desulphation and concurrent ring closure of the L-galactose-6-sulphate moiety supposedly present in agar. Percival (1939) and Forbes preferred to consider it an integral part of the agar molecule pointing out that the sulphate content was too low to account for the proportion of 3,6-anhydro-L-galactose present. Their belief was confirmed by the isolation of 3,6-anhydro-L-galactose dimethyl acetal on complete methanolysis of agar (Araki 1944) and further verified by mercaptolysis and separation of crystalline 3,6-anhydro-L-galactose diethyl dithiocacetal from commercial agar. (Araki 1953).

Chloroform extraction of both acetylated and methylated agar indicated that the polysaccharide was a mixture of at least two fractions (Araki 1937). The chloroform soluble fraction (70%) was named Agarose while the insoluble (30%) was designated Agaropectin. Agarose, the more thoroughly investigated polymer, consists of 3,6-anhydro-L-galactose and D-galactose. It contains no sulphate or pyruvic acid and is thought to constitute the principal polysaccharide of agar. Methylation of agarose has indicated that the D-galactose residues are connected through positions C1 and C3 with the neighbouring units in the agarose molecule whilst the anhydro-sugar linkages involve C1 and C4.
A major advance in the structure of agar involved the isolation of a disaccharide agarobiose (Araki 1944a) by partial acid hydrolysis of agar from Gelidium amansii. Since then agarobiose has been isolated as its diethyl dithioacetal (b) and dimethyl acetal (c) (Hirase 1954) by partial mercaptolysis and partial methanolysis respectively. Its structure has been published as 4-O-\(\beta\)-D-galactopyranosyl-3,6-anhydro-L-galactose (a).

![Chemical structure of agarobiose (fig. xiv)](image)

Agar digesting bacteria from Pseudomonas kyotoensis produced a different oligosaccharide neoagarobiose in which the 3,6-anhydro-L-galactose was the non-reducing function (Araki 1956). Neoagarobiose was shown to have the structure 3-O-(3,6-anhydro-L-galactopyranosyl)-D-galactose. (fig. xv) :-

![Chemical structure of neoagarobiose (fig. xv)](image)
The same workers were also able to isolate the neoagarotetraose. Thus it is probable that agarose is a linear polysaccharide composed of alternating residues of 1,3'-linked \( \beta-D \)-galactose and 1,4'-linked 3,6-anhydro-\( \alpha \)-galactose.

The structure of agaropeptin is much less clear and although it is known to contain the principal components of agarose these are associated with sulphate ester, pyruvic acid and glucuronic acid. Hirase (1957) was able to isolate a crystalline compound on partial methanolysis of commercial agar consisting of pyruvic acid and agarobiose dimethyl acetal thus providing some indication of the incorporation of pyruvic acid into the agar molecule, but the position of the glucuronic acid and sulphate entities remain as yet unsolved.

(b) Carrageenin

carrageenin, the water-soluble polysaccharide found in Chondrus crispus, Gigartina stellata and other related seaweeds, is similar to agar in many respects. There are, however, some important differences. The major components of carrageenin are
D-galactose (30-40%) ester sulphate (30%) (Mori 1953) and 3,6-anhydro-D-galactose (ca 12%) (Percival 1954, O'Neill 1955) whilst the presence of L-galactose (Johnston 1950) and D-glucose (Buchanan 1943) have also been reported although the latter is probably a contaminant. The presence of the anhydro-sugar expected from positive ketose reactions was confirmed by the isolation of the diethyl mercaptal of 3,6-anhydro-D-galactose (cf. agar L-form). (Percival 1954, O'Neill 1955).

The principal linkage in the carrageenin molecule was demonstrated to be 1,3', a result achieved by several workers who found the main product of methylation to be 2,4,6-tri-O-methyl-D-galactose. (Mori 1941, Johnston 1950 and Dillon 1951). The isolation of a fraction consisting of 2,4-di-O-methyl-D-galactose suggested that the carrageenin molecule must be branched. Dewar and Percival (1950; Buchanan 1943) indicated that the sulphate ester group which was approximately one per hexose residue, occupied position C_4.

Carrageenin has been fractionated into κ-carrageenin (40%) and λ-carrageenin (60%) by potassium chloride precipitation (Smith 1953). The fractionation appears to be analogous to that of agar. O'Neill (1955) has shown κ-carrageenin to be composed of D-galactose, 3,6-anhydro-D-galactose and sulphate residues in the approximate ratio 6:5:7.

Again partial mercaptolysis of κ-carrageenin led to the isolation of the diethyl dithioacetal of carrabiose, the structure of which was shown to be 4-0-β-D-galactopyranosyl-3,6-anhydro-
In the light of these findings O'Neill (1955) stated that \( \kappa \)-carrageenin consisted of alternating residues of \( \beta \)-\( \text{D} \)-galactopyranosyl-4-sulphate and 3,6-anhydro-\( \alpha \)-\( \text{D} \)-galactopyranose, connected through \( \text{C}_1 \) and \( \text{C}_3 \), and \( \text{C}_1 \) and \( \text{C}_4 \) respectively in a similar manner to the agarose molecule.

Every tenth \( \text{D} \)-galactose residue forms a branch point to \( \text{C}_6 \) of which is attached a bi-sulphated \( \text{D} \)-galactose unit as a non-
reducing end group.

That \( \lambda \)-carrageenin consisted almost entirely of sulphated \( D \)-galactose units was confirmed by acetolysis of \( \lambda \)-carrageenin which led to the isolation of \( D \)-galactose, 3-O-\( \lambda \)-D-galactopyranosyl-\( D \)-galactose and a crystalline trisaccharide tentatively identified as 3-0-\( \lambda \)-D-galactopyranosyl-(1-3)-0-\( \lambda \)-D-galactopyranosyl-\( D \)-galactose. (Morgan 1959).

Recently Rees (1961) working on the \( \lambda \)-component of carrageenin presented evidence for the presence of galactose-6-sulphate residues in the polymer and furthermore was able to convert these residues to 3,6-anhydro-galactose without marked degradation of the polysaccharide. It is therefore possible that \( \lambda \)-carrageenin contains a large quantity of that polysaccharide which is the biological precursor of \( K \)-carrageenin, differing from the latter in containing galactose-6-sulphate units in place of 3,6-anhydro-galactose.

While carrageenin differs from agar in consisting of \( D \)-linked galactose residues and the \( D \)-form of anhydro-galactose it is interesting that no reports of the isolation of a "neo-carrabiose" by enzymic hydrolysis analogous to neoagarobiose, have been intimated. It is probable that the enzyme would be inhibited by the 4-O-sulphate group, or by the presence of the different anomeric linkage.

(c) Galactan Sulphate of Dilsea edulis.

Barry and Dillon (1945) suggested that the main structural
feature of the dilute-acid soluble polysaccharide from \textit{D. edulis} consisted of a repeating unit of five \(D\)-galactopyranose residues: the first four linked 1,3'- while the fifth was linked 1,6' - and carried sulphate on C\(_6\). These suggestions were advanced on the basis of periodate oxidation studies and on the alkaline stability of the sulphate.

Dillon and McKenna (1950) subsequently showed that uronic acid was present to the extent of 10\% probably in the form of the lactone, and was associated with nine \(D\)-galactose residues and two sulphuric acid units. They showed that by complete methylation of a sulphate free polysaccharide acetate followed by hydrolysis, a high yield of 2,4,6-tri-O-methyl-\(D\)-galactose was obtained indicating a predominantly 1,3' - linked chain. The methylation experiments did not confirm the hypothesis of 1,6' - links advanced to explain the periodate oxidation results.

Later Barry (1957) intimated the presence of \(D\)-xylose (7\%) and traces of 3,6-anhydro-\(D\)-galactose. By successive applications of the method of periodate oxidation and phenylhydrazine degradation (Barry degradation) he demonstrated that the polymer was indeed composed of a backbone of 1,3'-linked \(D\)-galactose flanked by 6-sulphated 1,3'-linked galactose units which were removed by successive oxidation and degradation. Enhanced quantities of 3,6-anhydro-\(D\)-galactosazone in the neutralised hydrolysate indicated from experiments with barium galactose-6-sulphate that some of the sulphate residues in the polymer were located at C\(_6\).
The isolation of erythrosazone by Barry degradation showed for the first time the presence of 1,4'-linked galactose.

![Chemical structure](image)

(fig. xix)

Also, since the once-oxidised mucilage still contained xylose and glucuronic acid, these residues must be 1,3'-linked, their elimination on a second degradation indicating that they were apparently part of a side-chain although no indication of the position was given.

(d) Floridean Starch

The food reserve polysaccharide of *Uilsea* and *Furcellaria* spp. first isolated by Colin (1933) has been designated by many workers as a true starch. The evidence now available indicates that it also resembles glucans isolated from other algae (Meeuse 1954) e.g. *Odonthalia* and *Ulva expansa*. Early studies on *D. edulis* floridean starch by oxidation with potassium metaperiodate (Barry 1949) indicated a consumption of 0.6 moles periodate per glucose residue, suggesting the presence of 40%
1,3'-linked glucose residues. This result was, however, invalidated by the observation that the periodate-oxidised polysaccharide condensed with thiosemicarbazide and isoniazid to an extent indicative of an uptake of ca. one mole periodate per hexose unit (Barry 1954). Further, the acid hydrolysed oxopolysaccharide gave no chromatographic evidence for unoxidised glucose. Recent studies have revealed that the consumption is ca. one mole per glucose residue (Fleming 1956), the possible reason for the low results attained earlier being under-oxidation. Hence the polysaccharide appears to be devoid of units linked through C1 and C2.

Enzymic investigations with α- and β-amylase to both of which floridean starch was initially reported to be resistant (Barry 1949) have shown the polymer to have a $P_M$ value (apparent percentage conversion to maltose) of 65 with α-amylase (Fleming 1956); the effect of sweet potato β-amylase produced a 45% conversion to maltose, this being increased to 54% by initial incubation with yeast iso-amylase (an α 1,6'-glucosidase).

These properties, and the significantly short average chain length (C.L.9-13 units, cf. amylopectin ca. 21) suggest that floridean starch is similar to the animal reserve polysaccharide glycogen. In contrast recent physicochemical studies on floridean starch (Greenwood 1961) have shown it to have a structure of the amylopectin type.

The isolation of a small amount of nigerose (3-O-α-D-glucopyranosyl-D-glucose) on partial acid hydrolysis of floridean starch has recently been reported by Peat (1959), but later studies
in this laboratory indicate that this may be an artefact.

(e) Mannan from Porphyra umbilicalis

Mannose is a comparatively rare component of the algae found only until recently in the red seaweed *Porphyra umbilicalis* (Jones 1950). It has now been reported as a major constituent of the green seaweed *Codium fragile* (Iriki 1960, Love 1961). The Porphyra mannan was obtained by extraction of the weed with 20% sodium hydroxide solution after exhaustive treatment with dilute acid to remove galactose and fructose containing polysaccharides. Purification was achieved by the formation of an insoluble copper complex of the mannan. Periodate oxidation of the polymer led to a consumption of one mole periodate per anhydro-mannose unit, and this, combined with the results of methylation, indicated that the polysaccharide was composed of 1,4'-β-linked mannose residues. End group assay showed the polysaccharide to be branched with an average repeating unit of about 12-13 D-mannose residues.

(f) Xylan from Rhodymenia palmata

Dilute hydrochloric acid extraction of the red weed *Rhodymenia palmata* yields a polysaccharide composed entirely of D-xylose (Percival 1950). Periodate oxidation degraded 80% of the polymer suggesting the presence of 20% 1,3'-linked xylose and this was confirmed by methylation studies which showed that the ratio of 1,4'-linked to 1,3'-linked xylose units was 4:1, and that the average chain length was ca. 17.
The failure of all attempts to fractionate the polysaccharide suggested that both types of linkage (i.e. 1,4'- and 1,3') occurred in the same molecule. Support for this idea has been obtained from studies involving the Barry degradation (Barry 1954), and from partial enzymic and acid hydrolysis where oligosaccharides containing both 1,3' and 1,4'-linked xylose residues were isolated (Howard 1957). No indication of the relative arrangement of the 1,3'- and 1,4'-linked residues has been established.

5. POLYSACCHARIDES OF THE CHLOROPHYCEAE

The author feels that while certain aspects of the polysaccharides of the Chlorophyceae must be discussed in a general section, those introductions related to the different experimental sections of the thesis would be more conveniently described at the beginning of the appropriate section.

The structural studies of the green algal polysaccharides is much less advanced than those of the two preceding groups. Inspection of Table 1 (p. 4) indicates that these polysaccharides are complex in nature, rarely containing fewer than four different monosaccharides. In this respect they appear to resemble the higher land plants whose metabolism seems to require a number of different sugar-units in order to function effectively. In most cases the Rhodophyceae and Phaeophyceae utilise two, or sometimes three monosaccharides at the most.

The implications arising from the presence of sulphate or
Uronic acid residues have already been discussed. Many green seaweeds however, metabolise polysaccharides containing both these residues (e.g. *E. compressa, U. lactuca*). The contiguous presence of these units has conferred upon these polysaccharides two similarly charged negative groups and their reactions with regard to electrophoresis or complex-forming properties (e.g. "cetavlon", Fehling's solution, etc.) are alike. As already intimated, the sulphate groups, which are readily hydrolysed by acid, show marked stability to alkali, such hydrolysis causing degradation of the polysaccharide; methylation is hampered by their presence. Ideally, desulphation of the polysaccharide would solve many of the associated problems, but hitherto this has only been achieved with animal polysaccharides (Kantor 1957; Danishefsky 1960) such as chondroitin sulphate and heparin. Likewise, the interference of uronic acids could be removed by reduction to the corresponding free sugar. This however, requires the esterification of the carboxyl group which is impeded by the presence of sulphate. It is obvious therefore, that before the structure of such polysaccharides can be elucidated a means must be found by which one at least of the functional groups is deactivated.

Present investigations seem to indicate that the polysaccharides of the Chlorophyceae can be divided into three groups. One group contains those seaweeds which metabolise mainly arabinose, galactose and sulphate residues with little or no uronic acid content, such as species of *Cladophora* and
Chaetomorpha. Those having both uronic acid and sulphate and usually L-rhamnose, but no arabinose-galactose polymer constitute a second group and include Ulva and Acrosiphonia species. Finally there are those weeds which consist mainly of some monosaccharide other than arabinose or galactose e.g., xylose in Caulerpa sp. and mannose in Codium species and these polysaccharides appear to replace cellulose as the skeletal polysaccharide. In all cases there are indications of the presence of a small proportion of a polymer resembling starch, and indeed the isolation of one such polysaccharide has recently been reported (Mackie 1960) and is dealt with more fully in Section I of this thesis.

(a) Mucilage from Ulva lactuca (Brading 1954).

A preliminary study of the constitution of the water-soluble material from Ulva lactuca gave a first indication of the complexity of the problem faced by investigators of green algal polysaccharides.

Hydrolysis of the sodium carbonate extracted polysaccharide from this species produced as component monosaccharides D-xylose (9.4%) L-rhamnose (31%) and D-glucose (7.7%) accompanied by uronic acid (19.2%) and sulphate ester groups (15.9%). Protein contamination (ca. 25%) could be partially removed by the Sevag process (Sevag 1938). The acid was tentatively identified
as β-glucuronic acid by the isolation of methyl 2,3,4-trimethylglucuronosidamide.

The polysaccharide was difficult to methylate fully but chloroform extraction produced a fraction (17%) with a methoxyl content of 31% which upon hydrolysis afforded 2,3,4-tri-O-methyl-L-rhamnose, 2,3-di-O-methyl-D-glucose and 2,3,6-tri-O-methyl-D-glucose.

While chromatographic evidence only was obtained for the chloroform insoluble fraction, it appeared to contain 2,3,4-tri-O-methyl-D-xylose, 2,3-di-O-methyl-D-xylose, 2,3,4-tri-O-methyl-L-rhamnose and 2,3-di-O-methyl-L-rhamnose.

No further attempt was made to fractionate the polysaccharide. No evidence was presented for the mode of linkage of the uronic acid units and investigations on the acid fraction of U.lactuca constitute part II of this thesis.

(b) Mucilage from Cladophora rupestris.

(Fisher 1956, O'Donnell 1959)

The polysaccharide material extracted from C.rupestris by boiling water or dilute acid contained L-arabinose, D-galactose, D-xylose, L-rhamnose and D-glucose in the molar ratios of 3.7: 2.8: 1.0: 0.4: 0.2. The sulphate content was ca. 20% and protein contaminant was reduced from 25% to 7% by trichloroacetic acid treatment.

Chloroform extraction of an acetylated product separated a glucose-rich polymer (4%) resembling laminarin the properties of which are discussed in section I of this thesis. All attempts at further fractionation proved unsuccessful.
Methylation of the glucose-free polysaccharide yielded a complex mixture of products from which it was possible to deduce the presence of some $1,3'$-linked arabinose, galactose and rhamnose along with some $1,4'$-linked xylose. Further, it was evident that a proportion of the galactose and xylose occurred as end-group sugars.

These conclusions were supported by evidence obtained from periodate oxidation studies, all the xylose and 66% of the galactose residues being destroyed by this reagent. Xylose and galactose must therefore possess two adjacent hydroxyl groups; the xylose as $1,4'$-units and the galactose as $1,6'$-linked units both satisfy this condition. The residual polysaccharide (oxo-polysaccharide), sulphate ca. 20%, consisted of galactose and arabinose units in the ratio 1:3, suggesting that arabinose in addition to galactose may be sulphated.

Partial hydrolysis of the polysaccharide yielded di- and trisaccharides containing arabinose, xylose and galactose, indication of their existence as adjacent units within the polymer. In addition the separation of oligosaccharides containing only arabinose presented evidence for the presence of several contiguous arabinose residues in the polysaccharide. From this and other evidence it is not surprising that the glucose-free polymer resisted all attempted fractionations.

Application of the Barry degradation technique to the water-soluble extract from C. rupestris (O'Donnell 1959) produced a residual polysaccharide containing arabinose, galactose, and rhamnose residues thus confirming the fact that these units are
contained in the main chain of the polymer. A high proportion of sulphate groups were present in the residual material.

(c) *Acrosiphonia centralis* mucilage. (O'Donnell 1959a)

*Acrosiphonia centralis* (*Spongomorpha arota*), a closely related botanical species to *C. rupestris*, after removal of colouring matter and free sugars with aqueous ethanol, yielded a sulphated water-soluble polysaccharide which contained uronic acid (ca. 20%) and on hydrolysis gave the following molar proportions of free sugars:-

\[
galactose : xylose : rhamnose : glucose : mannose = 0.1 : 1.6 : 1.4 : 1.0 : 0.2
\]

The sulphate content was 7.8%.

The periodate consumption by the polysaccharide corresponds to ca. 1 mole and the formic acid release to ca. 0.6 mole for every anhydro-sugar residue. These results indicate that many of the residues have adjacent free hydroxyl groups.

The presence of both xylose and rhamnose residues in the hydrolysate of the oxopolysaccharide suggests that 1,3'-links or branch points, or both, also exist in the molecule.

As in *C. rupestris*, the isolation of a glucose-rich fraction (9%) was achieved by chloroform extraction of the acetylated polysaccharide. In contrast, however, this material resembled starch rather than laminarin and methylation, followed by isolation of the methylated sugars indicated that it consisted of chains of \(\alpha-1,4'\)-linked residues. 2,3,6-tri-O-methyl mannose
(ca. 10%) was also separated from the hydrolysate of methylated glucose-rich polysaccharide proving the presence of 1,4'-linked mannose residues and suggesting the presence of a small quantity of a mannan or gluco-mannan.

Methylation of the glucose-free material resulted, after hydrolysis, in the characterisation of 2,3,4-tri-O-methyl-D-xylose (1 part), 2,3-di-O-methyl-D-xylose (3 parts), 2,3-di-O-methyl-L-rhamnose (3 parts) and 2-O-methyl-L-rhamnose (5 parts).

The isolation of an acid-containing fraction was achieved by partial hydrolysis and this material is more fully discussed in Section II of this thesis.

Finally, the isolation of a large amount of mono-methyl-rhamnose is evidence either that rhamnose occurs at a branch point in the molecule or that it carries sulphate groups.

(d) Mucilage from Caulerpa filiformis (Mackie 1959; 1961)

The addition of cetethyltrimethylammonium hydroxide to the water-soluble sulphated polysaccharide mixture from the South African alga Caulerpa filiformis precipitated sulphated polymeric material and left in the supernatant liquid a pure unsulphated glucom $[\alpha_2^1]_D +184^0$ which is further described in Section I of this thesis.

The precipitated sulphated polysaccharide was shown to be similar to water-soluble polysaccharides of other green algae, containing a complex mixture of monosaccharides:

- Galactose : Mannose : Xylose
  - 3 : 2 : 1
The extract had $\left[\alpha\right]_D^{10.7°} = 10.4°$; ash 14.0%; total sulphate 15.4%.

Fractional precipitation of the polysaccharide with ethanol, acetone, Fehling's solution (Chanda 1951) cupric acetate (Erskine 1956), cetyl-trimethylammonium hydroxide at various pH (Scott 1955; Barker 1957) and cetyl-pyridinium chloride (Scott 1955; Barker 1957) all yielded precipitates which contained the same proportions of sugars as the original materials. Partial fractionation of the free acid polysaccharide was achieved with saturated barium hydroxide (Meier 1956) resulting in a partial fractionation into a sulphated galactose-rich polysaccharide and a mannose-xylose containing material.

After extraction of the water-soluble material mild treatment of the insoluble residual weed with chlorite (Wise 1945) was followed by extraction with dilute sodium hydroxide at room temperature. Acidification of the alkaline extract afforded a polymer $\left[\alpha\right]_D^{31°} = 31°$, consisting of xylose (90%) and glucose (10%). Aqueous leaching of this material removed all but 4% of the glucose. Repeated methylation of the leached xylan followed by hydrolysis produced 2,3,4-tri-O-methylxylose (2.9%), 2,4-di-O-methylxylose (95.2%) and a mixture of 2- and 4-mono-O-methylxylose (1.9%), clearly indicating that the polysaccharide was composed of linear chains of 1,3'-linked $\beta$-D-xylopyranose residues the $\beta$-configuration being inferred from the negative rotation. A small proportion of 2,3,6tri-O-methyl-D-glucose was also obtained, indication of slight contamination by a starch-type polymer. The contaminating starch could be removed by prolonged aqueous
leaching but this also removed about 20% of the xylan. The results from periodate oxidation confirmed the methylation experiments.

Xylose is a common constituent of the aqueous extracts of the green algae but experimental evidence in these instances indicates the presence mainly of 1,4'-linked xylose residues similar to those found in the hemicelluloses of land plants.

**SCOPE OF PRESENT WORK**

The present thesis describes the isolation, fractionation and structural investigation of polysaccharides from *C. rupestris*, *U. lactuca* and *Enteromorpha compressa* with a view to examining the following points:

I. The nature of the glucon in *C. rupestris.*
II. The structure of the acid fraction of *U. lactuca.*
III. The structure of the water-soluble polysaccharide from *Enteromorpha compressa.*
EXPERIMENTAL SECTION
GENERAL METHODS

The following general methods have been used throughout this work. Specific methods are described in the appropriate section.

1. Drying of Extracts.

Polysaccharides were either freeze-dried, or precipitated with alcohol and washed with alcohol and ether and finally evacuated in a desiccator. In every case, prior to estimation, samples were dried in a pistol drier at 60° over phosphorus pentoxide.

2. Optical Rotations.

Optical rotations were measured in a 1 dm. polarimeter tube at room temperature. Water was used as the solvent unless otherwise stated.

3. Ash Content.

Ash determinations were carried out by igniting polysaccharide (ca. 50 mg.) in a platinum crucible until constant weight was attained.


Nitrogen content was determined by a semi-micro modification of the Kjeldahl method (Belcher 1945) using a copper sulphate; sodium selenate; potassium sulphate 20 : 1 : 80 (w/w) catalyst.
5. **Sulphate.**

Sulphate estimations were executed (a) by digesting samples (30 - 50mg.) of polysaccharide in 8N hydrochloric acid (ca. 15ml.) containing 1-2 drops of concentrated nitric acid for 6 hours at 100°. The solution, after filtration, was diluted to ca. 100ml., 3% barium chloride was added and the precipitated barium sulphate estimated gravimetrically; or (b) by digesting samples (3 - 5mg.) of polysaccharide in analar concentrated nitric acid (ca. 1 ml.) in sealed tubes overnight at 100° followed by evaporation to dryness and two evaporations with analar concentrated hydrochloric acid. The residue was dissolved in water (0.5ml.) and 0.19% chloroamino-diphenyl (0.5ml.) added and the resultant precipitate centrifuged. A sample of the supernatant (0.2ml.) was diluted to 25ml. with analar 0.1N hydrochloric acid and the solution measured in a Beckmann S.P.500 Spectrophotometer at 254 m\(\mu\). A blank was run simultaneously.

6. **Methoxyl.**

Methoxyl determinations on methylated polymers were carried out by a micro-modification of the Zeisel method.

7. **Acetyl.**

Acetyl determinations were made according to the method described by Belcher (1945).

8. **Acid Hydrolysis.**

Hydrolysis of a polysaccharide sample (50 - 100mg.) was achieved by heating under reflux with N sulphuric acid (5ml.) at
100° for 8 hours. The resulting solution was cooled and neutralised with solid barium carbonate or 5% di-n-octyl methylamine in chloroform. The neutral solution was concentrated to a syrup.

In the case of amylose or amyllopectin control experiments showed that the necessary conditions for these polysaccharides were heating at 100° with 2N sulphuric acid for 1.5-2 hours. No destruction of glucose was observed even after heating with 2N sulphuric acid for 3 hours. Complete acid hydrolysis of these polysaccharides was therefore effected by heating samples at 100° for 2 hours with 2N sulphuric acid. Acid hydrolysates were neutralised with sodium hydroxide to phenolphthalein and then made just acid by the addition of 1 drop 0.2N sulphuric acid. The reducing sugar was then estimated by the Somogyi (1952) method.


Glucose and maltose were estimated with the Somogyi (1952) reagent calibrated against known quantities of the respective sugars. Concentrations of standard sugar solutions were determined polarimetrically. The ratio, mg. sugar: titre difference from blank (ml.0.01N sodium thiosulphate) was found to be 0.310 for glucose and 0.550 for maltose, these figures remaining constant to within ± 0.01 for different preparations of the reagent. The copper reagent was kept between 38° and 40° to prevent crystallization.
10. **Dialysis.**

Dialysis of a polysaccharide solution was effected in cellophane tubes suspended in running water for 3 days. Toluene was added to avoid bacterial action.

11. **Paper Chromatography.**

Whatman No.1 paper was used unless otherwise stated. In preparative work Whatman No.17 paper was employed, having first been washed with the separating solvent.

The following solvent systems were used:

E. Ethyl acetate : acetic acid : water (9 : 2 : 2).
F. Ethyl methyl ketone 50% saturated with water + 1 drop of concentrated ammonia.

 Sugars were located by means of the following reagents:

1. A saturated aqueous solution of aniline oxalate.
2. 1% (saturated) solution of silver nitrate in acetone followed by 0.5N sodium hydroxide in 70% ethanol followed by 2% sodium thiosulphate. The paper was dipped in each solution and dried between each operation.
3. 0.1% bromoresol green in ethanol, with alkali until just blue, the solution being filtered if necessary.

Unless otherwise stated solution 1 was used.
12. Electrophoresis.

Electrophoresis was carried out on Whatman No.1 paper at 750 volts for 5 hours, in the buffer solution indicated in the text. The papers were dried at 100° followed by development with No.1 solution above containing 5% acetic acid.

N.B. $R_g = \frac{\text{Distance travelled by sugar}}{2,3,4,6\text{-tetra-O-Me-glucose}}$

$R_{gl} = \frac{\text{Distance travelled by sugar}}{\text{glucose}}$

$R_{gal\text{-Acid}} = \frac{\text{Distance travelled by sugar}}{\text{galacturonic acid}}$
SECTION I

STARCH-TYPE POLYSACCHARIDES FROM CLADOPHORA RUPESTRIS AND ENTEROMORPHA COMPRESSA.

1 (a) INTRODUCTION

Starch is the food reserve polysaccharide of most green plants. It occurs as discrete granules showing distinct physical properties depending on the source. Starches generally consist of two structurally different polymers, Amylose and Amylopectin, the latter component comprising the major fraction in most plants and some like the waxy cereal starches are almost devoid of amylose.

Amylose is a linear $\alpha-1,4'$-glucan of D.P. (degree of polymerization) ca. 50 - 6000 depending on the source and the method of extraction. Periodate oxidation studies (Potter 1951) in conjunction with molecular weight determinations on various amyloses suggest the possibility of a low degree of branching in the amylose molecule while enzymic degradation studies by Peat (1952) reveal similar "anomalies". The nature of the anomalous linkage is still unknown, while the question of its origin (e.g. natural to amylose or introduced by oxidation during extraction and purification) has been the subject of much research (Cowie 1957; Gilbert 1958).

The amylopectin component of starch is composed of $\alpha-1,4'$-linked D-glucopyranose chains which may number from several hundred to several thousand. The average chain length varies from 20-25 glucopyranose units. Methylation (Hirst 1949) and periodate oxidation studies (Hirst 1948) show that the majority of the interchain linkages are of the $\alpha-1,6'$-type. An $\alpha$-configuration
Reducing end-group.
Non-reducing end-group.
1,4-linked glucose.
1,4,6-linked glucose.

**KEY**
- R Reducing end-group.
- • Non-reducing end-group.
- ○ 1,4-linked glucose.
- ◦ 1,4,6-linked glucose.
for the interchain linkage is indicated by the isolation of isomaltose \((\alpha-D\text{-glucopyranosyl-}(1 \rightarrow 6)-\alpha-D\text{-glucopyranose})\) and panose \((\alpha-D\text{-glucopyranosyl-}(1 \rightarrow 6)-\alpha-D\text{-glucopyranosyl-}(1 \rightarrow 4)-\alpha-D\text{-glucopyranose})\) from partial acid hydrolysates of amylopectin (Wolfrum 1951; Thompson 1951; Thompson 1953).

The isolation of nigerose \((\beta-D\text{-glucopyranosyl-}(1 \rightarrow 3)-\beta-D\text{-glucopyranose})\) in small yield from partial acid hydrolysis (Thompson & Wolfrum 1955) however, suggests the presence of other linkages in amylopectin although it has been pointed out that this may arise from acid catalysed transglucosidation. (Geerdes 1957).

Several structures have been proposed for the amylopectin macromolecule but it appears that the highly ramified structure of Meyer (1940) best fulfils the conditions imposed by the physical, chemical and enzymic evidence. (fig. 20).

The importance of enzymic synthesis and degradation in the elucidation of the structures of amylose and amylopectin cannot be exaggerated. It is, however, considered to be without the scope of this thesis to give in detail the known properties of each active enzyme. \(\alpha\) - and \(\beta\)-amylase bear description since they are included in the experimental work of this section.

(a) \(\alpha\)-Amylase.

The occurrence of \(\alpha\)-amylases is widespread in nature. Pure enzyme samples have been isolated from cereals, moulds, bacteria and mammalian pancreatic and salivary secretions. The exact specificity of \(\alpha\)-amylase for \(1,4'\)-linkages near a \(1,6'\)-interchain linkage and such properties as ptl optinrum and
specific inhibition varies according to the source. Largely, however, \( \alpha \)-amylase catalyses the random hydrolysis of \( \alpha \,-1,4' \)-linkages producing \( \alpha \)-maltose as the major product. The \( \alpha \)-amylase can circumvent \( \alpha \,-1,6' \)-linkages and thus degrade the interior parts of the amylopectin molecule giving maltose and a series of \( \alpha \)-limit dextrins. In the case of salivary \( \alpha \)-amylase the smallest of these is a tetrasaccharide \( 0-\alpha \,-D\)-glucopyranosyl-(1\( \to \)6)-0-\( \alpha \,-D\)-glucopyranosyl-(1\( \to \)4)-0-\( \alpha \,-D\)-glucopyranosyl-(1\( \to \)4)-0-D-glucopyranose (6\( ^3 \)-\( \alpha \)-glucosylmaltotriose) (Nordin 1958) although it appears likely that the product is dependent upon the concentration of enzyme since Walker and Whelan (1960) reported that by varying this concentration salivary \( \alpha \)-amylolysis proceeds in two stages. The first stage products from amylopectin are maltose, maltotriose and branched \( \alpha \)-limit dextrins, of which the smallest is the tetrasaccharide mentioned above.

(b) \( \beta \)-Amylase.

This enzyme which occurs in the seeds and other parts of higher plants, catalyses the stepwise hydrolysis of alternate linkages in chains of \( \alpha \,-1,4' \)-linked glucose residues starting at the non-reducing ends, producing \( \beta \)-maltose, it is classified as an exo-enzyme (cf. \( \alpha \)-amylase an endo-enzyme). The enzyme action is arrested by the presence of linkages other than \( \alpha \,-1,4' \). Thus linear amyloses are completely degraded by \( \beta \)-amyrase whereas branched \( \alpha \,-1,4' \)-linked glucosans such as amylopectin yield maltose and higher molecular weight limit dextrins with exterior
chain stubs which are 2-3 glucose units in length (Peat 1952; Manners 1953).

\[ \alpha \] -amylase has been used to detect anomalous linkages in amylloses (Peat 1952) and to determine exterior chain lengths of branched \( \alpha-1,4' \)-linked polymers.

**Starch-Type Polysaccharides Occuring in Algae.**

Many algae and protozoa metabolise starch-type polysaccharides as their food reserve material (Fogg 1953) but the structure of relatively few has been investigated in detail. Thus while it is generally accepted that the reserve carbohydrate of *Chlorella* is a starch precise structural details have not yet been reported.

X-ray diffraction studies by Meeuse and Kreger (1954) on an iodophilic polysaccharide from *Ulva expanse*, a member of the Chlorophyceae, suggests a starch-like polymer yet shows no appreciable birefringence and gives an abnormal X-ray powder diagram. Later X-ray studies by these authors (Meeuse 1959) on green and red algae have indicated that while many of these contain an apparently true starch, they differ in some respects from the starches found in higher plants. The samples investigated included *Gladophora* and *Enteromorpha* spp., both of which form part of the present work, and these were shown to have grain sizes of 5-12\( \mu \) and 2-3\( \mu \) respectively. However, while *Gladophora* showed the U-type spectrum typical of *Ulva* starch, *Enteromorpha* showed an A-type spectrum typical of the spectrum of cereal starches.

Polysaccharides, from *Oscillatoria* have been characterised by Hough Jones and Wadman (1952) as an amylopectin-type polyglucosan
of average chain length of 23 glucose units. Also these workers forwarded evidence for a $\beta$-glucan of the cellulose type in the blue-green alga *Nitella*, which has recently been shown to metabolise a starch-type polysaccharide as well. (King 1960).

Eady, Fleming and Manners (1958) have isolated a glucose-containing polysaccharide from the unicellular salt-water alga *Dunaliella bicuculata*. They reported that it resembled plant starches in many respects but contained only 13% of amylose (cf. 20-30% in the majority of plant starches), and the amylopectin component had a chain length of 15-16 glucose units (cf. 20-25). Both components were incompletely degraded by $\beta$-amylase, the barriers to this enzyme being hydrolysed by $\alpha$-enzyme and iso-amylase respectively. While the amylose content of this and other algal starches (Bourne 1950) appears to be low in comparison with those of land plants, the author feels that this may be partially accounted for by degradation during the methods of extraction employed. (e.g. with perchloric acid).

The isolation of an amylopectin-type glucan from the green seaweed *Caulerpa filiformis* (Mackie 1960) was achieved in this laboratory by the addition of "octaion" (cetyltrimethylammonium hydroxide) to the borate complexes of the mixture of water-soluble polysaccharides. The glucan could not be fractionated into two starch components, but in all, its properties resembled the amylopectin of land plant starches closely. Table II gives a comparison of this material with similar glucans from other sources.
### TABLE II

Comparison of the properties of starch-type polysaccharides.

<table>
<thead>
<tr>
<th>Property</th>
<th>Amylopectin (a)</th>
<th>Floridean Starch (a)</th>
<th>Glycogen (a)</th>
<th>G. filiformis glucan</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[\alpha]_D$ in H2O</td>
<td>212°</td>
<td>176°</td>
<td>196°</td>
<td>154°</td>
</tr>
<tr>
<td>Iodine colour</td>
<td>Purple</td>
<td>Deep reddish-brown</td>
<td>Reddish-brown</td>
<td>Purple</td>
</tr>
<tr>
<td>$\lambda_{max}$ of iodine complex</td>
<td>540</td>
<td>500</td>
<td>460</td>
<td>540</td>
</tr>
<tr>
<td>Optical density at $\lambda_{max}$</td>
<td>1.06</td>
<td>0.84</td>
<td>0.34</td>
<td>0.68</td>
</tr>
<tr>
<td>$\beta$-amylolysis limit</td>
<td>54</td>
<td>46</td>
<td>45</td>
<td>57</td>
</tr>
<tr>
<td>$\lambda$-amylolysis (Fm)</td>
<td>76</td>
<td>54</td>
<td>57</td>
<td>83</td>
</tr>
<tr>
<td>$\lambda$-amylolysis (Fm$^x$)</td>
<td>88</td>
<td>65</td>
<td>70</td>
<td>90</td>
</tr>
<tr>
<td>Reduction 10$^4$ (moles/anhydro glucose unit)</td>
<td>1.04</td>
<td>1.05</td>
<td>1.08</td>
<td>0.95</td>
</tr>
<tr>
<td>Average chain length</td>
<td>20</td>
<td>9</td>
<td>12</td>
<td>21</td>
</tr>
<tr>
<td>Limiting viscosity number</td>
<td>ca. 150</td>
<td>-</td>
<td>10</td>
<td>15</td>
</tr>
</tbody>
</table>

$x \quad F_m = \text{apparent conversion to maltose.}$

(a) Fleming, Hirst and Manners (1956); Manners and Wright 1960.

Methylation and periodate oxidation provided evidence that the *Gaulerpa* glucan contained $\alpha$-1,4'-linked glucose units with branches at C6 and had an average chain length of ca. 21 glucose units. Treatment with salivary $\alpha$-amylase gave a $F_m$ of 90 while $\beta$-amylolysis showed 57% conversion to maltose this value being increased to 83% by pre-treatment with isom-amylose.

Other green algae have been reported to contain starch-type...
polysaccharides (O'Donnell 1959) while there is evidence to suggest that laminarin-type glucans are also metabolised. Thus Fisher (1957) was able to isolate from C. rupestris a small glucose-rich fraction which gave no colour with iodine and was essentially resistant to periodate attack - furthermore it yielded a disaccharide with the same chromatographic speed as laminaribiose (Rf 0.81) on partial acid hydrolysis. There are indications that other weeds contain this small proportion of laminarin in addition to the more usual starch reserve.

The present thesis describes the isolation and characterisation of a starch-type glucan from the green seaweeds C. rupestris and E. compressa.

I (b) EXPERIMENTAL

A. CLADOPHORA RUPESTRIS

EXPT.1. EXTRACTION OF THE WEED.

Two samples of the green weed Cladophora rupestris were collected, one in October 1958 and the other in November 1959, both from the same site near Dunbar between high and low tide levels. The samples were steeped in commercial alcohol for 24 hours without previous drying treatment after which they were exhaustively extracted with 80% ethanol at 78°. The weed retained a high proportion of its colouring matter. The residual, alcohol extracted weed (1000g, wet weight) was further extracted
under the following conditions:

(A) Hot water extractions were repeated until the extract contained a negligible quantity of carbohydrate (Molisch's test).

(B) The residual weed from (A) was given a mild chlorite treatment at 70° according to the method of Wise (1946).

(C) The weed residue from (B) was washed with cold distilled water and extracted at room temperature with 4% sodium hydroxide solution in an atmosphere of nitrogen (Chanda 1951) for 12 hours.

(D) The weed remaining after extraction (C) was digested in 18% sodium hydroxide for 24 hours at room temperature.

(E) After removal of all alkali soluble carbohydrate the residual weed was washed free of sodium hydroxide and allowed to steep for 36 hours in 72% concentrated sulphuric acid (Monier-Williams 1921) followed by hydrolysis for 12 hours with H₂SO₄ at 100°.

Analysis and characterisation of extract (A) has been reported by Fisher (1957) and for this reason the water-soluble material was not examined further. Extracts (B), (C) and (D) were isolated after concentration and dialysis either by alcohol precipitation or freeze-drying.

**EXPT.2. PRELIMINARY EXAMINATION OF EXTRACTS (B), (C) AND (D)**

Polysaccharides (B), (C) and (D) were found to have the following properties:
<table>
<thead>
<tr>
<th></th>
<th>$[\kappa]_D$</th>
<th>N%</th>
<th>Sulphate%</th>
<th>Ash%</th>
<th>Uronic acid%</th>
</tr>
</thead>
<tbody>
<tr>
<td>(B)</td>
<td>+100</td>
<td>4</td>
<td>13.6</td>
<td>14.78</td>
<td>5.02</td>
</tr>
<tr>
<td>(C)</td>
<td>+54</td>
<td>7</td>
<td>1.85</td>
<td>11.15</td>
<td>6.56</td>
</tr>
<tr>
<td>(D)</td>
<td>-</td>
<td>8</td>
<td>-</td>
<td>35.5</td>
<td>-</td>
</tr>
</tbody>
</table>

Visual examination of a chromatogram (solvent B) of the hydrolysates of (B), (C) and (D) indicated the following sugars:

<table>
<thead>
<tr>
<th>Galactose</th>
<th>Glucose</th>
<th>Arabinose</th>
<th>Xylose</th>
<th>Rhamnose</th>
</tr>
</thead>
<tbody>
<tr>
<td>(B)</td>
<td>xxxxx</td>
<td>x</td>
<td>xxxxx</td>
<td>xx</td>
</tr>
<tr>
<td>(C)</td>
<td>xx</td>
<td>xxxxx</td>
<td>xx</td>
<td>x</td>
</tr>
<tr>
<td>(D)</td>
<td>xxxxx</td>
<td></td>
<td></td>
<td>x</td>
</tr>
</tbody>
</table>

xxxxx = major component
x = trace

The high glucose content of polysaccharide (C) together with its ability to produce a blue colour with iodine indicated the presence of a starch-like polymer. All further work in this section describes the glucan from polysaccharide (C).

**EXPT.2. PURIFICATION OF POLYSACCHARIDE (C)**

The nitrogen content (7%) polysaccharide (C) indicated high protein contamination. Treatment of a dilute alkaline solution of the polysaccharide (8.2g.) with 4% trichloroacetic acid to pH 4, (Stumpf 1948) precipitated a dark-green protein-rich material which was removed on the Sharples' high-speed centrifuge. The centrifugate was dialysed for 3 days, concentrated under reduced pressure and freeze dried to yield polysaccharide (F) (Yield 3.3g).
EXPT. 4. PHYSICAL PROPERTIES OF POLYSACCHARIDE (F)

The purified alkali-soluble material (F) had the following properties:

\[ \alpha_\text{D} = +57 \text{ (c.0.85 in alkali) N 2.83\%, sulphate 1.85\%, ash 6.56\%} \]

The uronic acid content as calculated by the yield of carbon dioxide on decarboxylation of an acid digest ca. 5.0% (Swenson 1946).


Standard solutions of control sugars were prepared by weighing out milli-molar quantities of these and diluting to 25ml. in a graduated flask. Accurately measured volumes of these solutions were spotted on to the starting line of a paper chromatogram and eluted for 60 hours in solvent B. After thorough air-drying the chromatogram was sprayed with a freshly prepared solution of aniline phthalate (1.66g. o-phthalic acid and redistilled aniline(0.91ml.) dissolved in butanol (48ml.) ether (48ml.) and water (4ml.) and developed at 100-110° for 5 minutes.

Each of the coloured spots which appeared was cut out and eluted from the paper by shaking with 0.7N hydrochloric acid in 80% ethanol (v/v 4ml.) (prepared by adding 36% hydrochloric acid (29ml.) to 95% ethanol (420ml.) and making up to 500ml. with distilled water in a graduated flask). The absorbance of the sugar solutions was measured in a Unicam Spectrophotometer S.P. 500 at the wavelength of maximum absorption for the particular sugar.
FIG. 21  COLOURIMETRIC ESTIMATION OF SUGARS

Weight of sugar (μg)

Absorbance (m/μ)

KEY
Galactose & Glucose.
Rhamnose.
Xylose.
Arabinose.
under examination (390m\( \mu \) for hexoses and deoxy-hexoses, 360m\( \mu \) for pentoses). A linear relationship was obtained on plotting absorbance against weight of each sugar. (fig. 21).

The relative weights of the sugars in a polysaccharide hydrolysate were then determined directly from the corresponding calibration graph for the particular sugar, each absorbance being compared with that of a blank solution. The method was found to be reproducible within ±5\% and it was found unnecessary to repeat the calibration with each estimation.

An examination of the neutral sugars present in the hydrolysate of polysaccharide (F) gave the results shown in Table III.

**TABLE III**

Relative proportions of the sugars in the hydrolysate of polysaccharide (F).

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Absorbance</th>
<th>Wt. of sugar (( \mu )g.)</th>
<th>Molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactose</td>
<td>0.123</td>
<td>57.5</td>
<td>8.4</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.520</td>
<td>202.0</td>
<td>34.0</td>
</tr>
<tr>
<td>Arabinose</td>
<td>0.091</td>
<td>22.5</td>
<td>4.3</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.05</td>
<td>6.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>0.03</td>
<td>tr.</td>
<td>tr.</td>
</tr>
</tbody>
</table>

**EXPT. 6. ACETYLATION OF POLYSACCHARIDE (F)**

The polysaccharide (2.9g.) was dispersed in dry pyridine (200ml.) and dimethyl formamide (25ml.) in a high-speed "Ato-mix" agitator. To this gel-like dispersion was added acetic anhydride
and the mixture was shaken for four days at room temperature in a dark bottle. The product was precipitated by addition of the dark brown solution to rapidly stirred ice-water and isolated after dialysis, by freeze drying a suspension in water. Two further acetylations under similar conditions yielded a pure white polysaccharide acetate (Wt. 1.1g. Found: acetyl 38.7%, sulphate nil, N 1.8%).

**Chloroform extraction of the acetylated polysaccharide.**

The acetylated polysaccharide (1.1g.) was continuously extracted with chloroform (150ml.) in a Soxhlet extractor for 24 hours at 60°C. The chloroform was concentrated to ca. 10ml. and a white flocculent precipitate was obtained on addition of the solution to light petroleum (b.p. 60°C-80°C) (4 vols.) (Yield 730 mg. 

$[\alpha]_D +160.0, 0.94$ in chloroform). Chromatographic investigation (solvent A) of a hydrolysate showed glucose with a trace of xylose.

**Deacetylation of the acetate.**

During the course of the work two methods of deacetylation were employed. (a) The acetate (98mg.) was treated with dry methanol (10ml.) and freshly cut potassium (ca. 20mg.), the solution being heated on a steam bath for 3 minutes. The mixture was allowed to stand at room temperature for 15 hours after which distilled water (5ml.) was added and the potassium precipitated by titration with commercial 60% perchloric acid to a potentiometrically determined end-point using a pH meter (pH8.15). The precipitated potassium perchlorate was filtered off, rinsed with water and the filtrate and washings freeze-dried. (Yield 48.8mg.)

(Bonner 1948.). (b) The same method as described in (a) except
that the potassium was removed by dialysis of an aqueous solution of the deacetylated material followed by concentration and freeze drying.

Total yield of glucan 416mg. from 715mg. acetate. Hydrolysis of a sample (10mg.) followed by chromatographic investigation indicated the presence of glucose only. This material isolated after acetylation and chloroform extraction of the acetate will henceforth be called Glucan I.

**EXPT. 7. IODINE PRECIPITATION OF A GLUCAN FROM POLYSACCHARIDE (F)**

A second series of extractions (160g. dry weed) under the same conditions as reported in Expt. 1 except that all the operations were carried out in an atmosphere of nitrogen, produced a polysaccharide (F) (Yield 2.8g.) having essentially similar properties to the material described in Expt. 4.

Polysaccharide (F) (2.7g.) was mixed with celite (3g.) and dispersed in 0.03 N ammonium carbonate (100ml.). The solution, in a conical flask, was placed in an oil-bath at 117-120° under nitrogen for 30 minutes with stirring. It was then cooled to room temperature and 20% aqueous sodium chloride (50ml.) together with 12% iodine in 20% potassium iodide solution (5ml.) introduced, the flask being filled with water (ca. 200ml.) and shaken vigorously. After 5 minutes the precipitate was centrifuged off and immediately redispersed in 20% sodium chloride (50ml.). The solution was stirred rapidly while the iodine complex was destroyed by dropwise addition of 0.5 N sodium thiosulphate
(ca. 5ml.), excess of the reagent being avoided. At this stage the celite was removed on the centrifuge.

To the starch solution was now added N hydrochloric acid (20ml.) and a further volume of the iodine, potassium iodide solution (10ml.). The re-precipitated starch-iodine complex was centrifuged off and suspended in 95% ethanol (200ml.) when the complex was again destroyed by dropwise addition of thiosulphate until the blue colour had disappeared (ca. 9ml.). The final concentration of ethanol was reduced to 70% by the addition of distilled water and the starch isolated after dialysis and concentration by freeze-drying. (Yield 200mg. 7.2% \(\left[\alpha\right]_D^{170^\circ} +170^\circ\) c,1.1 in alkali).

This material will be referred to as Glucan II.

B. ENTEROMORPHA COMRESSA

EXPT.8. IODINE PRECIPITATION OF A GLUCAN FROM POLYSACCHARIDE (G) (Steiner 1944).

The water-soluble polysaccharide (G) (15g.) from the green weed E. compressa, the properties of which are given in Expt.41 Section III, was treated in the manner described in Expt.7 with the following modifications in quantities: -

- 0.03 N Ammonium carbonate 500ml.
- 20% aqueous sodium chloride 200ml. in each application.
- Iodine-potassium iodide 25ml. " " "
- 0.5 N sodium thiosulphate ca. 25ml.
- N hydrochloric acid 60ml.

The polysaccharide was isolated as a pure-white material
(500mg. 3.5%) after dialysis and freeze-drying.

This glucan will be called Glucan III henceforth.

C. INVESTIGATION OF THE STRUCTURES OF GLUCANS I, II AND III


The glucan (e.g., 100mg.) was suspended in distilled water (10ml.) and added to vigorously stirred boiling water (40ml.). The solution was heated at 90° for 20 minutes and then allowed to cool, an atmosphere of nitrogen being maintained throughout. The small insoluble residue was filtered off and the clear solution heated at 60° with powdered thymol (0.5g.) for 30 minutes, again under nitrogen. Finally the mixture was set aside at room temperature for two days. The precipitate which formed was redispersed immediately after separation in distilled water (50ml.) at 95° under nitrogen and redistilled butanol (5ml.) added. The solution was maintained at this temperature for 30 minutes then allowed to cool slowly to room temperature and the butanol-amylose complex removed by centrifugation. The amylose was purified by two further precipitations with butanol and isolated by treatment of the butanol complex with butanol.

**TABLE IV**

**Weight of Amylose and Amylopectin in Glucans I, II and III.**

<table>
<thead>
<tr>
<th>Glucan</th>
<th>Weight</th>
<th>Amylose</th>
<th>Amylopectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>101mg.</td>
<td>-</td>
<td>65mg.</td>
</tr>
<tr>
<td>II</td>
<td>200mg.</td>
<td>46.6mg. (II(a))</td>
<td>110.6mg. (II(b))</td>
</tr>
<tr>
<td>III</td>
<td>500mg.</td>
<td>118.0mg. (III(a))</td>
<td>302.0mg. (III(b))</td>
</tr>
</tbody>
</table>
Henceforth, the glucans will be referred to by the above suffixes.

EXPT. 10. PRELIMINARY EXAMINATION OF THE PROPERTIES OF GLUCANS I, II and III.

The properties of the glucans obtained in the preceding experiments were found to be as shown in Table V.

<table>
<thead>
<tr>
<th>Glucan</th>
<th>$[\alpha]_D$</th>
<th>$N%$</th>
<th>Ash $%$</th>
<th>Reducing power $%$ glucose</th>
<th>Iodine colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>+187</td>
<td>0.78</td>
<td>1.0</td>
<td>92.5</td>
<td>blue</td>
</tr>
<tr>
<td>IIa</td>
<td>+176</td>
<td>0.31</td>
<td>0.90</td>
<td>83.0</td>
<td>blue</td>
</tr>
<tr>
<td>IIb</td>
<td>+182</td>
<td></td>
<td>0.31</td>
<td>90.0</td>
<td>blue</td>
</tr>
<tr>
<td>IIIa</td>
<td>+177</td>
<td>1.01</td>
<td>3.0</td>
<td>80.0</td>
<td>blue</td>
</tr>
<tr>
<td>IIIb</td>
<td>+190</td>
<td></td>
<td>0.92</td>
<td>91.0</td>
<td>purple</td>
</tr>
</tbody>
</table>

The glucans were readily soluble in dilute sodium hydroxide solution and remained in solution after dialysis. The amylose fractions IIa and IIIa showed a marked tendency to retrograde from acid solutions.

A sample of each glucan (ca. 20mg.) was heated with 2 N sulphuric acid under the stated hydrolysis conditions (p.43) neutralised with sodium hydroxide, made just acid with sulphuric acid and finally diluted to a known volume in a standard flask. Analysis of the reducing power of aliquots of these solutions by cuprimetric titration (Somogyi 1952) led to the estimation of the
percentage purity of each fraction (Table V). The remaining solution, containing the hydrolysed polysaccharide material, was concentrated to a syrup and analysed chromatographically (solvent A) when a single spot was revealed which was identical with glucose run as a control. The remainder of the hydrolysate was incubated with glucose oxidase for 24 hours at 30° when the sole product visible on a paper chromatogram was found to have the same speed as an authentic specimen of gluconic acid. (Spray 3)

EXPT.11. PROPERTIES OF THE IODINE COMPLEX.

(a) Examination of the Spectra.

Amylopectin solution containing 2.5mg. polysaccharide was introduced into a 25ml. graduated flask with one drop of 6 N hydrochloric acid and iodine solution (2.5ml; 0.2% iodine in 2% potassium iodide) and the mixture diluted to 25ml. with distilled water. The extinction (E) of the resulting polysaccharide-iodine complex was examined in a Unicam S.P.500 Spectrophotometer over the range 420-700 m/ against an iodine-water blank, in lcm. silica cells. The wavelength of maximum absorption (λmax.) was taken as the mid-point of the peak obtained by plotting wavelength against optical density, the optical density at λmax. being termed Emax.

The extinction coefficient (E) of amylose solutions (I-IIIA) under these conditions was too great to measure and the λmax. for these samples was studied under "Blue value" conditions as discussed in Expt.11(b).
FIG. 22 ABSORPTION SPECTRA OF THE GLUCAN - IODINE COMPLEXES.

KEY:
1. Glucan I.
2. IIa.
3. IIIa.
4. IIIb.
5. K.P. Amylose.

E

Wavelength. (m\u00b5)

400 500 600 700
\( \lambda_{\text{max}} \)

<table>
<thead>
<tr>
<th>Glucan</th>
<th>( \lambda_{\text{max}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>IIb</td>
<td>600</td>
</tr>
<tr>
<td>IIIb</td>
<td>550</td>
</tr>
</tbody>
</table>

The \( \lambda_{\text{max}} \) of Kerr's Pink potato amyllopectin studied under the above conditions was 545.

(b) **Blue value** *(Bourne 1948)*.

Amylose and amyllopectin were studied under "blue-value" conditions as follows:

Polysaccharide solution containing a known quantity of solute (5mg.) as determined by cuprimetric titration *(Somogyi 1952)* was transferred to a 500ml. standard flask and stained with the standard iodine solution (5ml.). The coloured solution was diluted to 500ml. with distilled water after addition of 0.25ml. 6 N hydrochloric acid. The spectrum was examined between 450 and 700 m\( \mu \) against an iodine-water blank. The blue value (B.V.) is defined as the \( E \) at 680 m\( \mu \) using 4cm. cells or the \( E \times 4 \) using 1cm. cells.

The \( \lambda_{\text{max}} \) of amyllose solutions was studied under the above conditions.

**TABLE VII(a)**

<table>
<thead>
<tr>
<th>Glucan</th>
<th>( \lambda_{\text{max}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>610</td>
</tr>
<tr>
<td>IIa</td>
<td>610</td>
</tr>
<tr>
<td>IIIa</td>
<td>610</td>
</tr>
<tr>
<td>Kerr's Pink potato amyllose</td>
<td>610</td>
</tr>
</tbody>
</table>
The "blue-value" of Kerr's Pink amylose and amylopectin controls were found to be 0.590 and 0.132 respectively. The plots of wavelength against extinction are shown in fig.22.

EXPT.12. POTENTIOMETRIC IODINE TITRATION.

(a) A sample of the C. rupestris Glucan I (5mg.) was examined by the potentiometric iodine titration method of Anderson and Greenwood (1955) when the curve shown in fig.23(a) was obtained indicating an iodine binding power of 1.05%.

(b) Glucan IIIa. A sample of the glucan (14.16mg.) dissolved in 0.5 N potassium hydroxide (10ml.) and neutralised with 0.5 N hydrochloric acid, was treated with 0.5 N potassium iodide (10ml.) and diluted to 100ml. with distilled water. The solution was then titrated potentiometrically against 0.000843 N iodine solution in 0.05 N potassium iodide using a saturated calomel electrode. The iodine solution was added in aliquots (2ml.), the galvanometer reading being taken 5 minutes after each addition; the aliquots were reduced to 1ml. as the end-point was neared (i.e. 13-14ml.). The true end-point was found by plotting the

<table>
<thead>
<tr>
<th>Sample</th>
<th>B.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucan I</td>
<td>0.800</td>
</tr>
<tr>
<td>IIa</td>
<td>0.475</td>
</tr>
<tr>
<td>IIb</td>
<td>0.300</td>
</tr>
<tr>
<td>IIIa</td>
<td>0.660</td>
</tr>
<tr>
<td>IIIb</td>
<td>0.140</td>
</tr>
</tbody>
</table>
FIG. 23 POTENTIOMETRIC IODINE TITRATIONS.

23a. Glucan I

poly saccharide.

E.m.f.

23b. Glucan IIIa

iodine affinity (mgs/100mgs)

Free I₂ (g/l.) x 10⁴
free iodine in solution against the percentage iodine bound per 100mg. polysaccharide. (fig. 23b).

**TABLE VI**

<table>
<thead>
<tr>
<th>Iodine added (ml.)</th>
<th>Potential (mV)</th>
<th>Free iodine g/1 x 10^4</th>
<th>Bound iodine g/1 x 10^4</th>
<th>z</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>182</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>227</td>
<td>0.6</td>
<td>22.2</td>
<td>1.68</td>
</tr>
<tr>
<td>4</td>
<td>232</td>
<td>1.5</td>
<td>43.1</td>
<td>3.22</td>
</tr>
<tr>
<td>6</td>
<td>235</td>
<td>1.9</td>
<td>63.3</td>
<td>4.86</td>
</tr>
<tr>
<td>8</td>
<td>238</td>
<td>2.2</td>
<td>83.7</td>
<td>6.50</td>
</tr>
<tr>
<td>10</td>
<td>243</td>
<td>3.2</td>
<td>102.2</td>
<td>8.10</td>
</tr>
<tr>
<td>12</td>
<td>251</td>
<td>6.0</td>
<td>118.2</td>
<td>9.55</td>
</tr>
<tr>
<td>14</td>
<td>261</td>
<td>15.0</td>
<td>127.4</td>
<td>10.42</td>
</tr>
<tr>
<td>15</td>
<td>264</td>
<td>20.0</td>
<td>131.2</td>
<td>10.85</td>
</tr>
<tr>
<td>16</td>
<td>268</td>
<td>29.5</td>
<td>131.5</td>
<td>11.00</td>
</tr>
<tr>
<td>17</td>
<td>270</td>
<td>34.0</td>
<td>134.0</td>
<td>11.30</td>
</tr>
<tr>
<td>18</td>
<td>272</td>
<td>40.0</td>
<td>137.0</td>
<td>11.60</td>
</tr>
<tr>
<td>19</td>
<td>275</td>
<td>51.5</td>
<td>134.0</td>
<td>11.60</td>
</tr>
</tbody>
</table>

**EXPT.12. VISCOSITY MEASUREMENTS ON THE GLUCANS.** (Bottle 1953; Greenwood 1957).

The specific viscosity (\( \eta_s \)) of a polymer solution is dependant upon the concentration of the solution, the ratio being designated the viscosity number. The limiting viscosity or intrinsic viscosity \([\eta]\) is the value obtained by extrapolation of \( \eta_s / c \) to infinite dilution.
\[ [\eta] = \lim_{c \to 0} \frac{\eta_{sp}}{c} \]

where \( c \) = concentration in g. per ml.

For the determination of \( \eta_{sp} \) and \([\eta]\) a modified Ubbelohde viscometer was used, the expression for the specific viscosity in dilute solution being given by:

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

where \( T \) is the solution flow time and \( T_0 \) the solvent flow time in seconds.

An approximately \( 1\% \) solution of the glucan in M potassium hydroxide was prepared and the exact concentration of polysaccharide determined by cuprimetric titration (Somogyi 1952) of a hydrolysed, neutralised aliquot of the solution. Measurements were made in a constant temperature bath at 25° with the viscometer clamped in a vertical position. Polysaccharide solutions were filtered through G4 sintered glass before use, as was the solvent.

Two methods for determining viscosities were used:

(a) Solution (10-15ml.) was placed in the viscometer and the flow time measured. Solvent (5ml.) was added and the flow time redetermined after each addition, approximately 30 minutes being allowed each time for the equilibration of temperature. The solvent flow time was determined separately.

(b) Solvent (10ml.) was placed in the viscometer and the flow time established. Aliquots (5ml.) of solution were added
FIG. 24a. UBBELOHDE VISCOMETER.

FIG. 24b. ISOTHERMAL DISTILLATION APPARATUS.
and the flow times measured after each addition.

The latter method was more practical as the solvent flow time could be measured without removing the solution from the viscometer. However, the former method lends itself to measurements in which only small quantities of material are available.

The following results were obtained:

**TABLE VIII**

Viscosities of Glucan samples.

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(g/ml.)</td>
<td>T (secs.)</td>
<td>T - To</td>
<td>η,</td>
<td>[η]</td>
</tr>
<tr>
<td>Glucan I</td>
<td>To = 237.30 seconds</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00303</td>
<td>251.90</td>
<td>14.60</td>
<td>0.0615</td>
<td>20.3</td>
</tr>
<tr>
<td>0.00202</td>
<td>246.81</td>
<td>9.49</td>
<td>0.0400</td>
<td>19.8</td>
</tr>
<tr>
<td>0.00152</td>
<td>244.60</td>
<td>7.30</td>
<td>0.0307</td>
<td>20.3</td>
</tr>
</tbody>
</table>

[η] was found graphically to be 20.0

<table>
<thead>
<tr>
<th>Glucan IIa</th>
<th>To = 237.00 seconds</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00390</td>
<td>262</td>
</tr>
<tr>
<td>0.00258</td>
<td>253.6</td>
</tr>
<tr>
<td>0.00194</td>
<td>249.2</td>
</tr>
</tbody>
</table>

[η] was found graphically to be 26.9
EXPT. 14. AMYLOLYSIS EXPERIMENTS ON THE GLUCANS.

(1) $\alpha$-amylolysis with salivary $\alpha$-amylase. (Liddle 1957).

The apparent conversion to maltose ($P_m$) of the glucan samples was determined using crystalline salivary $\alpha$-amylase. The polysaccharide sample was dissolved in dilute alkali and made...
neutral with hydrochloric acid. To an aliquot of the solution, containing a known amount of polysaccharide as determined by cuprimetric titration (Somogyi 1952), was added sodium chloride (5mg.) and freeze-dried salivary \( \alpha \)-amylase (5mg.) in a total volume of 25 or 50 ml. The solution was incubated at 30\(^\circ\) for 48 hours.

TABLE IX

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>Wt. of maltose (mg.)</th>
<th>PM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Glucan I</td>
<td>25.5mg ; ( \alpha )-amylase 2.5mg. ; Digest 50ml.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aliquots (5ml.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>15.6</td>
<td>63</td>
</tr>
<tr>
<td>10</td>
<td>17.9</td>
<td>71</td>
</tr>
<tr>
<td>24</td>
<td>19.85</td>
<td>78</td>
</tr>
<tr>
<td>(b) Glucan IIIa</td>
<td>3.875 mg. ; ( \alpha )-amylase 2mg. ; Digest 25ml.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aliquots (5ml.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.70</td>
<td>85.0</td>
</tr>
<tr>
<td>10</td>
<td>0.74</td>
<td>90.1</td>
</tr>
<tr>
<td>24</td>
<td>0.76</td>
<td>92.5</td>
</tr>
<tr>
<td>(c) Glucan IIIa</td>
<td>2.88mg. ; ( \alpha )-amylase 1mg. ; Digest 10ml.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aliquots (4ml.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.84</td>
<td>69.0</td>
</tr>
<tr>
<td>24</td>
<td>1.03</td>
<td>85.1</td>
</tr>
<tr>
<td>Time (hrs.)</td>
<td>Wt. of maltose (mg.)</td>
<td>$P_M$</td>
</tr>
<tr>
<td>------------</td>
<td>----------------------</td>
<td>------</td>
</tr>
<tr>
<td>(d) Glucan IIIb</td>
<td>3.98mg.</td>
<td>$\alpha$-amylase 2mg.</td>
</tr>
<tr>
<td>3</td>
<td>1.23</td>
<td>73.2</td>
</tr>
<tr>
<td>24</td>
<td>1.51</td>
<td>90.11</td>
</tr>
</tbody>
</table>

Control experiments using Kerr’s Pink potato amylose and amylopectin gave $P_M$ values of 93 and 88 respectively.

(ii) $\beta$-amylolysis.

A commercial sample of $\beta$-amylase from the Wallerstein Laboratories (New York) was used for this work. The enzyme was free from maltase but contained a trace of $\alpha$-enzyme. The activity of the preparation was 110 units/mg. as determined by the method of Hobson Whelan and Peat (1950).

$\beta$-amylolysis of amylose components was carried out by digesting the sample (10-30mg.) with 0.04 M acetate buffer at pH 3.6 and 4.6 with $\beta$-amylase (100 units/mg. at pH 3.6; 50 units/mg. at pH 4.6) at 37° for 48 hours. The digests were analysed for their maltose content as described in Expt. 14(1). At pH 3.6, $\beta$-amylase was preincubated with the buffer for 0.5 hours at room temperature prior to addition of the polysaccharide.

Toluene (1-2 drops) was added to all digests as an antiseptic. Polysaccharide concentrations were determined by acid hydrolysis of aliquots removed from the digests themselves, or from the polysaccharide solution before addition to the digests.
β-amyolysis of amylopectin (10-30mg.) was effected by incubation of the polysaccharide at 37° with 0.04 M acetate buffer at pH 4.6 with β-amylose (25 units/mg.). Aliquots (2-5ml.) were removed after 24 and 48 hours for estimation of the maltose produced, the result being expressed as a percentage conversion. It has been shown (Wright 1960) that when enzyme concentration is increased to 50 units/mg. or more at pH 4.6 a gradual increase in the maltose production is observed due to α-enzyme action and it was therefore imperative that the enzyme concentration was maintained below 25 units/mg. so that a true β-amyolysis limit could be obtained.

**TABLE X**

**β-amyolysis limits of Seaweed glucans.**

<table>
<thead>
<tr>
<th>Glucan</th>
<th>Digest</th>
<th>Time (hrs.)</th>
<th>Weight of maltose (mg.)</th>
<th>β-limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>25.81mg./20ml.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 3.6</td>
<td>24</td>
<td>9.11</td>
<td>40.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>11.12</td>
<td>49.1</td>
</tr>
<tr>
<td></td>
<td>pH 4.6</td>
<td>24</td>
<td>13.4</td>
<td>59.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>14.2</td>
<td>63.1</td>
</tr>
<tr>
<td>IIa</td>
<td>11.6mg./25ml.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 3.6</td>
<td>24</td>
<td>0.535</td>
<td>36.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>0.745</td>
<td>51.0</td>
</tr>
<tr>
<td></td>
<td>pH 4.6</td>
<td>24</td>
<td>0.760</td>
<td>52.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>0.970</td>
<td>66.2</td>
</tr>
</tbody>
</table>
### Glucan Digest *Time* Weight of maltose $\beta$-limit

<table>
<thead>
<tr>
<th>Glucan</th>
<th>Digest</th>
<th>Time (hrs.)</th>
<th>Weight of maltose (mg.)</th>
<th>$\beta$-limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>IIIa</td>
<td>14.4mg./25ml.</td>
<td>24</td>
<td>0.645</td>
<td>26.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>0.760</td>
<td>31.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH 4.6</td>
<td>24</td>
<td>1.70</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>48</td>
<td>1.85</td>
</tr>
<tr>
<td>IIIb</td>
<td>14.93mg./ml.</td>
<td>24</td>
<td>1.41</td>
<td>56.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>1.46</td>
<td>58.0</td>
</tr>
</tbody>
</table>

Control experiments using Kerr's Pink potato amylose at pH 3.6 and 4.6 and amylopectin at pH 4.6 gave 72.4 and 108% and 55% respectively.

**EXPT. 15. OXIDATION OF THE GLUCANS WITH SODIUM METAPERIODATE**

*Aspinall 1957*

Polysaccharide samples (10-30mg.) were oxidised using 0.015 M sodium metaperiodate in the dark at 20°. At intervals, the reduction of periodate was measured by removing an aliquot (1ml.) of the reaction mixture and diluting 250 times before reading the absorption on a Unicam S.P.500 Spectrophotometer at 222.5 m$\mu$ against a water blank. The fraction of periodate reduced was then found from a calibration graph obtained by measuring the absorbances of 0.015 M sodium meta-periodate and 0.015 M iodate under the same conditions.
<table>
<thead>
<tr>
<th>Sample and Concentration</th>
<th>Time (hrs.)</th>
<th>Absorbance</th>
<th>Frac. $10^4$ consumed</th>
<th>Moles $10^4$ reduced/mole C$_6$ anhydro-unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucan I</td>
<td>0</td>
<td>0.590</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12.20mg./50ml.</td>
<td>1</td>
<td>0.542</td>
<td>0.082</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.523</td>
<td>0.118</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.515</td>
<td>0.145</td>
<td>1.38</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.505</td>
<td>0.153</td>
<td>1.46</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>0.505</td>
<td>0.153</td>
<td>1.46</td>
</tr>
<tr>
<td>Glucan IIa</td>
<td>0</td>
<td>0.590</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>17.20mg./50ml.</td>
<td>1</td>
<td>0.575</td>
<td>0.022</td>
<td>0.156</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.546</td>
<td>0.076</td>
<td>0.540</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.540</td>
<td>0.087</td>
<td>0.615</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.537</td>
<td>0.097</td>
<td>0.682</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>0.537</td>
<td>0.097</td>
<td>0.682</td>
</tr>
<tr>
<td>Glucan IIIa</td>
<td>0</td>
<td>0.612</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12.80mg./25ml.</td>
<td>1</td>
<td>0.551</td>
<td>0.109</td>
<td>0.575</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.542</td>
<td>0.132</td>
<td>0.640</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.521</td>
<td>0.169</td>
<td>0.810</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.510</td>
<td>0.190</td>
<td>0.900</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.500</td>
<td>0.202</td>
<td>0.980</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>0.488</td>
<td>0.228</td>
<td>1.08</td>
</tr>
</tbody>
</table>
**Sample and Concentration** | **Time (hrs.)** | **Absorbance** | **Fractions 10^4 reduced/mole C₆ anhydro-unit**
--- | --- | --- | ---
Glucan IIIb | 0 | 0.612 | 0.144 | 0.450
19.8mg./25ml. | 1 | 0.532 | 0.144 | 0.450
 | 4 | 0.520 | 0.169 | 0.530
 | 24 | 0.488 | 0.228 | 0.530
 | 48 | 0.480 | 0.244 | 0.530
 | 72 | 0.468 | 0.266 | 0.530
 | 96 | 0.456 | 0.288 | 0.530

Oxidation of Kerr's Pink amylopectin samples were carried out simultaneously as control experiments, typical results for these being 0.900 and 0.970 moles per C₆ anhydro-unit.

After oxidation was complete (ca. 72-120 hours) the reaction was stopped by the addition of ethylene glycol (5ml.) after which the solution was dialysed, concentrated to dryness and examined chromatographically (solvent A). In no case was there evidence for glucose in the oxopolysaccharide hydrolysates.

Thus summarizing uptakes of periodate by the various glucan samples we have after four days oxidation at 20°:

<table>
<thead>
<tr>
<th>Glucan</th>
<th>Moles periodate/C₆ anhydro-unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1.46</td>
</tr>
<tr>
<td>IIa</td>
<td>0.682</td>
</tr>
<tr>
<td>IIIa</td>
<td>1.08</td>
</tr>
<tr>
<td>IIIb</td>
<td>0.900</td>
</tr>
</tbody>
</table>

and K.P. amylopectin | 0.970
FIG. 25 LIQUID AMMONIA METHYLATION APPARATUS
(FRUSH, ISBELL et al. 1957)
EXPT. 16. METHYLATION OF GLUCANS I and II(b).

Methylation was accomplished by the micro-technique of Isbell (1957) using the apparatus as shown in fig. 25.

The polysaccharide (I and IIb, 100mg. and 70mg. respectively) previously dried over phosphorous pentoxide was placed in reaction vessel (A) along with dry filter-cel (ca. 100mg.). The vessel was evacuated through tap 4 and liquid ammonia issuing from storage flask (F) was allowed to condense from the acetone/carbon dioxide cooled condenser (D) into (A) which was similarly cooled. After a suitable quantity (ca. 3ml.) of ammonia covered the polysaccharide-celite mixture, the ammonia storage vessel was re-immersed in an acetone/carbon dioxide bath and this part of the apparatus isolated by closing tap 3. By removing the cooling flask from (A) the ammonia was allowed to reflux onto potassium which was introduced into tap 1 through the side-arm (X) by means of a soft-glass capillary. The potassium was thus washed into the polysaccharide which was stirred by a magnetic stirrer. The addition of potassium in such a manner was continued until the polymer contained an excess, indicated by a permanent blue colour. The whole process was carried out under vacuum.

At this stage the ammonia in (A) was returned to the storage vessel by raising the cooling finger (E) and opening tap 3. Complete removal of the ammonia was essential and was best achieved by warming the reaction vessel and tubes with a hair drier. The reaction vessel (A) was then isolated by closing taps 1 and 2 and pure methyl iodide (ca. 3ml.) was introduced from (B) through tap 5.
by cooling (A) with liquid nitrogen. Finally, (A) was completely closed off and the methyl iodide allowed to reflux overnight, the heat from the magnetic stirrer being sufficient to ensure this. The methyl iodide was ultimately returned to (B) by cooling again in liquid nitrogen and simultaneously warming (A). This completed one methylation cycle.

The polysaccharide was given three such methylations, after which it was extracted from the filter-cel, glass residue by repeated shaking with dry chloroform (5 x 30ml.) and finally concentrated to a pale yellow mobile syrup (I 105.2mg.; IIb 50mg. [α]_D^160° +160° (c, 0.73 in chloroform) soluble in benzene methanol, and to a lesser extent in water. No blue colouration with iodine was observed.

EXPT.17. DETERMINATION OF THE NUMBER AVERAGE MOLECULAR WEIGHT OF THE METHYLATED GLUCAN I BY ISOHERMAL DISTILLATION. (Gee 1940).

The number average molecular weight of methylated polysaccharides has been determined by the method of isothermal distillation within the range 1,000-20,000 (Broatch 1956; Mackie 1960). The most reproducible results were obtained with benzene as the solvent.

A 1.0039% solution of the methylated glucan I (103.24mg.) previously dried over phosphorous pentoxide, was prepared by dissolution in dried "Analar" benzene (10.17894g.). The solution (ca. 4ml.) was introduced into one limb of an isothermal distillation apparatus and pure solvent (ca. 5ml.) was placed in the other limb. The liquids were degassed by the recommended method of
freezing in liquid air followed by evacuating the apparatus.
After repeating the process twice the apparatus was evacuated
and sealed. It was then immersed in a constant temperature
bath at 26.12° and after being allowed to equilibrate for one
hour it was inverted into the "distillation, position".

Readings of the increase in level of the solution and the
corresponding decrease in the level of the solvent were made by
means of a travelling microscope at intervals over a period of
six days.

If \( R \) = observed rate of distillation (mm. of measuring tube
per hour) and \( \frac{\Delta P}{P} \) = relative lowering of vapour pressure
calculated from the mole fraction of solute in solution,
the results are expressed as :-

\[
R \times K = \frac{\Delta P}{P}
\]

where \( K \) is the apparatus constant. A preliminary experiment
was carried out using triolein (M.Wt. 885.4) as the solute,
from which the constant (\( K \)) was found to be \( 2.4 \times 10^{-3} \). The
following results were obtained for the polysaccharide solution :-
FIG. 26  ISOTHERMAL DISTILLATION MEASUREMENTS ON
THE METHYLATED GLUCAN

KEY:
Solution  •
Solvent    ○
TABLE XII
Isothermal distillation of the methylated glucon.

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>Solution Height above zero</th>
<th>Increase</th>
<th>Solvent Height above zero</th>
<th>Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16.5</td>
<td>0.931</td>
<td>-</td>
<td>2.613</td>
<td>-</td>
</tr>
<tr>
<td>22.5</td>
<td>0.945</td>
<td>0.014</td>
<td>2.595</td>
<td>0.018</td>
</tr>
<tr>
<td>41.5</td>
<td>1.040</td>
<td>0.109</td>
<td>2.504</td>
<td>0.109</td>
</tr>
<tr>
<td>66.00</td>
<td>1.169</td>
<td>0.228</td>
<td>2.398</td>
<td>0.215</td>
</tr>
<tr>
<td>88.25</td>
<td>1.272</td>
<td>0.341</td>
<td>2.284</td>
<td>0.329</td>
</tr>
<tr>
<td>112.25</td>
<td>1.398</td>
<td>0.467</td>
<td>2.165</td>
<td>0.448</td>
</tr>
<tr>
<td>136.25</td>
<td>1.538</td>
<td>0.607</td>
<td>2.063</td>
<td>0.550</td>
</tr>
</tbody>
</table>

The graph obtained by plotting the change in the level of the liquid in either limb against time is shown in fig. 26. From the slope of this graph was found the rate of transfer (R) of the solvent (3.82/83 mm./hr.) and hence the number average molecular weight ($M_n$) was determined:

\[
\frac{3.82}{83} \times 2.4 \times 10^{-3} = \frac{1.0039}{M_n} = \frac{98.996}{78} + \frac{1.0039}{M_n}
\]

\[ \therefore M = 7200 \]
FIG. 27  COLOURIMETRIC ESTIMATION OF
METHYLATED SUGARS.

KEY.

2,3,4,6 - ME$_4$- Glucose.  ●

2,3,6 - ME$_3$ - Glucose.  ○

(a) Hydrolysis. The methylated glucan (72mg.) was hydrolysed with 90% formic acid (4ml.) in an atmosphere of carbon dioxide in a sealed tube at 100° for 6 hours. The solution was cooled and neutralised by passage through a column of Amberlite IR 4B(OH) resin followed by concentration to a pale yellow syrup (62.7mg.).

(b) Estimation of the relative proportions of methylated sugars.

Standard solutions of methylated sugars were prepared as described in Expt.5. Accurately measured volumes of the control solutions were spotted on the starting line of a paper chromatogram and eluted for 24 hours in solvent D.

In order to compile calibration graphs, the methylated sugar spots were located by means of control strips and the sugar-bearing areas thus indicated were cut out and extracted with 0.90% (w/v) tetraethylene glycol dimethyl ether (5ml.) under reflux in boiling tubes for 20 minutes. To the eluate thus obtained was added aniline phthalate in methanol (1ml.) and the methanol then removed at 30° under reduced pressure. The residual liquid was heated at 98° for 35 minutes and the residue dissolved in 95% ethanol (10ml.). The absorbance of the sugar solutions was measured in a Unicam S.P.500 Spectrophotometer at 415 mλ for methylated aldohexoses, against ethanol as a blank. The graph (fig.27) of the absorbance against the weight of each sugar gave a linear relationship.
The relative molar proportions of the methylated sugars in the hydrolysate of the methylated glucan I were then estimated by the method described above, the weight of each sugar being obtained by reference to the graph.

**TABLE XIII**

*Relative proportions of the methylated sugars.*

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Absorbance</th>
<th>Wt. of sugar</th>
<th>Molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 2,3,6-tri-O-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>methyl glucose</td>
<td>0.790</td>
<td>275</td>
<td>14.6</td>
</tr>
<tr>
<td>2,3,4,6-tetra-O-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>methyl glucose</td>
<td>0.190</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>2. 2,3,6-tri-O-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>methyl glucose</td>
<td>0.700</td>
<td>190</td>
<td>16</td>
</tr>
<tr>
<td>2,3,4,6-tetra-O-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>methyl glucose</td>
<td>0.190</td>
<td>11</td>
<td>1</td>
</tr>
</tbody>
</table>

**EXPT.19. GAS-LIQUID CHROMATOGRAPHIC ANALYSIS OF THE METHANOLISISED METHYLATED GLUCANS I AND IIb.**

The methylated glucans (15mg.) were heated under reflux with dry methanolic hydrogen chloride (2%; 6ml.) until the rotation was constant (6 hours). The solution was allowed to cool after which it was neutralized with dry silver carbonate, filtered and the solution de-ionised with IR 120(H) and IR 45(CH) resins. After removal of the resins the solution was concentrated to a syrup. (ca. 11mg. in each case).
FIG. 28  GAS CHROMATOGRAMS OF METHYLATED GLUCANS.

Glucan I

Glucan IIb

Oak starch.
### TABLE XIV
Gas liquid chromatography of methylated glucans.

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>Methylated sugar</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Glucon I (fig. 28)</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Methyl 2,3,4,6-tetra-O-methyl-(\beta)-glucoside</td>
<td>1.37</td>
</tr>
<tr>
<td>2</td>
<td>&quot; &quot; &quot; &quot; - (\alpha) - &quot;</td>
<td>5.96</td>
</tr>
<tr>
<td>3</td>
<td>Unknown</td>
<td>3.81</td>
</tr>
<tr>
<td>4</td>
<td>Methyl 2,3,6-tri-O-methyl-(\beta)-glucoside</td>
<td>26.81</td>
</tr>
<tr>
<td>5</td>
<td>&quot; &quot; &quot; &quot; - (\alpha) - &quot;</td>
<td>61.82</td>
</tr>
<tr>
<td>6</td>
<td>A dimethyl glucoside (not 2,3)</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td><strong>Glucon IIb (fig. 28)</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Methyl 2,3,4,6-tetra-O-methyl-(\beta)-glucoside</td>
<td>5.76</td>
</tr>
<tr>
<td>2</td>
<td>&quot; &quot; &quot; &quot; - (\alpha) - &quot;</td>
<td>10.60</td>
</tr>
<tr>
<td>3</td>
<td>Methyl 2,3,6-tri-O-methyl-(\beta)-glucoside</td>
<td>27.30</td>
</tr>
<tr>
<td>4</td>
<td>Unknown</td>
<td>2.20</td>
</tr>
<tr>
<td>5</td>
<td>Methyl 2,3,6-tri-O-methyl-(\alpha)-glucoside</td>
<td>55.0</td>
</tr>
<tr>
<td>6</td>
<td>Methyl 2,3-di-O-methyl-(\beta)-glucoside</td>
<td>trace</td>
</tr>
</tbody>
</table>

Comparison of the relative retention times of each glucoside with those of control substances indicated the respectively methylated sugars.

A sample of a methylated starch from oak sapwood was examined for comparison (fig. 28) and it was noted that besides giving a larger proportion of dimethyl glucosides it also gave an unknown peak intermediate between the \(\alpha\)- and \(\beta\)- trimethyl isomers.
EXPT 20. SEPARATION AND CHARACTERISATION OF THE METHYLATED SUGARS IN THE HYDROLYSATE OF THE METHYLATED GLUCAN I

The remainder of the hydrolysed glucan syrup (60.0mg.) was applied to the starting line of a paper chromatogram (Whatman 3 MM; 20 x 40cm.) and eluted for 24 hours in solvent D. The sugars were located as usual by developing side-strips and the sugar-retaining areas thus indicated were cut out and eluted with distilled water containing 10% methanol (100ml.). Care was taken to avoid the inclusion of overlapping fractions where the separation was not complete.

The eluate obtained from each strip was concentrated under reduced pressure and investigated as follows:

Fraction I. Crystalline 2,3,4,6-tetra-O-methyl glucose (7.0mg.) chromatographically and ionophoretically identical with an authentic specimen run as a control. It had a m.p. and mixed m.p. 84°, while that of the derived anilide was 137° undepressed by admixture with authentic 2,3,4,6-tetra-O-methyl glucose anilide. (Irvine 1908).

Fraction II. Crystalline 2,3,6-tri-O-methyl glucose (35mg.) \([\alpha]^D_{D+68}\) (c, 1.43 in water). It was identical with an authentic specimen both chromatographically \((R_{G} 0.85)\) and on electrophoresis in borate buffer \((M_{G} 0.0)\). It had m.p. and mixed m.p. 115° after recrystallisation from ether. The derived diethyl mercaptal (Wolfson 1937) had m.p. 70°.

Fraction III. Syrup dimethyl glucose (3mg.), \([\alpha]^D_{D+58}\) (c, 0.33 in water) \(R_{G} 0.51\) (solvent D). Ionophoretically identical with 2,6-di-O-methyl glucose \((M_{G} 0.05)\) and containing a trace of 3,6-di-O-meth-

\(\text{glucosa (Mg 0.65).}\)
APPENDIX TO C. RUPESTRIS SECTION

The major part of section I deals directly with the separation and investigation of starch-type materials from the alkaline extract of C. rupestris and from the water-soluble material from E. compressa. The former and other extracts from C. rupestris were subjected to fractionation which are best discussed in an appendix. The investigation of the residual E. compressa polysaccharide forms section 3 of this thesis.

EXPT.21. ATTEMPTED FRACTIONATION OF POLYSACCHARIDE (F) USING CETAVLON

Polysaccharide (F) (page 55) (500mg.) was dissolved in warm distilled water (100ml.) a small insoluble residue being removed by filtration. To the filtrate was added 0.6 M boric acid (150ml.) and an aqueous solution of 0.1 N cetyltrimethylammonium hydroxide (40ml.) and 0.5 N sodium hydroxide (1ml.) with vigorous stirring. A flocculent off-white precipitate was obtained which was removed on the centrifuge, washed with water and immediately suspended with rapid stirring in 10% sodium chloride solution (100ml.) to decompose the complex. The solution thus obtained was dialysed for 3 days after which the polysaccharide was isolated by concentration and alcohol precipitation. (185mg.).

The supernatant solution at the centrifuging stage was likewise dialysed and alcohol precipitated after concentration (205mg.). Chromatographic examination (solvent B) of the sugars present in the hydrolysate of the two fractions indicated the same mono-
saccharides as in the parent polysaccharide (i.e. galactose, glucose, arabinose, xylose and rhamnose). A slight decrease in the relative amount of glucose in the precipitated material was obvious from visual examination, but no absolute fractionation was achieved.

**EXPT.22. ATTEMPTED FRACTIONATION OF POLYSACCHARIDE (F) USING FEHLING'S SOLUTION. (Chanda 1951).**

Polysaccharide (F) (3g.) was dissolved in water (100ml.) and mixed Fehling's solution (40ml.) added with vigorous stirring. The mixture was shaken for 12 hours but no copper complex precipitate was obtained. The copper solution was treated with dilute hydrochloric acid until colourless, then with two batches of acetone to 40% and 60% concentration respectively, the precipitates being removed on the centrifuge after each addition, and dried in alcohol and ether.

Chromatographic analysis (solvent B) of the hydrolysates of each fraction indicated the presence of the same sugars in approximately the same molar proportions. Hence it was concluded that no fractionation was achieved either by copper complexing or fractional precipitation.

**EXPT.23. PRELIMINARY INVESTIGATION OF POLYSACCHARIDE (B).**

(a) Polysaccharide (B) (page 54), the material obtained from the mother liquor after treatment of *C. rupestris* water-extracted residue with sodium chlorite at 70° was shown to have the physical properties related in Expt.2.
Investigation of the molar ratios of sugars present in a hydrolysate of polysaccharide (B) by the method of Wilson (1959) gave the following results:

**Table XV**

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Absorbance m(\mu)</th>
<th>Wt. of sugar m(\mu)</th>
<th>Molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactose</td>
<td>0.820</td>
<td>298</td>
<td>18.6</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.020</td>
<td>16</td>
<td>1.0</td>
</tr>
<tr>
<td>Arabinose</td>
<td>0.630</td>
<td>150</td>
<td>11.2</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.160</td>
<td>46</td>
<td>4.0</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>trace</td>
<td>-</td>
<td>trace</td>
</tr>
</tbody>
</table>

(b) Treatment of polysaccharide (B) with salivary \(\alpha\)-amylase.

The polysaccharide (260mg.) was dissolved in distilled water (25ml.) and fresh salivary \(\alpha\)-amylase added. The solution was treated with toluene and allowed to incubate at 37\(^\circ\)C for 24 hours. After a second 24 hour treatment with fresh \(\alpha\)-amylase the solution was heated at 75\(^\circ\)C for 5 minutes to destroy the enzyme, filtered and the polysaccharide precipitated with alcohol and dried with alcohol and ether (202mg.) (Found \([\alpha]_D^{\text{\(\text{D}\)}} +94^0, \text{N} \, 4\%, \text{SO}_4 \, 13.8\%, \text{ash} \, 15\%) 

Investigation of the molar ratios of monosaccharides in the hydrolysate of this material gave:-
TABLE XVI

Molar ratios of sugars in a hydrolysate of \( \alpha \) -amylase treated Polysaccharide (B)

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Absorbance (m( \mu ))</th>
<th>Wt. of sugar (( \mu )g)</th>
<th>Approximate molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactose</td>
<td>0.500</td>
<td>200</td>
<td>6</td>
</tr>
<tr>
<td>Glucose</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Arabinose</td>
<td>0.472</td>
<td>92</td>
<td>3.25</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.170</td>
<td>28</td>
<td>1</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>0.035</td>
<td>trace</td>
<td>trace</td>
</tr>
</tbody>
</table>

Thus salivary \( \alpha \) -amylase completely removed the glucose containing polysaccharide leaving a material containing the same sugars in approximately the same molar-ratios.

(c) Attempted fractionation of polysaccharide (B).

(i) Barium hydroxide (Meier 1958). To polysaccharide (B) (400mg.) dissolved in water (500ml.) was added a saturated solution of barium hydroxide (50ml.) dropwise with vigorous stirring. The mixture was shaken for 24 hours but no precipitation was achieved.

(ii) Potassium chloride (O'Neill 1955). When N potassium chloride was added to a rapidly stirred solution of the polysaccharide (500mg. in 100ml. water), no precipitation occurred even after standing for several days at 0°.

EXPT. 24. INVESTIGATION OF EXTRACTS (D) and (E).

Polysaccharide (D) extracted from C. rupestris using 18% sodium hydroxide was found to consist mainly of glucose with a
trace of xylose. The extract was obtained in poor yield and had a high ash content (ca. 35%) and was not further investigated.

The weed residue after exhaustive alcohol, water, chlorite and alkali treatments in turn, was subjected to the Monier-Williams (1921) treatment with 72% concentrated sulphuric acid for 36 hours followed by 12 hours hydrolysis with H acid at 100°. After neutralisation the hydrolysate was investigated chromatographically in solvent C when mainly glucose with a trace of xylose was detected.
DISCUSSION

The Cladophora species of green seaweeds is fairly common to British coastal waters, growing in relatively small colonies, often found in close association with some species of brown seaweeds such as Fucus. The weed is small and filamentous in structure and very dark green in colour.

Preliminary extraction with 80% aqueous alcohol (Laidlaw 1952) for several days was found to remove only some of the chlorophyll and colouring matter, while other solvents were found to be no more successful.

The water-soluble polysaccharide obtained from the alcohol extracted weed by hot-water extractions, was set aside, its structure having been investigated by Fisher (1957) and described in the introduction to this thesis. It should be noted however, that this material contained a glucon similar to laminarin there being no evidence for a starch-like polymer at this stage of the extraction.

Mild chlorite treatment of the weed residue after exhaustive aqueous extraction completely removed the remaining colouring matter (Expt.1); furthermore, ca. 7% of a polysaccharide (B) composed of galactose, glucose, arabinose, xylose and rhamnose in the molar ratios 18.6 : 1 : 11.2 : 4 : trace was extracted. This polysaccharide which was not fully investigated owing to shortage of time, showed $\left[\alpha\right]_D +100^\circ$, N 4% sulphate 13.6%, ash 14.78% and uronic acid ca. 5.0% (Swenson 1946). A preliminary
investigation of this polysaccharide (B) (Expt.23) indicated that the glucose could be completely removed by treatment with salivary α-amylase, and the polysaccharide then obtained was found to consist of the following sugars in the given ratios:

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactose</td>
<td>6.0</td>
</tr>
<tr>
<td>Arabinose</td>
<td>3.25</td>
</tr>
<tr>
<td>Xylose</td>
<td>1.0</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>trace</td>
</tr>
</tbody>
</table>

It had $[\alpha]_D + 94^{\circ}$, N 4.0%, sulphate 13.8%, and ash 15%.

Thus, this polymer appears to be similar in constitution to the water-soluble polysaccharide and must constitute the major hemicellulose fraction of the weed. Chlorite extraction also removes a small proportion of an α-1,4-linked glucan.

Subsequent alkaline extraction of the chlorite treated material yielded a polymer which gave a deep blue iodine stain indicative of a starch polymer. A preliminary analysis (Expt.2) of this material revealed the presence of much protein (ca. 35%), this difficulty being encountered by Fisher during his investigation of the water-soluble material. Trichloroacetic acid treatment considerably reduced this contamination yielding a polysaccharide (F) composed of the following proportions of sugars:

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactose</td>
<td>8.4</td>
</tr>
<tr>
<td>Glucose</td>
<td>34</td>
</tr>
<tr>
<td>Arabinose</td>
<td>4.3</td>
</tr>
<tr>
<td>Xylose</td>
<td>1.0</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>trace</td>
</tr>
</tbody>
</table>

Polysaccharide (F) had $[\alpha]_D + 57^{\circ}$, ash 6.56%, sulphate 1.85%, N 2.33% and uronic acid ca. 5.0%, this latter figure possibly being derived not from uronic acid but being due to pentoses since the estimation was carried out by decarboxylation with 19% hydrochloric acid.
The determination of the relative sugar ratios of the hydrolysed polysaccharide (Expt. 5) was carried out by the recent method of Wilson (1959) involving aniline phthalate. This method was found to be superior to that of Pridham (1956) with respect to reproducibility and stability of the coloured complex. Measurements could be made several hours after elution of the spots from the chromatogram, in contrast to the 30 minutes maximum for pentoses, as required by the method of Pridham. The Wilson method was also found to be preferable to the method of Piper and Bernardin (1957) employing o-amino-diphenyl, aniline phthalate being much cheaper and more easily obtainable.

The persistence of contaminating protein in these polysaccharide extracts was thought to indicate the possibility of some form of linkage between the polysaccharide and the protein molecule (cf. the blood group polysaccharides where it has also been found impossible to remove the protein without serious destruction of the polysaccharide molecule). It was therefore decided to proceed with investigations without further attempting to remove protein.

The strong alkaline (18%) extract (Expt. 24) appeared to consist of glucose and trace amounts of xylose, but the yield and ash content were such that an analysis would yield little structural information. The final residual weed, however, after the Monier-Williams (1921) treatment revealed glucose only, thus confirming the work of Cronshaw, Myers and Preston (1958) in which X-ray examination of this weed residue gave a diagram typical of cellulose I.
In several cases separation of a glucose-rich material has been achieved from a parent polysaccharide by acetylation and chloroform extraction of the acetate (Fisher 1957, O'Donnell 1959) and this method was applied to polysaccharide (F) in an attempt to isolate a pure glucan (Expt.6). Three acetylations of the polysaccharide followed by chloroform extraction of the acetate produced a pure white material which had \([\alpha]_D +160^\circ\). A considerable amount (ca.30%) of a dirty brown residue remained after this extraction, probably mainly protein since the glucan ultimately separated had a very much reduced nitrogen content.

Deacetylation of the polysaccharide acetate by the method of Bonner (1949) yielded a pure white polysaccharide (glucan I) in 0.5% yield based on the initial weight of weed. This material was shown to contain only glucose on chromatographic investigation of a hydrolysate.

A subsequent investigation of the polysaccharide obtained by acetylation and chloroform extraction showed it to be a relatively degraded starch polymer with certain unusual properties. It was hoped that the less vigorous conditions of iodine precipitation might produce a starch more representative of the natural material present in the Cladophora spp.. Application of this technique (Expt.7), first used by Steiner (1944) in the isolation of pure tuber starches, was found to yield 7.2% of a starch (Glucan II) from Cladophora polysaccharide (F) (ca. 0.15% based on the dry weight of weed).
It was also found possible to isolate a starch polymer (Glucan III) from the water-soluble polysaccharide of *E. compressa* (Expt. 8), this material being obtained in 3.5% yield based on the water-soluble material (G). The method appeared to function reasonably well although it has been suggested that the starch polymer should constitute not less than 10% of the polysaccharide mixture.

The amylose of starches can be separated from the amyllopectin as an insoluble complex by the addition of an alcohol of low solubility such as butanol (Schoch 1942) or thymol (Haworth 1946) to an aqueous solution of the starch; an amylose-alcohol complex forms and slowly precipitates from solution, leaving the soluble amyllopectin in the supernatant.

Application of this technique to the two glucans (I and II) from *Cladophora* and the glucan (III) from *E. compressa* yielded complexes (IIa and IIIa) from samples II and III and, in addition, polysaccharides (IIb and IIIb) were precipitated from the respective supernatants (Expt. 9). It should be realized, however, that the failure to complex with thymol does not indicate the absence of an amylose fraction but may mean that the molecular size of the amylose is too small to form an insoluble complex. On the other hand, inability of Glucan I to form a complex is not surprising. The conditions necessary for its extraction and purification would most certainly degrade any amylose present in the natural polysaccharide and this degraded material would then probably be of a size and shape to pass through the dialysis sac.
during purification. Unfortunately omission of dialysis and isolation by precipitation with alcohol and ether invariably produced lower yields of more highly contaminated polysaccharide.

The properties of glucans I, IIa, IIb, IIIa, IIIb are shown in Table V (p. 61). It is seen that all the specific rotations are similar and somewhat low for a true starch \( [\alpha]_D^{20} +20^\circ \). Cuprimetric estimation of the purity of each sample indicated a certain degree of contamination in all samples, but the small amounts of polymer available discouraged further purification.

Glucan IIIb was soluble in water and gave a purple colour with iodine, similar to that exhibited by amylopectin, the branched fraction of the starch macro-molecule, whereas glucans I-IIIa all showed a reluctance to dissolve in water but were readily soluble in dilute alkali; they produced a deep blue colour when treated with iodine, resembling a normal starch.

Chromatographic analysis of a hydrolysate of the polysaccharide in each case revealed only glucose, this product being characterised by its conversion by oxidative degradation to gluconic acid. (Expt. 10).

The most characteristic reaction of any starch is its ability to form a coloured complex with iodine. The significant difference in the degree to which amylose and amylopectin bind iodine has long been a major factor in their distinction.

Apart from the obvious colour difference exhibited, the wavelength of maximum absorption of the complex is 515-550m\( \mu \) for
It has been suggested that the complex with iodine and amylose or amylopectin can be ascribed partly to the absorption of iodine molecules or triiodide ions and partly to the endwise axial arrangement of iodine molecules inside a series of helices of $\alpha$-1,4'-linked glucose residues (Higginbotham 1949). Each coil of the helix is believed to contain six glucose residues and one iodine molecule and it is most probable that a minimum of three coils are necessary to produce a stain. Thus an amylose type chain must contain eighteen or more glucose units. This has recently been substantiated by Bailey and Whelan (1961) who have shown, with synthetic amyloses, that the chains become iodine-staining when they are 18 units long. Thereafter, the blue value increases rapidly and linearly with the chain length until about 72 units at which point the rate of increase falls to about 5% of the initial value. The blue value of naturally occurring amyloses (ca. 1.4) is not reached until the chains exceed 400 units.

The wavelength of maximum absorption of the glucans was studied under standard conditions for the presumed amylopectin fractions (IIIb), and under "Blue-value" conditions for amyloses (I-IIIa) since the latter gave solutions which were too dark to measure under amylopectin conditions, (Expt. 11). The following results were obtained:

<table>
<thead>
<tr>
<th>Glucan I</th>
<th>IIa</th>
<th>IIb</th>
<th>IIIa</th>
<th>IIIb</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_{\text{max.}}$</td>
<td>610</td>
<td>610</td>
<td>600</td>
<td>610</td>
</tr>
</tbody>
</table>

It is apparent, therefore, that while IIIb definitely resembles
an amyllopectin, glucans I-IIIa display $\lambda_{max}$ more typical of small-chain amylose of the type described by Bailey and Whelan (1961), having a D.P. between 100 and 180 glucose residues. As far as the author is aware no reports of the value of $\lambda_{max}$ for degraded amyllopectins have been forwarded.

Similar results were indicated by the values obtained under the conditions of Bourne (1948) for the "Blue-value":

<table>
<thead>
<tr>
<th>Glucan</th>
<th>I</th>
<th>IIa</th>
<th>IIb</th>
<th>IIIa</th>
<th>IIIb</th>
</tr>
</thead>
<tbody>
<tr>
<td>B.V.</td>
<td>0.800</td>
<td>0.475</td>
<td>0.800</td>
<td>0.660</td>
<td>0.140</td>
</tr>
</tbody>
</table>

The B.V. of a true amylose is expected to be ca. 1.4 while of an amyllopectin is of the order 0.1 - 0.15. Although IIIb again has the correct value for an amyllopectin, the other four samples have blue values which are intermediate between a typical amyllopectin and a true amylose.

Estimation of the iodine binding power of Glucans I and IIIa (Bates 1943) gave values of 1.05% and 10.47% of bound iodine respectively. Schoch (1949) has found the iodine affinity for several amyloses to vary between 18.5 and 20.0% and an average value of 19.2% is often accepted as the percentage binding power of an amylose as compared with ca. 0.5% for an amyllopectin. On this basis Glucans I and IIIa have binding powers well below those of a typical amylose but many factors including molecular weight and its distribution, may influence these results.

In view of the limited supply of material and for reasons which will be discussed later it was decided to reserve the remaining portion of fraction IIIb for methylation studies.
The limiting viscosities of the glucans detailed below were measured in molar potassium hydroxide solution (Expt.13), the following values being obtained:

<table>
<thead>
<tr>
<th>Glucan</th>
<th>I</th>
<th>IIa</th>
<th>IIIa</th>
<th>IIIb</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[\eta]$</td>
<td>20.0</td>
<td>26.9</td>
<td>44.0</td>
<td>35</td>
</tr>
<tr>
<td>D.P.</td>
<td>192</td>
<td>324</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

From a consideration of the limiting viscosities of amylose and amyllopectin from other sources (ca. 400 and 150 respectively) it is obvious that all these samples investigated are much degraded molecules.

Cowie and Greenwood (1957) have shown the approximate relationship, \( \text{D.P.} = 7.4 \times \text{limiting viscosity} \), to hold for potato amyloses of reasonable chain length and this equation has been applied to the "supposed" amyloses separated from the seaweed glucans as shown above. It can be readily seen that they are very much smaller than the highly purified amylose of potato D.P.3,200, but amylose IIIa shows the same order of magnitude as that reported for amylose preparations from maize (ca.340) (Nussebaum 1951), malted barley (ca.330) (Aspinall 1959) and protozoal amylose from Chilomonas paramecium (ca.300) (Archibald 1960). In view of the methods of extraction of the glucan samples from \textit{C. rupestris} (involving Chlorite at 70° and alkali) it is not surprising as already mentioned that they are more degraded than the hot-water
extracted Glucan IIIa from *E. compressa*. The probability of
degradation is further enhanced by the necessity of trichloroacetic
acid purification of the original extracts.

It should be mentioned here that the limiting viscosity
number of amylose and amylopectin is dependant upon the shape
rather than the size of the molecule. Peterkin (1950) has shown
by viscosity measurements on amylose acetate that the results
for a linear molecule can be interpreted in terms of a random coil,
rather than the rigid rod advocated by some workers. The lower
intrinsic viscosity exhibited by amylopectin on the other hand,
indicates a more compact and probably spherical molecule, although
viscosity measurements on the $\beta$ -limit dextrin indicate that an
elongated molecule is possible. (Greenwood 1956).

The hydrolysis of a polysaccharide by a purified and tested
enzyme preparation has the advantage of specificity, and the
degradative action may follow paths which cannot be covered by
present chemical methods. It is essential to ensure that the
enzyme preparation is free from contaminating material. For
example, salivary $\alpha$ -amylase is often contaminated to a slight
extent with maltase which specifically hydrolyses maltose to
glucose, consequently its presence increases the $F_M$ value (apparent
conversion to maltose). The $\alpha$ -amylase used in these experiments
was produced by Nutritional Biochemicals Corp., and was free of
maltase activity.

Digestion of the glucan samples under investigation with
salivary $\alpha$ -amylase gave the following results. (Expt.14):
Control experiments with Kerr's Pink potato amylose and amylopectin gave $F_m$ values of 93 (theory requires 100) and 88 respectively. Thus glucan IIIb shows a result typical of an amylopectin. It is difficult to deduce anything of the finer details of structure of the other glucans from this experiment because of their tendency and also that of K.P. amylose to retrograde and precipitate out of solution during incubation with the enzyme, even under the conditions suggested by Meyer (1940). Nevertheless it does provide proof that all the glucans under investigation possess a basic starch-type structure comprising mainly $\alpha$-1,4- linked glucose units.

The general observation that purified $\beta$ -amylase causes only ca. 70% degradation of amylase has been confirmed by many workers (Neufeld 1955; Cowie et al 1957, 1958, and Eddy 1958). However, it is accepted that amorphous preparations of soya-bean $\beta$ -amylase cause ca. 100% conversion, the difference being accounted for by the presence of Z-enzyme (Peat 1952). The $\beta$ -amylase used in the present experiments was prepared by Wallerstein Laboratories and had Z-enzyme but no maltase activity (Expt.14). The results obtained were :

<table>
<thead>
<tr>
<th>Glucan</th>
<th>I</th>
<th>IIa</th>
<th>IIIa</th>
<th>IIIb</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F_m$</td>
<td>78</td>
<td>92.5</td>
<td>85.1</td>
<td>90.11</td>
</tr>
</tbody>
</table>

| pH 3.6 | 49.1 | 51.0 | 31.4 | - |
| pH 4.6 | 63.1 | 66.2 | 76.0 | 58 |
At pH 3.6 the Z-enzyme action is inhibited.

Glucan IIIb again shows a conversion similar to that obtained from the majority of amylopectins, i.e., 55%. Once again the difficulty of retrogradation was encountered for the other samples, especially at pH 3.6 but in keeping with many of their other properties they appear to have a $\beta$-amylolysis limit intermediate between an amyllose and an amylopectin. At the same time these results also support a basic $\alpha$-1,4'-linkage. It should be pointed out here that the treatment with chlorite prior to extraction besides degrading the starch-type polysaccharide, very probably also oxidises some of the residues in the molecule to oxo- or carboxy-units and the presence of such anomalous residues may be the explanation for the low amylolysis figures in these materials rather than a property of the native starch.

In aqueous solution, periodate will oxidise compounds containing $\alpha$-$\beta$-diol or $\alpha$-$\beta$-$\gamma$-triol groups with the formation of aldehydes, iodate and in certain cases formic acid, formaldehyde and carbon dioxide. The arrangement of diol or triol ($\alpha$-glycol) groups may be deduced from measurements of the reduction of periodate and the nature of the oxidation products. Thus the oxidation of a 1,4'-'linked glucose polymer involves the reduction of one mole of periodate for every unit in the polymer except at branch points and of two moles of periodate and the release of one mole of formic acid from each non-reducing end-group. Furthermore, if conditions are such that oxidation proceeds beyond the normal Malapradian oxidation with the hydrolysis of formyl esters then
instead of the reduction of one mole of periodate, one mole of formaldehyde and two moles of formic acid are released, with the reduction of three moles of periodate at the reducing end of the molecule. Hough and Perry (1956) have shown that this reaction will occur readily at pH 8.0, and that 1,3'- or 1,4'-linked aldohexose polymers are completely oxidised by a stepwise process with production of 1 mole formaldehyde per hexose residue. Oxidation under these conditions also makes it possible to detect the presence of 1,6- linkages which arrest the oxidation in a 1,3- or 1,4- linked polymer. Over-oxidation of this type occurs if the oxidation is carried out in the presence of a large excess of oxidant, at an elevated temperature (＞20°), in alkaline solution or in sunlight (Head and Hughes 1952). A careful control of the oxidation conditions is therefore essential.

Acid hydrolysis of the periodate-oxidised polysaccharide is a useful procedure for the identification of monosaccharide residues devoid of L-glycol groupings. (Hirst 1948). Thus glucose can be detected in the hydrolysate of the residual oxo-polysaccharide of a 1,3'- linked glucan oxidised under controlled conditions. A model starch should therefore reduce one mole of periodate for every anhydro-glucose unit if the reaction is carried out under carefully controlled conditions. In the present experiment (Expt.15) the following results were obtained on oxidation:

<table>
<thead>
<tr>
<th>Glucan</th>
<th>I</th>
<th>IIa</th>
<th>IIIa</th>
<th>IIIb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periodate reduced/06 anhydro unit</td>
<td>1.46</td>
<td>0.682</td>
<td>1.08</td>
<td>0.900</td>
</tr>
</tbody>
</table>
In spite of the standardised conditions it appears that over-oxidation has occurred in the case of Glucan I, whereas IIIa and IIIb have consumed ca. one mole per hexose residue as would be expected of an amylose or amylpectin. The low result obtained for IIa is not in keeping with the other glucans and cannot be explained by under-oxidation since no indication of unattacked glucose was obtained from this or any of the other oxopolysaccharides.

The micromethylation technique of Isbell (1957) was applied to glucan sample I (Exp. 15) and was found to have several advantages compared with the macro-method of Muskat (1934). Apart from the obvious advantage of the relatively small quantity to which the method is applicable, the fact that methylation is carried out under reduced pressure and hence virtually in the absence of oxygen, avoids oxidation of the potassium and thereby facilitates the reaction; furthermore, the polysaccharide is less likely to suffer degradation in the absence of oxygen, especially since it is known that sodamide and potassamide will degrade polysaccharides, (Hodge 1951). Finally, the reaction product does not have to be removed from the vessel after each methylation, thereby avoiding manipulative loss.

On completion of the methylation it was found difficult to effect complete removal of the filter-ocel from the methylated product. However, indication of the degree of methylation was obtained from the sugars found in the hydrolysed methylated glucan, the presence of only a trace of mono-methylglucose being
noted. The yield of methylated product was high (75%) in comparison with other methods but it must be remembered that a small proportion of filter-cel was present. The product was soluble in chloroform, methanol, benzene and to a lesser extent in water. The methylated product gave no blue colour with iodine in common with other amylopectins.

Measurement of the molecular weight of the methylated glucan I was carried out in benzene solution by the Isothermal Distillation method (Broatch 1956) (Expt.17). Such measurements are based on the rate of transfer of solvent to a solution of the polysaccharide under investigation, this being dependant on the molecular weight of the solute. It has been found that the method is best applied to benzene-soluble materials which have molecular weight within the range 1,000-20,000. In the present investigation on Glucan I a molecular weight of 7,200 was obtained, hence confirming that the algal starch has a very small molecule or more probably extensive degradation of the molecule had occurred.

Methanolysis of the methylated glucan I (Expt.19) produced a mixture of methyl glycosides of the methylated monosaccharide components. Analysis of these by gas-liquid chromatography on a polyester column at 200° very kindly carried out by Dr. Merz provided evidence for the following proportions of methylated sugars:—
Formic acid hydrolysis of the remainder of the methylated glucan I produced 2,3,4,6-tetra-O-methylglucose (1 part) and 2,3,6-tri-O-methylglucose (ca. 15 parts) (Schaeffer and van Cleve 1956) giving a ratio of tetra/tri methylglucoses of 1/15 a result within experimental error of that obtained by gas chromatographic analysis. Hence, we have further evidence that this is a relatively short chain molecule.

Since isothermal distillation gave a D.P. of ca. 45 glucose units for this material it follows from the proportion of tetra- to tri-O-methylglucose in the methylated hydrolysate that there must be a small degree of branching in this glucan. However, the apparent complete absence of 2,3-di-O-methyl glucose, the derivative obtained from the branch points of a methylated amylopectin, appears to contradict this conclusion. At the same time it must be pointed out that an amylose with a D.P. of 16 would not have the iodine staining properties of the glucan in question and it can only be concluded that some type of branching is present. It is true that gas chromatographic analysis of the methylated hydrolysate reveals ca. 4% of an unknown constituent which might conceivably be the units at the branch points of this
polysaccharide. This, however, does not seem to be very probable since the methylated methanolysate of oak sapwood starch gives an identical peak of unknown material on a gas chromatogram. This material could not be detected in an acid hydrolysate of the glucan and must therefore be there as a non-reducing substance or a derivative labile to acid.

In order to confirm the absence of 2,3-dimethyl-glucose in the methylated hydrolysate of the algal glucan the presumed amyllopectin IIb fractionated from Cladophora glucan II was subjected to methylation under conditions detailed for glucan I. Analysis by gas-liquid chromatography of the methanolysate of the derived material on a polyester column at 200° by the kindness of Dr. Aspinall gave the following proportions of methylated sugars:

- \(2,3,4,6\)-tetra-\(\alpha\)-methylglucose (ca. 16 parts)
- \(2,3,6\)-tri-\(\alpha\)-methylglucose (ca. 82 parts)
- \(2,3\)-di-\(\alpha\)-methylglucose (1 part)
- Unknown (2 parts)

This second methylation confirms the results of glucan I. Although a trace of \(2,3\)-di-\(\alpha\)-methylglucose is present in this second methylated material it is not nearly sufficient to accommodate the very high proportion of tetra-\(\alpha\)-methylglucose. It is more noteworthy that in this second methylated material the proportion of unknown material appears to have decreased approximately to the extent of the appearance of the \(2,3\) di-methyl derivative. It is tempting to conclude that there is some connection between the two and that both occur at the
branch points of the molecule. A final decision on this point must however, await characterisation of the unknown material.

While it is not possible to say with certainty whether the small proportion of 2,6- and trace of 3,6-dimethylglucoses in the hydrolysate of methylated glucan I arise from demethylation during hydrolysis or undermethylation, it is considered that the latter possibility is more likely. The same explanation may be applied to the occurrence of trace quantities of monomethylglucose.

2,3,4,6-tetra-0-methylglucose and 2,3,6-tri-0-methylglucose were obtained crystalline. The former was identified by chromatographic and ionophoretic mobility, melting point and preparation of the derived anilide, the latter by chromatographic and ionophoretic mobility, rotation, melting point and formation of the diethyl mercaptal derivative.

The high proportion of 2,3,6-tri-0-methylglucose in both methylated glucans further confirms the presence of 1,4'-linkages in the molecules.

The isolation of starch-type polysaccharides from *Gladophora rupestris* and *Enteromorpha compressa* is in keeping with accumulating evidence that this polymer is the food reserve material of the green algae, as discussed in the introduction to this section.

However, before a true estimation of the fine structure of
these polysaccharides can be made, it is evident that the techniques for their isolation must be improved. A variety of methods have been reported for extracting starches from other sources, such as 0.01 M mercuric chloride (Banks 1959; Greenwood 1959); aqueous leaching after liquid ammonia pretreatment (Greenwood 1961); perchloric acid digestion (King 1960), dimethyl sulphoxide extraction (Haaglund and Lindberg 195); and chloral hydrate extraction (Meyer 1940). The latter two methods were unsuccessful when applied to *E. compressa*. Much depends, naturally, on degree to which the starch polysaccharide is bound in the structural matrix of the plant. Thus, while tuber starches are readily separated from extraneous material in a relatively undegraded form starch polysaccharides in the algae invariably require fractionation from protein and other polysaccharides. One feature, for example, in the case of *C. rupestris* starch is the fact that it apparently cannot be extracted until after the material has undergone chlorite treatment, an indication of the manner in which it must be held within the cell walls. Thus it appears that until a method of cleaving the cell walls without degrading the starch has been found then it will always be degraded to a greater or lesser extent on extraction, as is evident from the structural results obtained. In the *C. rupestris* starch, however, there are many anomalies in the structure. That these can all be attributed to the drastic methods necessary for the isolation of the material is not at all certain. The apparent fractionation of glucan II into an amyllose and an amylopectin type molecule is not substantiated by their properties and
appears to be more probably a separation of polymers of similar constitution but different size.

The iodine binding properties are more in keeping with those of a degraded amylose-type molecule, whereas the enzymic experiments denote something between an amylose and amyllopectin and this is supported by methylation studies, which indicate small molecules of $\alpha-1,4'$-linked glucose molecules with some branch points of unknown constitution. While emphasis in this discussion has been laid on the probable alteration in constitution during extraction of these materials, it is significant that although Glucan II was separated from other polysaccharide material under milder conditions than Glucan I, there is no outstanding difference in their properties and it may be that this algal polysaccharide is different from a true starch.

The _E. compressa_ starch, on the other hand, is much more readily extracted, but protein contamination of the mixture of polysaccharides obtained was reduced by trichloroacetic acid treatment, and thus it is evident that some degradation has occurred. This polysaccharide much more closely resembles the starch of land plants comprising both an amylose and an amyllopectin fraction. The amylose fraction represents 28% of the whole starch, which is in keeping with the amylose content of most starches (ca. 25%), but very much lower than the values reported for starches from wrinkled pea (Senti 1959) and corn (Wolff et al 1955). While the amyllopectin has the characteristic iodine staining and amyllopectin properties of a land plant amyllopectin, it is unfortunate that time did not permit methylation studies of this material.
SECTION II
SECTION II

A. THE ACID FRACTION OF ULVA LACTUCA

II(a) INTRODUCTION

The stability of the uronosyl linkage in acid-containing polysaccharides is such that even after vigorous acid hydrolysis there remains a fraction which is incompletely hydrolysed. This fraction consists of aldobiuronic acids together with higher oligouronic acids and neutral sugars. Isolation of such acids and elucidation of their structure has provided considerable structural information regarding the mode of linkage present in the undegraded polymer. This technique has been applied with success to the structural analysis of the plant gums and mucilages. D-galacturonic, D-glucuronic and 4-O-methyl-D-glucuronic acids emerge as the most common acid components of gums and some of the neutral sugar residues to which they are linked is evident from Table XVII.

The presence of uronic acids is common in algal polysaccharides especially in the Phaeophyceae and Chlorophyceae. The occurrence of alginic acid, a polymer consisting of D-mannuronic and L-guluronic acids in certain species of brown seaweeds is characteristic of those sources and has been described earlier in the introduction to this thesis. Whereas these uronic acids have polymerised to form a major polysaccharide component in the Phaeophyceae, the same is not true of the polysaccharides of the Chlorophyceae and Rhodophyceae. Uronic acid containing polysaccharides have been
### Table XVII

Aldobiuronic acids from some plant gums and mucilages.

<table>
<thead>
<tr>
<th>Aldobiuronic acid</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha \text{-D-Gp A(1\to2)-D-Xylp}$</td>
<td>Corn (Zea mays) cob hemic.B.</td>
<td>Whistler 1953.</td>
</tr>
<tr>
<td>$\alpha \text{-D-Gp A(1\to3)-D-Xylp}$</td>
<td>Wheat straw</td>
<td>Bishop 1953; Roudier 1953.</td>
</tr>
<tr>
<td>$\alpha \text{-D-Gp A(1\to4)-D-Galp}$</td>
<td>Grapefruit gum</td>
<td>Connell 1950a</td>
</tr>
<tr>
<td>$\beta \text{-D-Gp A(1\to6)-D-Galp}$</td>
<td>Acacia Karroo gum</td>
<td>Charlson 1955</td>
</tr>
<tr>
<td></td>
<td>Gum arabie</td>
<td>(Challinor 1931</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Butler 1929</td>
</tr>
<tr>
<td></td>
<td>Black wattle gum</td>
<td>Stephen 1951</td>
</tr>
<tr>
<td></td>
<td>Acacia Karroo gum</td>
<td>Charlson 1955</td>
</tr>
<tr>
<td></td>
<td>Gum ghatti</td>
<td>Aspinall 1955</td>
</tr>
<tr>
<td>$4\text{-O-Me-}\alpha \text{-D-Gp A(1\to2)-D-Xylp}$</td>
<td>Corn (Zea mays) cob hemic B.</td>
<td>Whistler 1954.</td>
</tr>
<tr>
<td>$4\text{-O-Me-}\alpha \text{-D-Gp A(1\to6)-D-Galp}$</td>
<td>Mesquite gum</td>
<td>White 1947; Cunneen 1948</td>
</tr>
<tr>
<td>$4\text{-O-Me-}\alpha \text{-D-Gp A(1\to4)-D-Galp}$</td>
<td>Khaya grandifolia</td>
<td>Aspinall 1956.</td>
</tr>
<tr>
<td></td>
<td>Mesquite gum</td>
<td>Cunneen 1948</td>
</tr>
<tr>
<td>$\alpha \text{-D-Galp A(1\to2)-L-rhamn.}$</td>
<td>Flax seed</td>
<td>Anderson 1930</td>
</tr>
<tr>
<td></td>
<td>Slippy elm</td>
<td>Tipton 1929; Gill 1946</td>
</tr>
<tr>
<td></td>
<td>Khaya grandifolia</td>
<td>Aspinall 1956.</td>
</tr>
<tr>
<td>$\alpha \text{-D-Galp A 1 D-Xylp.}$</td>
<td>Plantago seed</td>
<td>Nelson 1942</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hostettler 1951.</td>
</tr>
</tbody>
</table>
reported to occur in *U. lactuca* (Brading 1954), *E. compressa* (Lowe 1956), *A. centralis* (O'Donnell 1959) and *D. edulis* (Dillon 1950) and in all these cases the uronic acid, which is D-glucuronic acid, is incorporated in a heteropolysaccharide containing at least two other monosaccharides and constituting not more than 20% of the polymer. Thus, for example, it is found along with xylose and rhamnose residues in the water-soluble extracts of *E. compressa* and *U. lactuca* to the extent of 16-18% and although no direct evidence of the position of the glucuronic acid in the polymer has been obtained indications are that at least some of those occupy end group positions. Owing to the stability of the uronosyl link, under the drastic conditions necessary for hydrolysis glucuronic acid is degraded as rapidly as it is liberated and therefore no report of the isolation of the acid or its lactone has been given.

The isolation of 4-O-β-D-glucuronosyl-L-rhamnopyranose by O'Donnell (1959) from the acid hydrolysate of the water-soluble extract from *A. centralis* (*Spongomorpha arctica*) was the first report of the separation and characterisation of an aldobiuronic acid from a green algal polysaccharide. The acid, had high chromatographic mobility (Rf 1.0 cf. 2-O-D-glucuronosyl-L-rhamnose Rf 0.24) and it seemed likely that in the free state it existed as the lactone. Evidence in favour of this presumption was attained by the production of a second slower spot on treatment of the acid with ammonia prior to elution on a paper chromatogram. Its constitution
was established by reduction and hydrolysis of the glycoside ester which afforded equimolar proportions of glucose and rhamnose, while hydrolysis of the methylated disaccharide glycoside yielded crystalline 2,3,4,6-tetra-0-methyl glucose and syrupy 2,3-di-0-methyl rhamnose. Periodate uptake by the glycoside was in accordance with these results and in agreement with the above structure.

In view of the similarity in the constituents of the water-soluble extracts from \textit{Aerosiphania centralis}, \textit{Ulva lactuca} and \textit{Enteromorpha compressa}, it was decided to investigate the acid fractions of the latter two materials and the next part of this thesis describes the isolation and characterisation of aldobiouronic acids from partial acid hydrolysates of the water-soluble extracts from these two green seaweeds.

II b EXPERIMENTAL

EXPT.25. EXTRACTION OF THE ACID POLYSACCHARIDE.

Two batches of \textit{Ulva lactuca} were collected at Kames Bay, Millport in May 1958 and June 1959, total dry weight 600g. The weed was extracted with 85\% aqueous acetone at room temperature for 24 hours to yield practically colourless tissue. In a pilot experiment the weed was given a preliminary dimethyl sulphoxide treatment prior to 85\% acetone extraction, but this was found to remove polysaccharide material and was not repeated on the larger bulk of weed. That the dimethyl sulphoxide extract contained all
the monosaccharides present in the water-soluble polysaccharide hydrolysate (i.e. glucose, xylose, rhamnose and uronic acid) was shown by chromatographic analysis in solvent A.

The acetone-extracted residual weed was extracted with cold distilled water (4 x 2l.), the combined extracts being dialysed and concentrated. The concentrated solution, which formed a stiff gel on standing, was freeze dried yielding an off-white flaky solid (35.9g., 6%).

**EXPT.26. PROPERTIES OF THE WATER-SOLUBLE POLYSACCHARIDE.**

The above water-soluble polysaccharide material had the following properties:

\[ [\alpha]_D^{50^\circ} (c, 1.1 \text{ in water}) \text{ ash, 16.0\%}, \text{ total sulphate 18.2\%}, \]

\[ N 0.43% \text{ Uronic acid, as calculated from the yield of carbon dioxide on decarboxylation 15.9\% (Swenson 1946).} \]

Chromatographic analysis (solvent A) of an acid hydrolysate of this material indicated the following sugars: Glucose, xylose, rhamnose and a number of oligosaccharides.

**EXPT.27. EQUIVALENT WEIGHT DETERMINATIONS.**

The polysaccharide (171.7mg.) was dissolved in distilled water (10ml.) and passed through a column of Amberlite IR 120(4) resin six times. The resultant acid solution (pH 2-3) was freeze dried to a white powder (ash 1.9\%, sulphate 18.2\%). The equivalent weight was then determined by dissolving samples (20mg.) in water (5ml.) and titrating against 0.0123N sodium hydroxide solution using phenolphthalein indicator. The mean of several determinations gave a value of 313g. for the free-acid
polysaccharide.

EXPT.28. SMALL SCALE HYDROLYSIS OF THE POLYSACCHARIDE.

The polysaccharide (0.5g.) was hydrolysed at 100° with N sulphuric acid (40ml.) until a constant rotation was achieved:

<table>
<thead>
<tr>
<th>Time</th>
<th>0</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\left[\alpha\right]_d$</td>
<td>-52.3°</td>
<td>-28.5°</td>
<td>-23.0°</td>
<td>-17.6°</td>
<td>-7.6°</td>
<td>3.7°</td>
<td>4.0°</td>
<td>4.1°</td>
</tr>
</tbody>
</table>

The solution was cooled and neutralised by the addition of barium hydroxide then barium carbonate, the precipitate being filtered off. The filtrate was shaken with IR 120(H) resin and, after removal of the resin, was concentrated to a syrup. Chromatographic investigation in solvent A revealed the presence of rhamnose, xylose, glucose and slower spots; investigation in solvent C, spraying with solution 3 indicated three acidic spots with $R_{Gal,Acid}$ 1.06, 0.52 and 0.27.

EXPT.29. LARGE SCALE SEPARATION OF AN ALDABIURONIC ACID FROM THE WATER-SOLUBLE POLYSACCHARIDE.

The water-soluble polysaccharide (10g.) was hydrolysed with N sulphuric acid (300ml.) at 100° for 5.5 hours. The hydrolysate was cooled and neutralised with barium hydroxide and barium carbonate, filtered and concentrated to a syrup (ca. 8.4g.).

The syrup was applied to the top of a cellulose column (4 x 50cm.) and the neutral sugars (6.05g.) were eluted with a solution of redistilled butan-1-ol half saturated with water (ca. 161). The eluate finally gave a negative Molisch test.

The column was then eluted with butan-1-ol : acetic acid : water (2 : 1 : 1), and fractions (20ml.) collected on a fraction
cutter. Every 5th tube was analysed by paper chromatography (solvent C) like fractions being combined.

**TABLE XVIII**

Separation of acids from U. lactuca on cellulose column.

<table>
<thead>
<tr>
<th>Tubes</th>
<th>Fraction</th>
<th>R_{Gal,Acid}</th>
<th>Colour of Spot</th>
<th>Weight (mg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-49</td>
<td>I</td>
<td>1.06</td>
<td>Yellow brown</td>
<td>892.0</td>
</tr>
<tr>
<td>50-62</td>
<td>II</td>
<td>1.06; 0.53</td>
<td>&quot;</td>
<td>190.0</td>
</tr>
<tr>
<td>63-95</td>
<td>III</td>
<td>1.06; 0.53</td>
<td>&quot;</td>
<td>23.1</td>
</tr>
<tr>
<td>96-120</td>
<td>IV</td>
<td>0.53</td>
<td>Red brown</td>
<td>12.6</td>
</tr>
<tr>
<td>131-170</td>
<td>V</td>
<td>0.53; 0.27</td>
<td></td>
<td>18.3</td>
</tr>
<tr>
<td>171-200</td>
<td>VI</td>
<td>0.53; 0.27</td>
<td>trace.</td>
<td></td>
</tr>
</tbody>
</table>

In a second separation the syrup (9.4g.) as the free acid mixture was pipetted on to a column (4 x 50cm.) containing Amberlite CG-45 resin in the formate form. The neutral sugars (7.30g.) were removed by eluting with water until the eluate gave a negative Molisch test. The acid sugars were then removed from the column by gradient elution with formic acid (0-2N). The fractions were collected as before, every 5th tube being analysed chromatographically (solvent C), like fractions being combined.
TABLE XIX
Separation of acids from U. lactuca, on CG-45 resin column.

<table>
<thead>
<tr>
<th>Tubes</th>
<th>Fraction</th>
<th>R\textsubscript{Gal. Acid}</th>
<th>Colour of Spot</th>
<th>Weight (mg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-50</td>
<td>I</td>
<td>1.06</td>
<td>Yellow brown</td>
<td>765.0</td>
</tr>
<tr>
<td>51-120</td>
<td>II</td>
<td>1.06; 0.53</td>
<td>Yellow &amp; red brown</td>
<td>142.5</td>
</tr>
<tr>
<td>121-170</td>
<td>III</td>
<td>0.53</td>
<td>Red brown</td>
<td>51.4</td>
</tr>
<tr>
<td>171-200</td>
<td>IV</td>
<td>0.53; 0.26</td>
<td>Red brown</td>
<td>18.3</td>
</tr>
<tr>
<td>201-250</td>
<td>V</td>
<td>streak</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

Mixed fractions were separated by application of the free-acid oligosaccharide to the starting line of Whatman No.17 paper chromatograms (ca. 400mg. per paper) followed by development in solvent C for 48 hours. The papers were either sprayed with reagent 3 and the yellow acid sections cut out, eluted with water and treated with charcoal in 15% ethanol to remove the indicator; or the sugars were located by blotting the thick paper with Whatman No.1 paper immediately after removal from the chromatography tank, the positions of sugars being indicated on the No.1 paper by spraying with aniline oxalate. The positions were then marked off on the thick paper, the areas cut out and eluted with water.

\textbf{EXPT.30. PROPERTIES OF THE ALDOBIURONIC ACID.}

Fraction I (390.7mg.) had $[\alpha]_D^{22.1} = -22.1^\circ$; $R\text{Gal. Acid} = 1.06$ (solvent C) (cf. 4-O-glucuronosyl-rhamnose ($R_g = 1.07$) from \textit{A. centralis}.)

No second spot could be obtained by treating a spot of this syrup on a paper chromatogram with ammonium hydroxide before elution.
Further, treatment of the acid (8mg.) with dry pyridine (2ml.) at 120° for one hour produced no change in the mobility of this acid and gave no evidence for a second spot when run on a chromatogram in solvent C. It had Mg 0.80 (cf. Mg 0.75 for A. centralis acid) in borate buffer (pH 10); in acetate buffer (pH 5.0) run under the same conditions, it gave two spots one of which diffused backwards for ca. 2cm., by electroendosmosis; glucurone behaved similarly. The major component travelled forward at approximately the same speed as galactacturonic acid.

Determination of the equivalent weight of the acid was carried out by titration against 0.0126 N sodium hydroxide solution using phenolphthalein as indicator:

81.0mg. aldobiuronic acid 18.5ml. 0.0126 N alkali

i.e. Equivalent weight 347.

Calculated for a glucuronosyl-rhamnose 340.

EXPT. 31. CONVERSION OF THE ALDOBIURONIC ACID TO THE ESTER GLYCOSIDE AND REDUCTION TO THE NEUTRAL DISACCHARIDE

The aldobiuronic acid (504.7mg.) was converted to the methyl ester methyl glycoside by refluxing with 2.5% dry methanolic hydrogen chloride (30ml.) until the rotation was constant:

\[
\begin{array}{cccccc}
\text{Time} & 0 & 1 & 3 & 5 & 7 & 8 \\
\left[\alpha\right]_D & -31^\circ & -28^\circ & -25^\circ & -23^\circ & -21^\circ & -22^\circ \\
\end{array}
\]

The solution was neutralised with dry silver carbonate and filtered. The residue was exhaustively extracted with dry methanol, and the filtrate and extracts on evaporation to dryness yielded the ester glycoside as a pure syrup (515mg.).
Two methods were used for the reduction of the acid to the disaccharide methyl ester:--

(a) Lithium aluminium hydride (Nystrom 1947). The syrup (e.g. 100mg.) was suspended in dry tetrahydrofuran (20ml.) which had been carefully dried by distillation from sodium and from lithium aluminium hydride, and a saturated suspension of the hydride in tetrahydrofuran (5ml.) was then added dropwise and the mixture refluxed for 90 minutes. Addition of water (30ml.) precipitated aluminium hydroxide which was removed on the centrifuge. The centrifugate was treated with Amberlite IR 120(H) resin to remove dissolved cations after which the organic solvent was removed under reduced pressure. The disaccharide glycoside thus obtained was a clear syrup. (Yield 70.2mg. $[\alpha]_D^{20} = -12.6^\circ$ (c, 1.6)).

(b) Potassium Borohydride. (Frush 1956). The ester glycoside (300mg.) in 0.4M boric acid (40ml.) pH 4.4 was cooled to 2° in an ice-water bath and the addition of an aqueous solution of potassium borohydride (300mg. in 10ml.) made dropwise with stirring. The solution was allowed to stand at 2° overnight. The final pH of the reaction mixture was pH 9.

After standing the solution was adjusted to pH 7 with glacial acetic acid to destroy residual potassium borohydride and then shaken with IR 120(H) resin followed by repeated evaporation with methanol until all methyl borate was removed, finally being concentrated to a clear syrup. (244mg.).
EXPT. 32. HYDROLYSIS OF THE DISACCHARIDE GLYCOSIDE AND CHARACTERISATION OF THE NEUTRAL SUGARS.

The disaccharide glycoside (30mg.) was hydrolysed with N sulphuric acid (3ml.) for 4 hours at 100°. After cooling the solution was neutralised by treatment with Amberlite IR 120(H) and IR 45(OH) resins and concentrated to a syrup (26.4mg.). Chromatographic analysis in solvent A revealed the presence of glucose and rhamnose. The molar proportions of the sugars in this hydrolysate were determined by the Wilson (1959) method as described in Expt.

\[\text{Table XX}\]

Molar ratios of sugars present in the hydrolysate of the reduced \textit{aldobiuronic acid}.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Absorbance (mm.)</th>
<th>Weight (µg.)</th>
<th>Molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.300</td>
<td>130</td>
<td>1.09</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>0.432</td>
<td>111</td>
<td>1.0</td>
</tr>
</tbody>
</table>

The remaining syrup (ca. 25 mg.) was treated with glucose oxidase in the presence of two drops of hydrogen peroxide (100 vols.) (Keilin 1948). The digest was incubated at 30° for 36 hours after which the removal of the enzyme was effected by treatment with cadmium sulphate and barium hydroxide solutions followed by filtration of the resultant precipitate. Methanol extraction of the concentrated residue afforded a pale yellow syrup which showed only rhamnose on a paper chromatogram (solvent A). That it was indeed rhamnose was confirmed by preparation of the phenyllosazone derivative (Freudenberg 1929) which had m.p. and mixed m.p. 190°.
FIG. 29  PERIODATE OXIDATION OF DISACCHARIDE.

KEY.

Disaccharide. ●
Control. ○
EXPT. 32. PERIODATE OXIDATION OF THE DISACCHARIDE GLYCOSIDE.
(Aspinall 1957).

The disaccharide glycoside (5.42mg.) was dissolved in distilled water (5ml.) and made up to 10ml. in a standard flask with 0.03 M sodium metaperiodate. The solution, and a control solution containing β-methyl cellobioside (4.04mg.) and sodium metaperiodate, were incubated in the dark at 30°. Aliquots (1ml.) were removed and analysed as detailed in Expt. 15 Section I b.

TABLE XXI
Peridate uptake by disaccharide glycoside.

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>Absorbance $^{223}$ (m$^2$)</th>
<th>Fraction $10^4$ consumed</th>
<th>Moles $10^4$ reduced/mole sugar.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.521</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>0.525</td>
<td>0.174</td>
<td>1.62</td>
</tr>
<tr>
<td></td>
<td>0.570</td>
<td>0.093</td>
<td>1.17</td>
</tr>
<tr>
<td>1</td>
<td>0.513</td>
<td>0.200</td>
<td>1.87</td>
</tr>
<tr>
<td></td>
<td>0.560</td>
<td>0.109</td>
<td>1.37</td>
</tr>
<tr>
<td>3</td>
<td>0.490</td>
<td>0.245</td>
<td>2.30</td>
</tr>
<tr>
<td></td>
<td>0.529</td>
<td>0.174</td>
<td>2.20</td>
</tr>
<tr>
<td>24</td>
<td>0.450</td>
<td>0.315</td>
<td>2.80</td>
</tr>
<tr>
<td></td>
<td>0.500</td>
<td>0.217</td>
<td>2.63</td>
</tr>
<tr>
<td>48</td>
<td>0.436</td>
<td>0.348</td>
<td>3.10</td>
</tr>
<tr>
<td></td>
<td>0.496</td>
<td>0.235</td>
<td>2.98</td>
</tr>
</tbody>
</table>
The first reading at each time refers to the disaccharide, the second to the control of \(\beta\)-methyl celllobioside.

Extrapolation of the graph (fig. 29) of time against moles of sodium periodate reduced, indicated an uptake of periodate of 3 moles per mole of sugar.

**Expt. 34. Methylation of the Disaccharide Glucoside.**

The remaining weight of disaccharide glucoside (235mg.) was dissolved in distilled water (25ml.) and cooled to 2\(^\circ\). The solution was treated with 30\% sodium hydroxide (20ml.) and dimethyl sulphate (8ml.) added dropwise over a period of 6 hours. The mixture was allowed to attain room temperature and stirred overnight, after which a further quantity of the methylating reagents was added as before. Following a third methylation the mixture was brought to pH 8 with sulphuric acid and exhaustively extracted with chloroform (4 x 100ml.). On evaporation of the organic solvent the methylated disaccharide glucoside was obtained. (Yield 264mg.).

An aliquot (15mg.) of the syrup was hydrolysed with N hydrochloric acid in 20\% methanol and neutralised with silver carbonate. The residue was extracted with methanol and the filtrate and washings combined and concentrated to afford a clear syrup. Chromatographic analysis in solvent F indicated the presence of 2,3,4,6-tetra-O-methyl-\(D\)-glucose, 2,3-di-O-methyl-L-rhamnose \(R_g 0.66\) and some mono-methyl rhamnose, \(R_g 0.32\).

Methanolysis of an aliquot (15mg.) of the methylated
disaccharide glycoside was effected with dry 3% methanolic hydrogen chloride for 6 hours at 100°, followed by neutralisation with silver carbonate and extraction of the methylated sugar glycosides with chloroform. Finally the chloroform extract was concentrated to a syrup (13.2mg.). The syrup was analysed by gas-liquid chromatography or an Apiezon M column at 150° when the mixture was shown to contain:—

<table>
<thead>
<tr>
<th>Peak</th>
<th>Glycoside</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2,3,4,6-tetra-O-methyl-(\beta)-glucoside</td>
<td>13.0</td>
</tr>
<tr>
<td>2</td>
<td>&quot;   &quot; 0 &quot;   &quot; -(\alpha)&quot;</td>
<td>55.0</td>
</tr>
<tr>
<td>3</td>
<td>2,3-di-O-methyl-(\beta)-rhamnose</td>
<td>23.0</td>
</tr>
<tr>
<td>4</td>
<td>&quot;   &quot;  &quot;   &quot; -(\alpha)&quot;</td>
<td>2.5</td>
</tr>
<tr>
<td>5</td>
<td>2,3,4 tri-O-methyl-rhamnose</td>
<td>5.0</td>
</tr>
</tbody>
</table>

The presence of mono-O-methyl rhamnose suggested that the disaccharide was not fully methylated. The material was therefore further methylated under conditions used by Kuhn (1955). The partially methylated disaccharide (210mg.) was dissolved in dry dimethyl formamide (6ml.) and methyl iodide (2.5ml.). The addition of dry silver oxide (2.5g.) was made over a period of 45 minutes with vigorous stirring. The temperature was not allowed to rise above 30°. After addition of the silver oxide, the mixture was shaken for 12 hours in a well-stoppered flask and then extracted with chloroform (2 x 5ml.) and dimethyl formamide. To the combined extracts was added successively distilled water (50ml.) and potassium cyanide (0.5g.) and the mixture finally extracted four times with chloroform (4 x 50ml.). Ultimately
the chloroform was washed with water, dried over anhydrous sodium sulphate and concentrated to a syrup. (130mg.).

**EXPT.35. HYDROLYSIS OF THE METHYLATED DISACCHARIDE AND SEPARATION OF THE METHYLATED SUGARS.**

The methylated disaccharide (130mg.) was hydrolysed at 100° for 5 hours using N hydrochloric acid in 20% methanol (5ml.), cooled, neutralised with silver carbonate and extracted with methanol. The removal of silver salts was completed by precipitation as silver sulphide, and the filtrate concentrated to a pale yellow mobile syrup. (154 mg.)

The methylated sugar mixture was applied to the starting line of Whatman 3MM chromatogram (20 x 40cm.) and eluted overnight in solvent D. The major fractions were located by developing control strips and the sugar positions thus indicated were cut out and eluted with 50% methanol. The eluates were concentrated to ca. 10ml. filtered through hard filter paper and finally concentrated to syrups.

**Fraction I** Crystalline 2,3,4,6-tetra-C-methyl-D-glucose (78.5 mg.)

D 78°A(25) chromatographically (solvents D and F) identical with an authentic specimen run as control. It had m.p. and mixed m.p. 84°.

**Fraction II** Syrupy 2,3-di-O-methyl-L-rhamnose (54.0 mg.)

It showed the same chromatographic mobility (solvents D and F) Rf 0.66 as authentic 2,3-di-methyl rhamnose and had MgO.04 which also compared favourably. Furthermore, this material showed the same properties as the sample obtained from the green alga _A. centralis_ (O'Donnell 1959) by a similar series of experiments.
Demethylation of a sample (ca. 5mg.) of each of these fractions by the method of Allen (1958) was effected by treatment with dichloromethane (2ml.) and redistilled boron trichloride (5ml.) at -80°C (cardice and acetone) for 30 minutes after which the reaction mixture was allowed to attain room temperature in a vacuum desiccator. The remaining solvent was removed under vacuum and aqueous methanol (5ml.) added to decompose the residue. The solution was taken to dryness and investigated on a paper chromatogram (solvent A). Fraction I showed glucose with a trace of rhamnose (visible only under ultra violet light) while fraction II showed only rhamnose.

**EXPT. 36. INVESTIGATION OF HIGHER ACID FRACTION.**

The higher acid fractions (III-V) containing an acid R gal. Acid 0.53 were combined and purified by separation on Whatman No. 17 paper as described in Expt. 29 (Yield 41mg. $[\alpha]_D^20$ (c. 10)

The syrup which had R gal. Acid 0.53 in solvent C was found to have an equivalent weight of 270g. (calculated for diglucuronosyl rhamnose 275g.). Conversion to the ester glycoside followed by reduction (potassium borohydride) and hydrolysis as described for the aldobiuronic acid yielded a syrup (30mg.). Chromatographic investigation of the molar ratios of sugars (Wilson 1959) gave the following results:

**TABLE XXII**

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Absorbance (m. u.)</th>
<th>Weight (µg.)</th>
<th>Molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.462</td>
<td>184</td>
<td>2.4</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>0.307</td>
<td>76</td>
<td>1.0</td>
</tr>
</tbody>
</table>
B. THE ACID FRACTION OF ENTEROMORPHA COMPRESSA.

During investigation of the water-soluble polysaccharide from *E. compressa* (this thesis Section III) it was noticed that acid hydrolysates of this material contained an acid component of similar chromatographic mobility to the aldobiuronic acid of *U. lactuca* and it was thought that a preliminary investigation might reveal certain similarities between the two materials.

**EXPT. 37. SEPARATION OF AN ACID FRACTION FROM *E. compressa*.**

**WATER-SOLUBLE POLYSACCHARIDE AND PRELIMINARY INVESTIGATION**

The polysaccharide (2g.) whose properties are fully described in Expt. Section III of this thesis, was hydrolysed with N sulphuric acid (40ml.) for 4 hours at 100°. The cooled solution was neutralised with barium carbonate and filtered, the filtrate being passed through a column of Amberlite IR-120(H) resin six times, to a final pH 2. The free-acid polysaccharide solution was then passed down a column (4 x 30cm.) containing Amberlite IR-45(OH) resin, and washed continuously with water until the eluate gave a negative Molisch test.

The column was then washed with 2N Ammonia (ca. 200ml.) and the eluate taken to a syrup (Wt. 163mg.) after removal of the ammonia by repeated distillations with distilled water under reduced pressure. Chromatographic analysis (solvent C) revealed a major component \( R_{Gal. Acid} 1.06 \) and three slower moving acids.

The main component was separated from the other acids by paper chromatography on Whatman 3MM paper using solvent C. (Yield 52mg.). \( [\alpha]_D ^{20} -12^\circ \). It had \( R_{Gal. Acid} 1.06 \) identical with the aldobiuronic acid reported. It was converted to the methyl ester
methyl glycoside, and reduced with potassium borohydride in 0.4M boric acid. (Frush 1956) as described in Expt. 31 (Yield 40mg.). Hydrolysis of an aliquot (10mg.) of this syrup yielded an equimolar mixture of rhamnose and glucose as shown chromatographically in solvent C.

SECTION II (c)

DISCUSSION

*Ulva lactuca* is an algal species common to the British coastline and is therefore readily available. It is easily recognised by its broad leaf-like fronds and is frequently termed the "sea-lettuce". Supplies of the weed collected in May 1958 and June 1959 at Kames Bay, Millport were kindly presented by Mr. Harry Powell and I take this opportunity of recording my thanks to him.

Preliminary structural examination of the water-soluble polysaccharide from *U. lactuca* by Brading (1954) and her colleagues has been discussed in the introduction to this thesis. (Page 34). While these authors were able to show the uronic acid to be D-glucuronic acid, characterised by isolation of methyl 2,3,4-tri-O-methyl-D-glucuronosidate and formation of the derived amide, they were unable to provide any indication of the mode of linkage of the uronic acid residue. From the results obtained in this work it is shown that it is linked to L-rhamnose in position C4.
Preliminary extraction of the dried milled weed with 80\% aqueous ethanol (Laidlaw 1952) removed practically all the chlorophyll and other colouring matter. (Expt. 25). An attempt to increase the yield on subsequent extraction of water-soluble material, by decolourising and swelling with dimethyl sulphoxide (Haaglund and Lindberg 1956) was found to remove a proportion of the water-soluble polysaccharide and for this reason the procedure was not repeated.

The decolourised weed was extracted repeatedly with cold water and the extract isolated, after dialysis and concentration, by freeze-drying (Yield 6\%). The cold and hot water extracts were found to have the same composition of neutral sugars and uronic acid but the former had a considerably lower protein content and there being no shortage of the weed, only the cold water extract was investigated. (Expt. 26).

This material had [\alpha]_D -50^\circ and contained D-glucose, D-xylose and D-rhamnose residues, 16\% ash, 18.2\% sulphate and 15.9\% uronic acid. The free-acid polysaccharide had an equivalent of 313. Assuming the absence of sulphate and ash a polysaccharide containing 15.9\% uronic acid would have an equivalent weight of 1100. The neutral polysaccharide contained 18.2\% sulphate which corresponds to 200g. of sulphate in 1100g., or 2.08 equivalents of sulphate. Thus an equivalent of 1100 corresponds to 3.08 equivalents, and hence a polysaccharide with 18.2\% sulphate and 15.9\% uronic acid should have an equivalent weight of 1100/3.08
Since analyses for sulphate and uronic acid content were made on a polysaccharide containing 16% ash the final calculated value for the equivalent becomes 300 which is in relatively good agreement with that obtained by direct titration.

Prolonged acid hydrolysis of the polysaccharide failed to yield any indication of glucuronic acid or its lactone, leading only to degradation of the polysaccharide. However, partial acid hydrolysis led to the isolation of a hydrolysate 25-30% of which consisted of the barium salts of acid oligosaccharides and an optimum amount of an aldobiuronic acid was attained after hydrolysis with N sulphuric acid at 100° for 5 hours. The barium uronates from such a hydrolysate were separated from the neutral sugars on a cellulose column and on Amberlite CG-45 resin. (Expt.29). In the former case 72% of neutral sugars and 17% of barium uronates were separated and in the latter 78% of neutral sugars and 13% of acidic oligosaccharides were isolated. From both methods a pure aldobiuronic acid was obtained constituting 75-78% of the acidic material. A small quantity of a triuronic acid was separated on thick paper from the mixed fractions containing higher oligo-saccharides.

Two methods of locating the acids in the preparative paper chromatography were investigated; that using an acid indicator which was subsequently removed by charcoal was superior to blotting the wet chromatogram and detecting the acids on the blotter. The latter gave indistinct interfaces between the fractions.
The aldobiuronic acid was degraded on severe methanolysis and hydrolysis, but was shown to comprise glucuronic acid and rhamnose by reduction of the ester glycoside followed by hydrolysis (Expt. 31). The biuronic acid, \([\alpha]_D -22^0\) had an equivalent weight of 347g., the calculated weight for glucuronosyl-0-rhamnose being 340g. It had chromatographic mobility, \(R_{\text{Gal.Acid}} 1.06\), indistinguishable from that recorded by O'Donnell and Percival (1959) for the aldobiuronic acid 4-O-glucuronosyl-rhamnose (\(R_{\text{Gal.Acid}} 1.07\)) isolated from Acrosiphonia centralis water-soluble polysaccharide. Furthermore, both acids had comparable \(M_g\) values 0.30, on electrophoresis in borate buffer (pH 10). In contrast to the acid from \(A.\ centralis\) however, the present acid failed to yield a second spot when treated with ammonium hydroxide prior to elution on a paper chromatogram. Similarly, treatment with dry pyridine did not yield a component with a smaller \(R_g\) value; on the other hand ionophoresis in acetate buffer (pH 5.0) produced two spots, one which diffused backwards at the same rate as glucurone and the other forwards at approximately the same speed as galacturonic acid, thus confirming the presence of both charged and uncharged groups at this pH. It is difficult to correlate these findings with the apparent identity of the two acids.

The ester glycoside was insoluble in tetrahydrofuran and reduction in this manner was therefore less satisfactory than the
aqueous phase reduction with potassium borohydride. The hydrolysed product as stated, revealed chromatographically the presence of equimolar proportions of glucose and rhamnose, the former being characterised enzymatically and the latter by formation of the authentic crystalline phenyllosazone. The glucose must have arisen from the reduction of glycosidically-linked glucuronic acid.

Measurement of the periodate uptake of the disaccharide glycoside (Expt. 33) by the method of Aspinall and Ferrier (1957) gave a value of 3.1 moles per mole at 30°. A control sample of β-methyl cellotrioside consumed 2.98 moles per mole under the same conditions. That the oxidation was complete after 48 hours was indicated by the uptake of the control sample, and other experiments with disaccharide glycosides (Ferrier 1956). Thus the mode of linkage is through C₂ or C₄ of the rhamnose residue. Methyl-2- or 4-glucuronosyl rhamnose (fig. xxxiii) requires 3 moles of periodate for complete oxidation, whereas methyl 3-glucuronosyl-rhamnose (fig. xxxiv) consumes only 2 moles.

fig. (xxxiii)  
fig. (xxxiv)
Methylation of the disaccharide glycoside was effected by three applications of the Haworth technique and the partially methylated dimer finally treated with methyl iodide and silver oxide according to Kuhn (1955) (Expt. 34). Gas liquid chromatography of a methanolised aliquot of the methylated disaccharide on a polyester column at 200° indicated the presence of methyl 2,3,4,6-tetra-O-methyl-D-glucoside, methyl 2,3-di-O-methyl-L-rhamnoside and methyl 2,3,4-tri-O-methyl-L-rhamnoside indicating the main linkage in the disaccharide to be on C4 of rhamnose.

This was confirmed by hydrolysis and thick paper separation of the hydrolysed syrup. Crystalline 2,3,4,6-tetra-O-methylglucose and syrupy 2,3-di-O-methylrhamnose were separated; the former was characterised by its rotation, melting point chromatographic and ionophoretic mobilities, the latter by its rotation and chromatographic and ionophoretic mobilities. Both methylated sugars were further characterised by demethylation to the parent sugars. The quantity of tri-O-methylrhamnose was too small to be isolated. It is considered to have arisen from a small portion of the aldobiuronic acid and/or disaccharide which was inadvertently hydrolysed prior to methylation.

The aldobiuronic acid separated from U. lactuca is therefore 4-O-β-gluconosyl-L-rhamnose, the same as that reported to be present in the hydrolysate from the water-soluble polysaccharide.
from *Acrosiphonia centralis* (O'Donnell and Percival 1959). That its rotation is slightly more negative may be due to the purity of the sample.

The triuronic acid had an equivalent weight of 270; a structure such as glucuronosyl-0-rhamnosyl-0-glucuronic acid or diglucuronosyl-rhamnose would require 257. Reduction and hydrolysis afforded rhamnose and glucose in the approximate molar ratios of ca. 2 : 1 lending weight to the equivalent value and indicating that the triuronic acid was a diglucuronosyl-rhamnose.
SECTION III

THE WATER-SOLUBLE POLYSACCHARIDE FROM ENTEROMORPHA COMPRESSA.

III(a) INTRODUCTION.

Enteromorpha spp. belong to the class Ulvaceae, a group of algae occurring commonly in British waters, both in fresh and salt-water forms. Members of this class show a preference for estuarine growth, especially where there is an influx of sewage. Enteromorpha spp. grow in the form of long tendrils, frequently interwoven with other groups of weed, and the collection of any one member of this genus is therefore difficult.

An investigation of the carbohydrate components of a mixture of E. torta, E. compressa and E. intestinalis (Lowe 1956) has shown that dilute acid extracted polysaccharide consists of at least two polymers. Hydrolysis of the polysaccharide indicated the presence of D-glucose, L-rhamnose, D-xylose, 3-O-methyl-L-rhamnose, D-glucuronic acid and sulphate residues. Fractionation of this material by chloroform extraction of the methylated polymer yielded a soluble fraction containing a glucan, and a residue consisting of a mixture of the methyl ethers of the other monosaccharide components, practically devoid of glucose. Examination of the proportion of methylated sugars showed fully methylated rhamnose (18%), partially methylated rhamnose and xylose (65%) and unmethylated rhamnose (15%), indication of a high degree of branching in the polymer. No evidence was given for the position of the sulphate group and no other work on Enteromorpha, as far as the author is aware, has been recorded.

The present thesis describes the investigation of a water-
soluble polysaccharide from a pure sample of *E. compressa*, with particular reference to the possible position of the sulphate group.

The glucose containing polysaccharide separated from this water-soluble material is discussed along with the glucan from *C. rupestris* in Section I of this thesis.
III(b) EXPERIMENTAL.

Three batches of the green seaweed *Enteromorpha compressa* were procured from Mr. H. Powell of Millport for this investigation, each sample yielding approximately the same polysaccharide material.

**EXPT.38. PRELIMINARY ALCOHOLIC EXTRACTION**

Prior to extraction of the polysaccharide material from the weed, each sample was exhaustively extracted with 85% aqueous ethanol in a Soxhlet apparatus. This treatment removed most of the colouring matter together with mono- and di-saccharides.

**EXPT.39. EXAMINATION OF THE ALCOHOLIC EXTRACT.**

The alcoholic chlorophyll containing solution was clarified with barium hydroxide and cadmium sulphate solutions (Laidlaw 1952) and then de-ionised by ion exchange electrodialysis (Anderson 1950) using Permutit Permaplex A-10 and C-10 membranes. The resultant clear solution was concentrated and on paper chromatographic investigation (solvent B) was found to contain glucose and a trace of sucrose.

**EXPT.40. EXTRACTION OF THE POLYSACCHARIDE MATERIALS.**

The residual alcohol extracted weed (170g.) was successively treated with the following solvents under the conditions stated:-

(G) Distilled water at $70^\circ$ in an atmosphere of nitrogen until further treatment gave a negative Molisch test.

(H) The weed from the above treatment was stirred at $70^\circ$ in aqueous acetic acid (4ml. glacial acetic acid per litre) with
addition of batches of sodium chlorite (10g.) (Wise 1945), until the weed was decolourised.

(I) The residual weed from (H) was washed thoroughly with cold water then treated, under nitrogen, with 4% sodium hydroxide solution (Chanda 1951) at room temperature for 24 hours.

(J) The residue from (I) was further extracted with 18% sodium hydroxide under the same conditions.

(K) 72% sulphuric acid (Monier-Williams 1921) on the weed residue from (J) (see page 53).

In each case the polysaccharide was isolated by freeze-drying the solution obtained after dialysis and concentration. Chromatographic investigation of the hydrolysed polysaccharides provided a visual estimation of the amounts of the constituent sugars, as shown in Table XXIII together with the respective yields.

<table>
<thead>
<tr>
<th>Extract</th>
<th>% Yield</th>
<th>Glucose</th>
<th>Xylose</th>
<th>Rhamnose</th>
<th>Uronic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>16.8</td>
<td>xx</td>
<td>xx</td>
<td>xxxxx</td>
<td>xx</td>
</tr>
<tr>
<td>H</td>
<td>6.0</td>
<td>x</td>
<td>xxx</td>
<td>xxxxx</td>
<td>x</td>
</tr>
<tr>
<td>I</td>
<td>10.1</td>
<td>xx</td>
<td>xx</td>
<td>xxxx</td>
<td>xxx</td>
</tr>
<tr>
<td>J</td>
<td>4.5</td>
<td>xxxxx</td>
<td>xx</td>
<td>xx</td>
<td>-</td>
</tr>
<tr>
<td>K</td>
<td></td>
<td>xxxxx</td>
<td>xx</td>
<td>xx</td>
<td>-</td>
</tr>
</tbody>
</table>

xxxxxx major component; x trace.
EXPT. 41. PRELIMINARY EXAMINATION OF THE EXTRACTS.

The physical properties of the above polysaccharides were shown to be:

<table>
<thead>
<tr>
<th>Extract</th>
<th>$\left[\alpha\right]_D$</th>
<th>ash%</th>
<th>sulphate%</th>
<th>N%</th>
<th>Uronic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>-41</td>
<td>13.0</td>
<td>9.6</td>
<td>3.0</td>
<td>17.0</td>
</tr>
<tr>
<td>H</td>
<td>-79</td>
<td>23.4</td>
<td>11.9</td>
<td>2.86</td>
<td>12.8</td>
</tr>
<tr>
<td>I</td>
<td>-46</td>
<td>10.0</td>
<td>8.3</td>
<td>2.49</td>
<td>16.6</td>
</tr>
<tr>
<td>J</td>
<td>-</td>
<td>40.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Since the best yield was the hot-water extracted polysaccharide, and there was no apparent fractionation in the other extracts, all subsequent investigations refer to extract (G) which will henceforth be designated the "Water-soluble Polysaccharide".

EXPT. 42. REMOVAL OF PROTEIN FROM THE WATER-SOLUBLE POLYSACCHARIDE. (Stumpf 1948).

The water-soluble polysaccharide (G) (23.6g.) from the dried weed was treated, with stirring at room temperature, with trichloroacetic acid to give a final concentration of 4%. After standing for two days the denatured precipitated protein was removed on the high speed centrifuge (12,000r.p.m.) and the polysaccharide recovered as a friable white solid after dialysis and concentration, by freeze-drying.

Yield of purified polysaccharide, 26g..
**EXPT.43. PROPERTIES OF THE PURIFIED WATER-SOLUBLE POLYSACCHARIDE.**

The above freeze-dried polysaccharide had the following properties:

\[ [\alpha]_D ^{20} -49^\circ \ (c,1,1), \text{ ash} \ 11.0\%, \text{ sulphated ash} \ 13.2\%, \text{ total sulphate} \ 11.45\%, \text{ N} \ 1.4\%. \text{ Uronic acid by decarboxylation} \ 17.2\% \ (Swenson \ 1946). \]

Conversion to the free-acid polysaccharide was achieved by repeated passage of a solution of the water-soluble polysaccharide (G) through a column of Amberlite IR-120(H) resin to pH 2-3.

The equivalent weight of the free-acid material (200.4mg., ash 2.3%, sulphate 9.7%) was determined by titration of aliquots against standard sodium hydroxide solution using methyl red as indicator.

21.80mg. polysaccharide (G) = 5.01ml. 0.0123 N sodium hydroxide

*: Equivalent 346.

The polysaccharide gave a negative Seliwanoff test, and stained blue-purple on addition of dilute iodine solution.

**EXPT.44. QUANTITATIVE ESTIMATION OF THE RELATIVE PROPORTIONS OF THE SUGARS IN THE HYDROLYSATE OF WATER-SOLUBLE POLYSACCHARIDE.**

The estimation of the relative proportions of the monosaccharides present in a N sulphuric acid hydrolysate of the water-soluble polysaccharide was carried out by the method of Wilson (1959) as described in Section I Expt.5.
TABLE XXV

Molar ratios of the sugars present in the hydrolysed water-soluble polysaccharide.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Absorbance (μμ)</th>
<th>wt. of sugar (μg.)</th>
<th>approximate molar ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.040</td>
<td>24</td>
<td>1.00</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.175</td>
<td>28</td>
<td>1.34</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>0.625</td>
<td>168</td>
<td>6.70</td>
</tr>
</tbody>
</table>

Estimation of the uronic acid content of the hydrolysate provided erratic results due to degradative hydrolysis of the uronosyl linkage.

EXPT. 45. ALKALINE HYDROLYSIS OF THE WATER-SOLUBLE POLYSACCHARIDE

Removal of the sulphate residues was attempted by heating the polysaccharide (1.55g.) in N sodium hydroxide solution (200ml.) at 100°. Aliquots (10ml.) were removed at known intervals, filtered, made acid with concentrated hydrochloric acid and the sulphate estimated gravimetrically with 3% barium chloride.

After 4 hours it appeared from the weight of barium sulphate that more than 100% of the sulphate residues had been liberated and the reaction was stopped. The solution was cooled, dialysed and concentrated, and alcohol precipitated to yield 0.35g. (21%) of a dirty polysaccharide material containing only 4% sulphate. Hydrolysis of this material followed by chromatographic analysis (solvent C) revealed a complicated mixture of reducing sugars including glucose, xylose, rhamnose and a number of other spots with $R_{\text{Glucose}}$ 0.77, 1.2 and 1.36.
EXPT. 46. **ATTEMPTED FRACTIONATION OF THE WATER-SOLUBLE POLYSACCHARIDE WITH A XYLANASE.** (Howard 1957).

The above polysaccharide (51.5mg.) was dissolved in a buffer solution pH 6.8 [0.2 M disodium hydrogen phosphate (15.45ml.) and 0.1 M citric acid (4.55mg.)]. To this solution was added the xylanase (2.2mg., from sheep's rumen very kindly supplied by Dr. B.H. Howard of the Rowett Research Institute) and the flask flushed out with nitrogen and incubated at 37° for 26 hours. The solution was heated to 60° to deactivate the enzyme followed by dialysis in a closed system. Hydrolysis of the solution retained in the dialysis sac and chromatographic examination of the derived syrup (solvent A) showed the monosaccharide constituents to be unchanged and the proportions of each approximately the same as in the original polysaccharide. Chromatographic examination (solvent A) of the concentrated dialysate failed to reveal any xylose.

EXPT. 47. **TREATMENT OF THE WATER-SOLUBLE POLYSACCHARIDE WITH SALIVARY α-AMYLASE.**

The water-soluble polysaccharide (45.2mg.) was dissolved in distilled water (10ml.) and fresh salivary α-amylase added. The mixture, with a few drops of toluene, was incubated at 37° overnight, after which a second introduction of the enzyme was made. Following a further 24 hours incubation, the enzyme was deactivated by heating to 70° for ten minutes, and the solution dialysed in a closed system. Analysis by chromatography (solvent A) of an hydrolysate of the undialysable material indicated a large decrease in the glucose content of the polysaccharide, although not complete.
removal. In contrast, xylose and rhamnose were present in the same proportions relative to each other. The concentrated dialysate was found to contain maltose with a trace of glucose. (paper chromatogram solvent A).

**EXPT. 48. ATTEMPTED CETAVOLON FRACTIONATION OF THE WATER-SOLUBLE POLYSACCHARIDE.**

(a) Cetyltrimethylammonium bromide (C.T.A.) fractionation. (Bera 1955).

To the polysaccharide (250mg.) in water (20ml.) was added dropwise 20% aqueous C.T.A. (0.5ml.) with stirring. No precipitate was obtained even after prolonged stirring.

(b) Cetyltrimethylammonium hydroxide (C.T.A.-OH) in Boric acid solution. (Bouveng 1958).

To an aqueous solution of the polysaccharide (600mg. in 100ml. water) was added with stirring 0.6 M boric acid (60ml.). Dropwise addition of a solution of C.T.A.-OH (8ml.; 0.1 N) containing sodium hydroxide (C.2ml.; 0.5 N), yielded a white precipitate. Further addition of the precipitating reagents produced no further precipitate. Removal of the complex (a) was effected by filtration and the filtrate on treatment with ethanol (4 volumes) produced a small precipitate (B) which was washed thoroughly with ethanol and dried in ethanol and ether (58mg.).

After washing with water to remove contaminating C.T.A.-OH, the precipitate (A) was treated with excess molar sodium chloride in order to decompose the complex. This was found to be extremely difficult as was a similar attempt using 2 N acetic acid. The polysaccharide was isolated eventually by alcohol precipitation of the partially decomposed complex. (323mg.).
(c) Treatment with C.T.A. at pH 0.5 (Scott 1955).

To a buffered solution of the polysaccharide (100mg. in 50ml. N sodium acetate/N hydrochloric acid, pH 0.5) was added C.T.A. (15ml. 10% w/w), with vigorous stirring. The solution became turbid, but no precipitation occurred. Further addition of C.T.A. had no effect.

(d) Arquad 16-(50%) precipitation.

Precipitation under conditions described in (b) above was made using commercially pure Arquad 16 - 50% (a hexadecyltrimethylammonium chloride) when a flocculent white precipitate (A) was obtained. Addition of ethanol to the supernatant produced a precipitate (B) which was dried with alcohol and ether. Decomposition of complex (A) was even more difficult than previously.

Chromatographic examination of the hydrolysates of the different fractions obtained under (b) and (d) indicated that the "cetavlon" complexes contained a smaller relative proportion of glucose, while the alcohol precipitated un-complexed material contained glucose with only traces of xylose and rhamnose. Thus it would appear that the polysaccharide can be fractionated into two components using "cetavlon". However, the low yield of glucon material and the relative difficulty in regaining the precipitated polysaccharide from the complex made this method impracticable from the point of view of a preparative fractionation.
EXPT. 49. FRACTIONATION OF A GLUCAN FROM THE WATER-SOLUBLE POLYSACCHARIDE BY IODINE PRECIPITATION. (Steiner 1944).

This method of fractionation was applied with effect to the alkali-soluble polymer from C. rupestris and the experimental details are described in Section I Expt. 7. Only the relative weights of material, therefore, are given here.

Polysaccharide (G) 15g.
0.03 N ammonium carbonate 500ml.
20% aqueous sodium chloride 2 x 200ml.
0.5 N sodium thiosulphate ca. 25ml.
N hydrochloric acid 60ml.

Yield of iodine precipitated glucan III 500mg. (3.5%).
Yield of residual polysaccharide 9.6g. (65%).

The characterisation of glucan III is fully discussed in Section I of this thesis.

EXPT. 50. PROPERTIES OF POLYSACCHARIDE (L). THE RESIDUE AFTER REMOVAL OF GLUCAN III.

Polysaccharide (L) had the following properties: -

\([\alpha]_D \text{ -}87^\circ (c, 0.89), \text{ ash } 13.7\% \text{ sulphate in ash } 7.8\%, \text{ total sulphate } 16.0\%, \text{ N } 0.86\%. \text{ The uronic acid content calculated by decarboxylation (Swenson 1946) } 18.3\%.

The polysaccharide was soluble in water, and did not give a blue colour with iodine. The equivalent weight estimated by titration of the free-acid polysaccharide (95mg.) with standard sodium hydroxide was 310.
EXPT. 51. QUANTITATIVE ESTIMATION OF THE RELATIVE PROPORTIONS OF SUGARS IN THE HYDROLYSATE OF POLYSACCHARIDE (L).

Hydrolysis of the polysaccharide (50 mg.) in the usual manner followed by paper chromatographic separation of the sugars (solvent C) in the neutralised hydrolysate and estimation of the relative proportions (Wilson 1959) indicated the following:

<table>
<thead>
<tr>
<th>Sugars</th>
<th>Absorbance (mG)</th>
<th>Wt. of sugar (mg)</th>
<th>Approximate molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.059</td>
<td>34</td>
<td>1.0</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.142</td>
<td>76</td>
<td>2.7</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>1.12</td>
<td>275</td>
<td>8.1</td>
</tr>
</tbody>
</table>

Thus glucose containing polymers were not completely removed by iodine precipitation.

EXPT. 52. SEPARATION AND CHARACTERISATION OF THE COMPONENT SUGARS OF POLYSACCHARIDE (L).

Polysaccharide (L) (650 mg.) was hydrolysed for 8 hours with N sulphuric acid. After neutralisation with barium carbonate, the solution was de-ionised with Amberlite IR-120(H) resin. The resulting solution was then concentrated to a syrup (520 mg.) and applied to the starting line of a Whatman No. 17 paper chromatogram (20 x 40 cm.). After eluting with solvent A for 48 hours the sugar bands were located by means of control strips and the respective fractions cut out and eluted from the paper with water (100 ml.). Subsequent concentration of these solutions produced the sugar components as separate entities.
**Fraction I.** Crystalline L-rhamnose (281mg.) \([\alpha]_D^+7.8\ (c, 0.98)\) had m.p. and mixed m.p. 92°. The derived phenyllosazone (Freudenberg 1929) had m.p. 190° undepressed by admixture with a sample prepared from authentic L-rhamnose.

**Fraction II.** Crystalline D-xylose (68mg.) \([\alpha]_D^{+19.4}\ (c, 1.0)\) had m.p. and mixed m.p. 143°. The derived dibenzylidene dimethyl acetal (Breddy 1945) had m.p. and mixed m.p. 211°.

**Fraction III.** Syrupy D-glucose (42mg.) \([\alpha]_D^+52\ (c, 0.6)\). Incubation of a portion with glucose oxidase at 37° for 30 hours caused complete conversion to D-gluconic acid (Keilin 1948) as revealed by chromatographic resolution in solvent C, spraying with 0.1% bromoresol green solution.

**Fraction IV.** Syrup, acidic sugar (42mg.) Rg 0.29 (solvent A) \([\alpha]_D ^{-12.2}\ (c, 0.84)\). Chromatography in solvent C revealed an RGalAcid 1.06 identical with the 4-0-β-glucuronosyl-L-rhamnose isolated from Ulva lactuca (Section II).

**EXPT. 53. ATTEMPTED SEPARATION OF A SULPHATE-RICH FRACTION FROM POLYSACCHARIDE (L) BY CHROMATOGRAPHY.** (Spolter 1960).

Polysaccharide (L) (100mg.) was dissolved in a minimum of water and applied to the starting line of a Whatman 3MM paper chromatogram (15 x 40cm.). The chromatogram was irrigated for 24 hours in an ammonium formate buffer/propan-2-ol mixture (65/35). Polysaccharide material carrying charged groups was located by spraying control strips with azure II when two areas were revealed as dark blue spots on a light blue background, one on the starting line, one as a streak towards the solvent front. The appropriate
sections of the main paper were cut out and extracted with distilled water (50ml.), the solutions being concentrated to syrups. Hydrolysis of these materials in the normal manner followed by paper chromatographic investigation (solvent A) showed both fractions to contain xylose and rhamnose with a trace of glucose.

Both unhydrolysed fractions gave positive tests for sulphate when sprayed with sodium rhodizonate. (Lloyd 1960).

**EXPT.54. TREATMENT OF POLYSACCHARIDE (L) WITH SALIVARY α -AMYLASE.**

Polysaccharide (L) (50mg.) was incubated at 37° with salivary α-amylase as described in Expt.47, Section III. After dialysis, the polysaccharide was isolated by alcohol precipitation then hydrolysed in the usual way. Chromatographic evidence (solvent B) for the presence of glucose was obtained.

**EXPT.55. ATTEMPTED FRACTIONATION OF POLYSACCHARIDE (L) WITH BEHILLING’S SOLUTION.** (Chanda 1951).

To the polysaccharide (500mg.) dissolved in water (70ml.) was added Fehling’s solution (30ml.) with rapid stirring. After standing overnight, the precipitate which formed was centrifuged off. The complex was decomposed by addition of dilute hydrochloric acid to a suspension of the material in acetone, leaving a white precipitate (I). Addition of ethanol to the supernatant in two stages first to 40% concentration and then to 60% yielded precipitates (II) and (III).
Visual examination of the hydrolysed precipitates of polysaccharide (L) with Fehling's solution.

<table>
<thead>
<tr>
<th>Precipitate</th>
<th>Weight</th>
<th>Glucose</th>
<th>Xylose</th>
<th>Rhamnose</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>60mg.</td>
<td>x</td>
<td>xx</td>
<td>xxxxx</td>
</tr>
<tr>
<td>II</td>
<td>205mg.</td>
<td>x</td>
<td>xx</td>
<td>xxxxx</td>
</tr>
<tr>
<td>III</td>
<td>180mg.</td>
<td>x</td>
<td>xx</td>
<td>xxxxx</td>
</tr>
</tbody>
</table>

**EXPT.56. ATTEMPTED BARIUM HYDROXIDE FRACTIONATION OF POLYSACCHARIDE (L) (Meier 1958).**

Polysaccharide (L) (460mg.) dissolved in distilled water (100ml.) was re-cycled through Amberlite IR-120(H) resin to a pH 2-3. The solution was diluted to 500ml. with water followed by dropwise addition of a saturated solution of barium hydroxide. The solution was allowed to stand for 48 hours but no precipitate was obtained. Variation of the polysaccharide concentration and strength of the barium hydroxide still failed to yield a precipitate.

**EXPT.57. ATTEMPTED POTASSIUM CHLORIDE FRACTIONATION. (O'Neill 1955).**

Addition of a solution of N potassium chloride solution to a rapidly stirred aqueous solution of the polysaccharide (500mg. in 50ml. water) produced no precipitate even after standing for several days. Variation in the concentration of the polysaccharide and of the potassium chloride did not alter the result.

To two separate samples of Polysaccharide (L) (each 200mg.) in distilled water (50ml.) was added dialysed samples of commercial Taka-diastase (40mg.) and hemicellulase (35mg.). The solutions were incubated at 37° in dialysis sacs immersed in distilled water (200ml.). After stirring for 24 hours half the dialysis water was removed from each experiment and concentrated to syrups (8mg. and 12mg. respectively). Chromatographic investigation (solvent C) revealed one spot in each case, both having R<sub>Gal</sub>Acid 1.10 and giving a positive reaction to bromo-oresol green indicator spray.

The residue of the two syrups derived from the respective dialysates were converted to the ester glycosides and reduced under the conditions given in Expt. 31, Section II.

Chromatographic analysis of the hydrolysates of the reduced acids (solvent A) revealed the presence of glucose and rhamnose in the relative proportions shown below:

| Relative proportions of sugars in the hydrolysate of the enzyme digests. |
|----------------|----------------|-----------------|
| Sugar          | Absorbance (μg.) | Weight of sugar (μg.) | Molar ratio |
| (a) Glucose    | 0.219            | 90               | 4.5         |
| Rhamnose       | 0.097            | 20               | 1           |
| (b) Glucose    | 0.302            | 120              | 40          |
| Rhamnose       | 0.148            | 30               | 1           |

(a) Taka diastase extract (b) Hemicellulase digest.
The polysaccharide remaining in the dialysis sacs was investigated after hydrolysis by chromatography when the presence of glucose, xylose, rhamnose and uronic acid was detected.

**EXPT. 59. SODIUM PERIODATE OXIDATION OF POLYSACCHARIDE (L)**

*Fleury 1933.*

The polysaccharide (273.0mg.) was dissolved in 0.1564 M sodium metaperiodate and made up to 50ml. in a graduated flask with the same solution. The oxidation was allowed to proceed at room temperature in the dark, a blank experiment being carried out simultaneously.

The reduction of periodate was measured by taking an aliquot (1ml.) and adding it to a solution of sodium arsenite (10ml., 0.0490 N), followed by addition of sodium bicarbonate (1g.) and 25% potassium iodide (1ml.). After standing for 30 minutes, the residual arsenite was titrated against standard iodine solution (0.0199 N).

**Results:**

10ml. arsenite solution (0.0490 N) ≡ 24.7ml. iodine (0.0199 N).

1ml. periodate (0.1564 M) ≡ 3.91ml.

10ml. arsenite solution ≡ 8.91ml.

<table>
<thead>
<tr>
<th>Time (Hrs.)</th>
<th>0</th>
<th>12</th>
<th>36</th>
<th>60</th>
<th>84</th>
<th>108</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iodine titre (ml.)</td>
<td>8.91</td>
<td>12.40</td>
<td>13.00</td>
<td>13.30</td>
<td>13.65</td>
<td>13.68</td>
</tr>
<tr>
<td>i.e.</td>
<td>0</td>
<td>0.21</td>
<td>0.243</td>
<td>0.262</td>
<td>0.280</td>
<td>0.283</td>
</tr>
</tbody>
</table>

After 108 hours, 1 mole of periodate was reduced per 460g. polysaccharide (assuming that the contaminating protein had no effect). The reaction was stopped by the addition of ethylene.
FIG. 30 INFRARED SPECTRA OF POLYSACCHARIDES (L) & (M).
glycol (10ml.). After dialysis and freeze-drying the oxo-
poly saccharide was obtained as a fluffy white solid. (190.0mg.).

Examination of the oxo-poly saccharide.

Chromato graphic examination of a hydrolysat e showed that
 glucose, xylose and rhamnose were present. Furthermore, a
 longer oxidation period failed to remove any one sugar residue
 completely. Colorimetric estimation of relative amounts of
 sugars present in a hydrolysate (Wilson 1959) provided the
 tabulated results:

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Absorbance (m/μ )</th>
<th>Weight of Sugar (μg.)</th>
<th>Approx. molar ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.02</td>
<td>36</td>
<td>1.6</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.125</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>0.200</td>
<td>85</td>
<td>3.6</td>
</tr>
</tbody>
</table>

EXPT.60. DESULPHATION OF POLYSACCHARIDE (L) USING DRY METHANOLIC
HYDROGEN CHLORIDE. (Kantor 1957).

A preliminary account of this work was read at the 407th
meeting of the Biochemical Society. (see back of thesis)

Infrared analysis of a sample of polysaccharide (L) in a
"nujol" mull on the Perkin-Elmer "Infrachord" Spectrophotometer
revealed the presence of axial sulphate with a peak at 845cm⁻¹
(fig.30) (Lloyd 1961) together with a peak at 1240cm⁻¹
indicative of a C-O-S stretching frequency.
The polysaccharide (330mg.) was shaken with 0.09 M methanolic hydrogen chloride (50ml.) for 24 hours at room temperature, after which the undissolved polysaccharide was removed on the centrifuge and washed with dry methanol until acid-free. It was then dissolved in distilled water (50ml.) and dialysed for 3 days. This was followed by concentration and freeze-drying. (Yield 230mg.; 70%). Hydrolysis of a sample of this material showed chromatographically (solvent B) glucose, xylose, and rhamnose to be present. The sulphate content (Jones 1954) was 2.1% cf. 16% in the original polysaccharide (L).

If one takes into consideration this reduction in sulphate content, then the yield of sulphate-free polysaccharide corresponds to 81.5%. A second infrared analysis as described above, confirmed this decrease in sulphate content (fig.30). The two peaks detailed above had practically disappeared.

The methanolic hydrogen chloride solution used for the desulphation was neutralised with dry silver carbonate and filtered, the filtrate yielding a syrup (19.8mg.) on concentration. This was shown to contain glucose, xylose and rhamnose by paper chromatography (solvent B).

A second desulphation (900mg.) for the longer period of 48 hours yielded 545mg. (71%) of desulphated polymer (M) having a sulphate content (Jones 1954) of 0.75%. The sulphate peaks were no longer evident. An attempt was made to follow the release of sulphate by analysing aliquots of the supernatant solution at known intervals for its sulphate content. This gave inaccurate results, however, due probably to the dissolution of a small quantity of polysaccharide.
EXPT. 61. PRELIMINARY EXAMINATION OF POLYSACCHARIDE (M)

The polysaccharide (M) had the following properties: 
\[ \left[ \alpha \right]_D = -38^\circ, \text{ash } 2.6\%, \text{total sulphate } 0.75\%, \text{N } 0.9\%.

Uronic acid as determined by decarboxylation (Swenson 1946), 23.5%. The polysaccharide was soluble in water.

The equivalent weight by titration of the free-acid polysaccharide with standard sodium hydroxide solution was found to be 655.


The relative proportions of the sugars present in the hydrolysed polysaccharide were estimated colorimetrically with aniline phthalate. The following results were obtained:

**TABLE XXX**

Relative proportions of sugars in Polysaccharide (M).

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Absorbance (m\textmu)</th>
<th>Wt. of sugar (\textmu g.)</th>
<th>Approximate molar proportions.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.124</td>
<td>56</td>
<td>1.0</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.370</td>
<td>67</td>
<td>1.4</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>1.082</td>
<td>280</td>
<td>5.0</td>
</tr>
</tbody>
</table>

EXPT. 63. PERIODATE OXIDATION OF POLYSACCHARIDES (L) AND (M). (Aspinall 1957).

(a) Periodate uptake. The polysaccharides (L) and (M) (46.70 mg. and 43.8 mg.) were dissolved in 0.2 M sodium acetate buffer, pH 3.6 (25 ml.) (Hough 1956, Cantley 1959) and made up to 50 ml. in a
FIG. 31
PERIODATE OXIDATION OF POLYSACCHARIDES
(L) & (M).

Moles Periodate/6 anhydride unit

Time (hrs)

10 20 30 40 50 60 70 80

0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
standard flask with 0.03 M sodium periodate dissolved in the same buffer. The periodate oxidation of polysaccharide (L) was repeated here in order that a direct comparison of results could be made, although its uptake has already been studied by the method of Fleury and Lange (1933) (Expt. 59).

The samples were kept at 20° in the dark and the oxidation of periodate was followed as described in Expt. 33 (Section II). The following results were obtained:

**TABLE XXXI**

**Periodate uptake by Polysaccharides (L) and (M)**

<table>
<thead>
<tr>
<th>Time</th>
<th>Absorbance (μ)</th>
<th>Fraction of 10^4 Moles 10^4 reduced/ C₆ anhydride unit.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.620</td>
<td>-</td>
</tr>
<tr>
<td>0.5</td>
<td>0.585</td>
<td>0.065</td>
</tr>
<tr>
<td></td>
<td>0.551</td>
<td>0.130</td>
</tr>
<tr>
<td>1.75</td>
<td>0.578</td>
<td>0.082</td>
</tr>
<tr>
<td></td>
<td>0.540</td>
<td>0.146</td>
</tr>
<tr>
<td>3.0</td>
<td>0.570</td>
<td>0.093</td>
</tr>
<tr>
<td></td>
<td>0.520</td>
<td>0.184</td>
</tr>
<tr>
<td>5.0</td>
<td>0.552</td>
<td>0.124</td>
</tr>
<tr>
<td></td>
<td>0.515</td>
<td>0.195</td>
</tr>
<tr>
<td>24</td>
<td>0.547</td>
<td>0.136</td>
</tr>
<tr>
<td></td>
<td>0.515</td>
<td>0.196</td>
</tr>
<tr>
<td>48</td>
<td>0.540</td>
<td>0.147</td>
</tr>
<tr>
<td></td>
<td>0.515</td>
<td>0.196</td>
</tr>
<tr>
<td>72</td>
<td>0.540</td>
<td>0.147</td>
</tr>
<tr>
<td></td>
<td>0.515</td>
<td>0.196</td>
</tr>
</tbody>
</table>
In each case the first figure refers to (L), the second to (M).

Results:

After 72 hours oxidation at 2° we find:

1 mole periodate = 300g. polysaccharide (M)
and " " " = 425g. " (L) (cf. 460g by titration)

After 72 hours the solutions were allowed to rise to room temperature when it was found that oxidation was complete after a further 5 hours. One mole of sodium periodate was then found to be equivalent 240g. polysaccharide (M) and 328g. polysaccharide (L).

(b) Isolation of the oxo-polysaccharide. The reaction was stopped by passing sulphur dioxide through solution for 15 minutes and the oxo-polysaccharides were isolated by freeze-drying after dialysis and concentration. Yields: (M) 3 0.2mg., 69%; (L) 3.6mg., 81%.

Chromatographic analysis of the hydrolysed oxo-polysaccharides (solvent C) indicated the presence of glucose, xylose and rhamnose. Colourimetric estimation (Wilson 1959) of the relative proportions of sugars in this hydrolysate gave the results shown below:

| Table XXXII |
|---|---|---|---|
| **Relative proportions of sugars in the oxo-polysaccharide (M).** | | | |
| **Sugar** | **Absorbance** | **Wt. of sugar (µg.)** | **Approximate molar ratios** |
| Glucose | 0.012 | 18 | 2.5 |
| Xylose | 0.050 | 6.3 | 1.00 |
| Rhamnose | 0.108 | 20 | 2.75 |
FIG. 32  CYSTEINE - H₂SO₄
ESTIMATION OF
METHYL
PENTOSES (1948).

Methylation of pentoses in cysteine.
Rhamnose reacts with cysteine hydrochloride in concentrated sulphuric acid to form a reaction mixture which, although not coloured, shows a sharp absorption maximum at 400\text{nm}. All other classes of saccharides, viz. pentoses, hexoses, hexosamines etc., produce coloured products in the primary cysteine reaction which differ considerably from methyl pentoses in their absorption curve.

A calibration graph (fig. 32) was prepared by reacting aliquots of a rhamnose solution of known concentration with 6:1 sulphuric acid : water mixture (4.5ml.) while cooling in ice, followed by heating the solution at 100\(^\circ\) for 3 minutes. After being cooled in tap water a 3\% solution of cysteine hydrochloride (0.1ml.) was added and the mixture well shaken. The absorbance of the solution was read after 15 minutes in a Unicam S.P. 500 Spectrophotometer, the value \((E_{396\text{nm}} - E_{427\text{nm}})\) being compared with that of a blank. A straight line relationship was obtained for the plot of \(\Delta E\) against concentration.

The estimation of the rhamnose contents of polysaccharides \((L)\) and \((M)\) and oxo-polysaccharides \((L)\) and \((M)\) was carried out on an aqueous solution containing known weights of the respective materials, the procedure being as described for the calibration graph, from which the weight of rhamnose was obtained by comparison. (see Table XXXIII).
Concentration of polysaccharide solutions:

Polysaccharide (L) = 100.6 μg. per ml.

" (M) = 98.5 " " "

Oxo-polysaccharide (L) = 98.6 " " "

" (M) = 88.56 " " "

Estimation 1 was carried out on the polysaccharide solutions above. Estimation 2 refers to the same solutions treated with potassium borohydride at 2° for 12 hours, thus reducing the free aldehydic groups present in the oxo-polysaccharides.
### TABLE XXXIII

Estimation of the Relative Weights of Rhamnose in Polysaccharides (L) and (M) and their oxo-polysaccharides.

<table>
<thead>
<tr>
<th>Polysaccharide</th>
<th>Volume of solution (ml.)</th>
<th>$E_{406} - E_{387}$</th>
<th>Weight (g.)</th>
<th>Theoretical polysaccharide</th>
<th>% Rhamnose</th>
<th>Average %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. (L)</td>
<td>0.5</td>
<td>0.220 0.044 0.176</td>
<td>19.2</td>
<td>50.3</td>
<td>38.0</td>
<td>37.3</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.452 0.070 0.382</td>
<td>36.5</td>
<td>100.6</td>
<td>35.5</td>
<td></td>
</tr>
<tr>
<td>(M)</td>
<td>0.5</td>
<td>0.388 0.060 0.278</td>
<td>27.5</td>
<td>49.5</td>
<td>56.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.600 0.091 0.509</td>
<td>46.5</td>
<td>98.5</td>
<td>47.0</td>
<td>51.0</td>
</tr>
<tr>
<td>Oxo (L)</td>
<td>0.5</td>
<td>0.200 0.040 0.160</td>
<td>18.0</td>
<td>49.3</td>
<td>36.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.310 0.050 0.260</td>
<td>26.5</td>
<td>98.6</td>
<td>27.0</td>
<td>31.7</td>
</tr>
<tr>
<td>(M)</td>
<td>0.5</td>
<td>0.212 0.044 0.168</td>
<td>18.0</td>
<td>44.33</td>
<td>40.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.340 0.040 0.300</td>
<td>30.0</td>
<td>88.66</td>
<td>34.0</td>
<td>37.0</td>
</tr>
<tr>
<td>2. (L)</td>
<td>0.4</td>
<td>0.164 0.035 0.129</td>
<td>15.5</td>
<td>40.24</td>
<td>38.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>0.240 0.045 0.195</td>
<td>21.3</td>
<td>60.36</td>
<td>35.5</td>
<td></td>
</tr>
<tr>
<td>(M)</td>
<td>0.4</td>
<td>0.271 0.081 0.190</td>
<td>21.0</td>
<td>39.40</td>
<td>53.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>0.400 0.121 0.279</td>
<td>28.0</td>
<td>59.10</td>
<td>47.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.572 0.157 0.415</td>
<td>39.3</td>
<td>98.50</td>
<td>44.0</td>
<td>48.0</td>
</tr>
</tbody>
</table>
### Estimation of the Relative Weights of Rhamnose in Polysaccharides (L) and (M) and their oxo-polysaccharides

<table>
<thead>
<tr>
<th>Polysaccharide</th>
<th>Volume of solution (ml.)</th>
<th>$E_{396}$</th>
<th>$E_{427}$</th>
<th>$\Delta E$</th>
<th>Weight (μg.)</th>
<th>Theoretical polysaccharide</th>
<th>% Rhamnose</th>
<th>Average %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxo (L)</td>
<td>0.4</td>
<td>0.110</td>
<td>0.019</td>
<td>0.091</td>
<td>12.5</td>
<td>39.44</td>
<td>31.70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>0.158</td>
<td>0.028</td>
<td>0.130</td>
<td>16.0</td>
<td>59.16</td>
<td>27.20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.270</td>
<td>0.052</td>
<td>0.218</td>
<td>23.0</td>
<td>98.60</td>
<td>23.50</td>
<td>27.0</td>
</tr>
<tr>
<td>Oxo (M)</td>
<td>0.4</td>
<td>0.125</td>
<td>0.041</td>
<td>0.084</td>
<td>12.0</td>
<td>35.46</td>
<td>34.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>0.190</td>
<td>0.065</td>
<td>0.125</td>
<td>15.5</td>
<td>53.20</td>
<td>28.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.342</td>
<td>0.135</td>
<td>0.207</td>
<td>22.0</td>
<td>88.66</td>
<td>25.0</td>
<td>29.0</td>
</tr>
</tbody>
</table>
EXPT. 65. CARBAZOLE/SULPHURIC ACID ESTIMATION OF URONIC ACID CONTENT OF POLYSACCHARIDES (L) AND (M) AND THEIR RESPECTIVE OXOPOLYSACCHARIDES.

An attempt was made to estimate colorimetrically (McComb 1952) the uronic acid content of the four polysaccharides using carbazole and sulphuric acid. The method incurred the interaction of a sample (2 ml.) of a solution of the unknown (as in Expt. 64) with a similar concentrated sulphuric acid (12 ml.) at 100° for 10 minutes. The solution was allowed to cool and then treated with 0.1% carbazole solution (1 ml.) and shaken, the absorbance being estimated in an Unicam S.P.500 Spectrophotometer at 540 μm against a blank, after 20 minutes. In theory, comparison of the absorbance obtained with a standard graph for glucuronic acid gave the weight of acid present.

<table>
<thead>
<tr>
<th>Polysaccharide</th>
<th>Absorbance (μm)</th>
<th>Wt. of acid (μg.)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>(L)</td>
<td>0.063</td>
<td>11.0</td>
<td>6.00</td>
</tr>
<tr>
<td>(M)</td>
<td>0.125</td>
<td>220.0</td>
<td>10.2</td>
</tr>
<tr>
<td>oxo (L)</td>
<td>0.030</td>
<td>5.5</td>
<td>2.7</td>
</tr>
<tr>
<td>oxo (M)</td>
<td>0.032</td>
<td>5.8</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Detailed investigation of the extracts (H)-(J) was not pursued since they appeared to be similar in monosaccharide composition to polysaccharide (G), except for (J), which had a high ash content.
A preliminary examination of the 4% sodium hydroxide extract (I) however, showed a considerably reduced rhamnose content the ratios of glucose : xylose : rhamnose being 1 : 1.12 : 1.88 (cf. (G) 1 : 1.34 : 6.7).

Examination of the sugar content of the 72% sulphuric acid digest of the weed residue, after extractions (G)-(J) by the Monier-Williams (1921) technique indicated the presence of glucose, xylose and rhamnose.
III(d) DISCUSSION

The preliminary treatment of the green weed *E. compressa* with 85% aqueous ethanol removed a large proportion of the colouring matter. Various solvents, including benzene: ethanol (2:1), acetone, methanol and methylated spirits were used in de-colouration of the weed, but in no case was the colouring matter completely removed, 85% aqueous ethanol being the most efficient extractive. In view of the fact that dimethyl sulphoxide was found to extract polymeric carbohydrate material from *U. lactuca* to the extent of 6%, without preferential fractionation of the polysaccharide, it was felt that omission of this treatment was justifiable, although it was found to be an excellent solvent for organic colouring matter, and is known to effect fractionation of certain polysaccharide mixtures. (Hägglund 1956).

Exhaustive hot-water extraction of the alcohol treated residue yielded 16.8% of a flaky off-white polysaccharide material, hereinafter called the water-soluble polysaccharide. This yield compares favourably with those achieved from other green seaweeds (cf. *U. lactuca* only 6%) and it was deemed unnecessary to investigate further means of aqueous extraction, all work being carried out on this hot water extract. The weed residue after exhaustive hot water treatment was, however, given a hypochlorite treatment (which removed 6% polysaccharide) prior to extraction with 4% alkali in the cold. This was found to extract 10.1% of a heterogeneous polysaccharide containing glucose, xylose and rhamnose with some uronic acid which in contrast with the hot
water extract, showed a relatively low rhamnose content. Further extraction of *E.* compressa with 18% alkali yielded an impure polysaccharide (ca. 35-40% ash) which was composed largely of glucose residues along with traces of xylose and rhamnose.

Final treatment of the fibrous weed residue after the foregoing sequence of extractions produced, under the conditions first described by Monier-Williams (1921) for commercial cellulose, a syrup which showed glucose, xylose and rhamnose on a paper chromatogram. In this respect, therefore, *E.* compressa is similar to *U.* lactuca (Cronshaw 1958) and *A.* centralis (O’Donnell 1959) in that the fine structure of the cell walls is not based solely on cellulose I. Indeed Nicolai and Preston (1952) have shown *Ulva* and *Enteromorpha* spp. to give X-ray diagrams basically different from that of cellulose I. In contrast, however, the cell walls of *Cladophora rupestris* and *Chaetomorpha meiagonium* have been shown to be built up of many lamellae each of which consists of straight microfibrils of cellulose I arranged parallel to each other. This finding increases the points of similarity found to exist between *Ulva,* *Enteromorpha* and *Acarosiphonia* spp.

The water-soluble polysaccharide $[\alpha]_D^{-49^\circ}$ (cf. *U.* lactuca $-50^\circ$; *A.* centralis $-31^\circ$), was isolated mainly as the sodium salt and was found to comprise about 8.75% protein, 11% ash and 11.45% sulphate. The uronic acid content (17.2%) corresponds to about
one uronic acid residue per five sugar units which is comparable to that of *Ulva lactuca* (15-20%) but markedly different to polysaccharides isolated from *C. rupestris* and *Caulerpa filiformis* (ca. 5% and 7% respectively). The presence of uronic acid in the polymer presents much difficulty with respect to its complete hydrolysis and as a result it was found that comparative estimations of the proportions of sugars present in any hydrolysate of the polysaccharide were erratic unless the same conditions were used throughout for each hydrolysis.

The free-acid polysaccharide had an equivalent weight of 346 (cf. *A. centralis* 459). The equivalent calculated on the basis of a uronic acid content of 17.2% would be expected to be 963. If one considers, however, that the neutral polysaccharide contains 11.45% sulphate residues, which corresponds to 114g. of sulphate or 1.14 equivalents, then it follows that 963 corresponds not to 1 but to 2.14 equivalents. Therefore, the true equivalent weight of the neutral polymer will be 963/2.14 = 450. Further, the uronic acid and sulphate estimations were made on a material containing 11.0% ash and 8.75% protein, consideration of which reduces the value to 360 and thus agrees favourably with the experimental results.

The molar proportions of the neutral sugars in the hydrolysate of the polysaccharide (Expt.44) were determined by the colourimetric method of Wilson (1959), which was found to be more reproducible and less cumbersome than the ε-amino-diphenyl method
of Piper (1958). The polysaccharide was shown to contain glucose, xylose and rhamnose in the molar ratios 1: 1.34: 6.7. Efforts to estimate the uronic acid content colourimetrically were hindered by the existence of the uronosyl link, which does not break under normal hydrolysis conditions, and by the difficulty encountered in separating the acidic material from the free sugars. It was found that in acid solvents this ran at approximately the speed of glucose, while in neutral solvents it showed the typical streaking effect of uronic acid.

No indication was obtained for the presence of 3-O-methyl-L-rhamnose in the polysaccharide hydrolysate. This methyl sugar was reported to occur in the polysaccharide obtained from a mixture of Enteromorpha spp. (Lowe 1956) and its absence from the E. compressa polymer suggests that it originates either from E. torta or E. intestinales.

A wide variety of techniques for the fractionation of polysaccharides was applied to this water-soluble extract. Howard (1957) has shown that a xylanase from sheep's rumen could completely hydrolyse the pentosan from wheat flour and that the xylan from Rhodymenia palmata yielded a trisaccharide containing both a $\beta$-1,3' and $\beta$-1,4' linkage. If the xylose units in the present water-soluble material comprised a separate xylan it was hoped to remove them by incubation with this enzyme under the conditions described by Howard (Expt. 46). Since, however, the polysaccharide recovered after this treatment
contained the same proportion of xylose it may be presumed that
this sugar forms an integral part of the hetero-polysaccharide
and/or that $\beta\cdot-1,4^\prime$-linked xylopyranose residues are largely
absent from this material.

The reaction of this extract with iodine was indicative
of the presence of a starch polymer but attempts to remove all
the glucose units with salivary $\alpha$-amylase were unsuccessful
(Expt.47). After two consecutive treatments the glucose content
had only decreased from one in nine to one in twelve, which
suggests the presence of a laminarin-type glucan similar to that
found in the brown algae. There is accumulating evidence that
several of the green algae metabolise a small amount of
laminarin. It has been reported in the water-soluble fraction
of *C. rupestris* (Fisher 1957) and of *Acrosiphonia centralis*.
However, there also rests the possibility that the glucose
residues are incorporated with the other sugar units in a hetero-
polysaccharide in such a manner as to be immune to $\alpha$-amylase.
Stronger evidence for the presence of a laminarin-type polymer
is advanced for *Caulerpa filiformis* (Mackie 1959), where the
$1,3^\prime$-linked glucan is extracted together with a $1,3^\prime$-linked
xylan by aqueous alkali.

The use of quaternary ammonium salts to effect fractionation
of mixtures of acidic and neutral polysaccharides has been
reported in many instances in recent years (Scott 1955; 1960,
Barker 1957; Wolstenholme 1958). The addition of a solution
of the quaternary ammonium salt (e.g. "octavlon") to the poly-
saccharide solution, precipitates the "polyanion" while leaving
neutral polysaccharides in solution.

Several attempts were made (Expt. 48) to obtain a fractionation using "cetavlon" and this was found to give a partial separation in two experiments. In these instances the supernatant material contained mainly glucose with traces of xylose and rhamnose after one precipitation, but it was found necessary to add more "cetavlon" to get a pure glucan; further since some of the glucan appeared to co-precipitate with the rest of the material the final yield of unprecipitated glucose-rich material was very low. (ca. 1%).

Furthermore, the quaternary ammonium salt-polysaccharide complex was found to be extremely stable and in many cases could not be completely decomposed. The method, therefore, although not practicable in this instance, served to show that the water-soluble polysaccharide consists of at least two components, one a neutral glucose polymer, the other an acid xylose-rhamnose polysaccharide.

The addition of an iodine, potassium iodide solution to a starch containing polysaccharide mixture has been shown to preferentially precipitate the starch while leaving the other polymers in solution (Steiner 1944). Under the conditions described, the starch from sweet potatoes was found to be relatively undegraded, exhibiting an optical rotation $[\alpha]_D^{20} + 200^\circ$. The method was found to be applicable to materials containing 10% or more starch calculated on a moisture-free basis. The water-soluble polysaccharide of *E. compressa* contained ca. 11%
of glucose containing polymer calculated from the molar proportions of glucose in a hydrolysate but enzyme studies have indicated that only a proportion of this has a starch type structure. Attempted removal of all the glucose by precipitation with iodine was unsuccessful (Expt.49). Only 3.5% of a pure starch, \([\alpha]_D^+182^\circ\), was separated leaving a residual polymer (L) 65%, which still contained glucose units but which no longer stained blue with iodine, confirming the earlier findings with salivary \(\alpha\)-amylase. The investigation of the structure of the iodine-precipitated starch type polysaccharide has already been described in Section I of this thesis.

Investigation of the Residual water-soluble Polysaccharide.

The polysaccharide (L) had \([\alpha]_D^+ -87^\circ\) and was found to contain ash 13.7%, total sulphate 16.0%, sulphate in ash 7.8%, and nitrogen 0.86% (ca. 5.4% protein) (Expt.50). The relative increase in the percentage sulphate and decrease in the protein content suggests that considerable purification of the polymer has been achieved during the dispersion and iodine precipitation, and this would partially account for the considerable loss of material (ca. 30%). That the sulphate residues are present as ethereally linked residues follows from the fact that the sulphate in the ash was half the total sulphate, ignition resulting in the loss of half the sulphate as sulphur trioxide.

A polysaccharide analysing according to the figures given in the previous paragraph can be calculated (see page 152) to have an equivalent of 301. The value found by titration was 310 which agrees with the calculated result.
Treatment of polysaccharide (L) with salivary α-amylase (Expt.54) did not further reduce the glucose content of the polymer which once again confirms the fact that some glucose occurs other than the starch entity.

Quantitative estimation of the percentage relative proportions of sugars present in the polysaccharide gives:

Polysaccharide (L) Glucose 12, Xylose 16.0, Rhamnose 72.0

cf. Polysaccharide (G) " 8.5, " 23.0, " 68.2

Besides the removal of glucose units it appears that a small proportion of rhamnose units were lost during the fractionation of the starch.

The character of each sugar component was confirmed by isolation on thick paper chromatograms, followed by determination of rotational and chromatographic data, and formation of characteristic crystalline derivatives.

A variety of fractionation methods were investigated in an attempt to isolate a homo-polysaccharide from this water-soluble polysaccharide (L). As has already been stated, the application of "octavlon" at various pH's and concentrations to solutions of the original polysaccharide (G) indicated that the xylose, rhamnose, glucuronic acid material might form a single hetero-polysaccharide. This was supported by attempts to fractionate the material using Fehling's solution and organic solvents (Expt. 55), barium hydroxide (Expt.56) potassium chloride (Expt.57) and paper chromatographic separation (Expt.53). This latter technique using varying ratios of ammonium formate buffer
(pH 4.3)/iso-propanol has been employed to effect separation of mixtures of sulphated mucopolysaccharides (Spolter 1960). When applied to the algal polysaccharide in question it was found that although a separation occurred, it appeared to be a differentiation between two polysaccharides of different molecular weights rather than an actual separation of differently constituted polymers, since both fractions contained the same sugars and sulphate content. The question of a molecular weight distribution is an interesting consideration since it has been shown in this laboratory that the water-soluble polysaccharide from U. lactuca, which appears to be very similar to that of E. compressa, can be separated into three fractions which have the same rotation and constitution but which differ in their intrinsic viscosities. (Wold 1961).

Commercial Taka-diastase is a highly complex mixture of enzymes and in consequence it shows activity towards a variety of polysaccharide systems. Courtois, Keda and Petch (1958) observed that it will break down a galacto-mannan into galactose, mannose and mannosylmannose. Likewise commercial hemicellulase has been shown to preferentially degrade certain polysaccharides (Perila and Bishop 1961) with the production of oligosaccharides. It was hoped that incubation of these enzyme systems with the water-soluble polysaccharide (Expt. 58) would result in the production of neutral and/or different acidic oligosaccharides from those isolated after acidic hydrolysis. An apparently single aldobiuronic acid comprising glucuronic acid and rhamnose with
similar chromatographic mobility \( R_{\text{Gal,Acid}} 1.10 \) to the aldobiuronic acid \( R_{\text{Gal,Acid}} 1.06 \) liberated by acidic hydrolysis (see Expt. 52) appeared to be the only cleavage product from both enzyme systems. Furthermore, the residual polysaccharide isolated after incubation still contained rhamnose, glucuronic acid, xylose and glucose units. The molar ratios of glucose and rhamnose in the cleaved fragments, however, suggests the possibility of a mixture of two oligosaccharides, a glucuronosyl-glucose and a glucuronosyl-rhamnose and further investigation, by ionophoresis may clarify this point. While failing to yield the desired results it may be inferred from these experiments that the glucuronosyl-rhamnose occurs at the end of the polysaccharide chains and at the same time lends further support for the conclusion that the water-soluble material (L) comprises a single heteropolysaccharide is provided.

Normal acid hydrolysis of the polymer failed to produce any evidence of sulphated monosaccharides. The acid hydrolysis (0.25 N hydrochloric acid at 100°C) of the \textit{E. compressa} polysaccharide (L) has been studied, along with other algal sulphated polysaccharides, by Dr. Rees (1961) in this laboratory. After an initial rapid liberation of sulphate, the reaction has been shown to follow 1st order kinetics and yield 90% of the sulphate in 3.25 hours. The rate constant for the reaction (0.56 hours\(^{-1}\)) was found to be similar to that for other secondary hydroxyl sulphates. (e.g. \( \kappa \)-carrageenan 0.71 hours\(^{-1}\)) but quite different to the value given by primary sulphate release (e.g. Porphyran 0.26 hours\(^{-1}\)). Thus although the \textit{E. compressa}
sulphate residues showed some acid stability, it was felt that it was not such that the isolation of monosaccharide sulphates could be achieved effectively by the method of Turvey and Rees (1961). It was suggested by Dr. Rees that the rapid initial liberation of sulphate from *E. compressa* could be accounted for by the existence of some sugar di-sulphate residues in the polymer.

No evidence for the site of the sulphate groups in the heteropolysaccharide was derived from acidic or enzymic hydrolysis experiments. Alkaline hydrolysis of the unfractionated polysaccharide (Expt. 45) resulted in the simultaneous removal of sulphate and degradation of the polysaccharide. The apparent lability of the sulphate residues towards this reagent indicates the presence of hydroxyl groups so placed as to favour anhydro-ring formation (Percival 1948b). However, the fact that aliquots contained more sulphate than theory indicated, suggested some error in the method of determination of sulphate liberated, and hence, an indication of whether such ring formation had occurred is impossible. This error may be due to the dissolution of sulphated degraded polysaccharide in the aliquots; any sulphate in this material would be liberated under the conditions for sulphate determination and consequently give enhanced values for the sulphate present. Furthermore, an interpretation of the results is difficult in the light of the state of the residual polysaccharide, its low yield and the mixture of products obtained on hydrolysis.
Kantor and Schubert (1957) reported the complete removal of sulphate groups from chondroitin sulphate by the action of 0.06 M methanolic hydrogen chloride without degradation of the polysaccharide, while similar treatment of heparin resulted in a partial removal of the sulphate groups and the production of an undegraded polysaccharide containing equal amounts of N and S (Danishefsky 1960). Application of this technique to fucoidin, the sulphated polysaccharide of the brown algae, caused concomitant desulphation and degradation, giving a low yield of polymer with slightly higher sulphate content than the starting material.

In view of the fact that some ester sulphate hydrolysis of the water-soluble polysaccharide from *H. compressa* was achieved with alkali and there were indications of a fraction of easily liberated sulphate, it was thought that it might be possible to reduce the sulphate content by this less drastic treatment without degradation of the polysaccharide. In practice after hydrolysis with 0.09 M methanolic hydrogen chloride for 24 hours an 81.5% yield of polysaccharide material was recovered in which the sulphate content had been reduced to 2.1%. A second desulphation carried out on fresh material for 48 hours led to the isolation of a polysaccharide (M) containing only 0.75% sulphate in 71% yield.

Analysis of the hydrolysate of polysaccharide (M) showed a percentage monosaccharide composition very similar to the original sulphated polysaccharide (L):-
<table>
<thead>
<tr>
<th>Glucose</th>
<th>Xylose</th>
<th>Rhamnose</th>
<th>Sulphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polysaccharide (M)</td>
<td>13.4</td>
<td>19.6</td>
<td>68</td>
</tr>
<tr>
<td>Polysaccharide (L)</td>
<td>8.5</td>
<td>22.5</td>
<td>68</td>
</tr>
</tbody>
</table>

Chromatographic analysis of this hydrolysate failed to reveal any unusual sugars such as might be expected from the trans-elimination of the sulphate group and it is suggested that the sulphate residues are located such that this trans-elimination has not occurred, a result which disagrees with those of alkaline hydrolysis. This could be fulfilled by any of the following structural units:

(xxxv) 1,3'-linked xylose.
(xxxvi) 1,4'-linked rhamnose.
(xxxvii) 1,3'-linked rhamnose.

This assumes that the sulphate occurs on either xylose or rhamnose or on both residues.

The polysaccharide (M) had $\left[\alpha\right]_D^{25^\circ} -98^\circ$ and contained ash 2.6%, total sulphate 0.75%, N 0.9% (Expt. 61). The uronic acid had risen proportionately to 23.5% (cf. 18.3% in the
sulphated polymer). The equivalent weight estimated by titration of a sample with standard alkali was 655. Calculation of the equivalent based on the analytical figures (see page 162) gives a value of 642 in reasonable agreement with the experimental value.

Subjection of the sulphated and desulphated (SO₄ 2.1%) polysaccharides to periodate oxidation (Expt. 63) showed that for every C₆ sugar residue the reduction of periodate was 0.38 mole and 0.54 mole respectively. Under similar conditions the polymer (M) obtained from the second desulphation (SO₄ 0.75%) reduced 0.68 mole of periodate for every C₆ sugar residue. The low reduction of periodate by all these polysaccharides indicates a high proportion of 1,3'-linked residues or that it is highly branched. The higher consumption of periodate by the desulphated material reveals that the removal of sulphate produced additional units with adjacent free hydroxyl groupings.

Estimation (Wilson 1959) of the relative proportions of the unattacked monosaccharides in the hydrolysates of the two periodate oxidised polysaccharides gave the following results: -

Oxopolysaccharide (L) Glucose 26, Xylose 16.2, Rhamnose 58.
Oxopolysaccharide (M) Glucose 40, Xylose 16, Rhamnose 44.

The lower proportion of rhamnose units in the oxidised desulphated polysaccharide suggests that the sulphate groups are mainly attached to rhamnose. In the 101 conformation of rhamnose only C₂ carries an axial hydroxyl group, and since infrared
analysis of the sulphated polysaccharide revealed a peak at 850 cm\(^{-1}\) indicating the presence of axial sulphate (Lloyd 1961), it would appear, assuming that the rhamnose is present in the polysaccharide in its stable conformation that \(C_2\) of rhamnose carries at least some of the sulphate residues. This is in agreement with isolation of a 4-O-glucuronosyl-rhamnose unit (see Section II).

Apart from the galactan sulphate of *Porphyra umbilicalis* (Turvey 1961), this is the first direct evidence of the site of the sulphate group in algal sulphated polysaccharides.

As confirmation of the larger decrease of rhamnose in the desulphated oxo-polysaccharide compared with that of the sulphated material, estimation of the absolute amount of rhamnose in each polysaccharide and its oxidised derivative was made by the method Dische and Shettles (1948, 1949) (Expt. 64). This reaction is based on the fact that all saccharides, with the exception of hexosamines, produce furfural or one of its derivatives on mixing with concentrated sulphuric acid. The addition of cysteine to the reaction mixture produces new compounds which have characteristic absorption curves depending on the particular furan derivatives present. Furthermore,
The speed at which these derivatives form and the stability of the final reaction products differs from one class of sugar to another. On this basis it was found possible to measure the rhamnose content of the polysaccharides in the presence of other saccharides (in this case xylose, glucose and glucuronic acid) by measuring the difference in absorption at 396mμ and 427mμ at which wavelength the optical density of the interfering compounds is equal, but at which that of rhamnose at 427mμ is only a fraction of the 396mμ value. The accuracy of the method was helped by the large amount of this sugar present in the polysaccharide.

It was considered that the free aldehydic groups present in the oxopolysaccharides might give increased values for the absorption of the cysteine complex and thus result in high values. Two sets of results were prepared therefore, one on the initial polysaccharides and their derived oxo-derivatives and the second on the products after borohydride reduction of the materials. Results of the estimation of rhamnose content were:

<table>
<thead>
<tr>
<th></th>
<th>Estn.1</th>
<th></th>
<th>Estn.2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Polysaccharide</td>
<td>% rhamnose</td>
<td></td>
<td>% rhamnose</td>
<td></td>
</tr>
<tr>
<td>(L)</td>
<td>37.3</td>
<td></td>
<td>35.0</td>
<td></td>
</tr>
<tr>
<td>(M)</td>
<td>51.0</td>
<td></td>
<td>48.0</td>
<td></td>
</tr>
<tr>
<td>Oxopolysaccharide</td>
<td>31.7</td>
<td></td>
<td>27.0</td>
<td></td>
</tr>
<tr>
<td>(L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(M)</td>
<td>37.0</td>
<td></td>
<td>29.0</td>
<td></td>
</tr>
</tbody>
</table>

All the reduced materials gave lower results and this difference is greater in the two oxo-polysaccharides. Estimation 2 is considered therefore to be the more accurate.
Comparison of these results with those calculated for rhamnose in the sulphated and desulphated polysaccharides gave results in good agreement with each other. (see Table XXXV).

### Table XXXV

<table>
<thead>
<tr>
<th>Polysaccharide (L)</th>
<th>Polysaccharide (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhamnose by cysteine</td>
<td>35%</td>
</tr>
<tr>
<td>Calculated:</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>5.4</td>
</tr>
<tr>
<td>Sulphate</td>
<td>16.0</td>
</tr>
<tr>
<td>Ash</td>
<td>13.0</td>
</tr>
<tr>
<td>Uronic</td>
<td>18.3</td>
</tr>
<tr>
<td>Total</td>
<td>52.7%</td>
</tr>
</tbody>
</table>

..: Free sugars | 47.3% | 67% |
Molar proportions | 1 : 3 : 8 | 1 : 1.4 : 5 |
..: Rhamnose | 34.5% | 45.2% |

Hence, from the rhamnose estimations it is possible to say that while 77.3% of the rhamnose remained un-oxidised in the sulphated polysaccharide, only 60.5% was unattacked in the desulphated polymer, which represents an increase of 16.8% free hydroxyl positions on the rhamnose residues. The increase in periodate (0.38 mole - 0.68 mole) reduction is not fully accounted for by this increase in free hydroxyl groups and it is therefore likely that other residues than rhamnose carry sulphate. A comparison of the proportions of unattacked glucose and xylose (page 173) in the two oxopolysaccharides indicates
besides rhamnose an additional small proportion of the xylose units are also cleaved in the desulphated material and it is possible that these units also carry sulphate groups.

A decision concerning the immunity of the uronic acid units to periodate was difficult. Only small quantities of unattacked uronic acid could be detected by paper chromatography of the hydrolysates of the oxopolysaccharides. Quantitative determination by decarboxylation was obviously valueless since this is based on the number of carboxyl groups in the material and therefore the colorimetric method of McComb and McCready (1952) utilizing carbazole and sulphuric acid was investigated. While the results obtained bore no relationship to those obtained by decarboxylation for the unoxidised or oxidised polysaccharide, they were similar and considerably lower for both the oxidised materials. While this cannot be regarded as proof that these units in the original polysaccharide (L) are oxidised by periodate, it at least indicates that this is probable and that the uronic acid residues may be present as end groups.
CONCLUSION

When a comparison of the water-soluble polysaccharide of *E. compressa* is made with those metabolised by *U. lactuca* and *A. centralis*, certain striking similarities are revealed. (see Table XXXVI).

<table>
<thead>
<tr>
<th>Molar proportions of sugars</th>
<th><em>U. lactuca</em> (a)</th>
<th><em>A. centralis</em> (b)</th>
<th><em>E. compressa</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactose</td>
<td>0</td>
<td>0.1 (G)</td>
<td>0 (L)</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.0</td>
<td>1.0 (G)</td>
<td>1.0</td>
</tr>
<tr>
<td>Mannose</td>
<td>0</td>
<td>0.2 (G)</td>
<td>0 (L)</td>
</tr>
<tr>
<td>Arabinose</td>
<td>0</td>
<td>0 (L)</td>
<td>0 (G)</td>
</tr>
<tr>
<td>Xylose</td>
<td>1.3</td>
<td>1.6 (G)</td>
<td>1.34 (G)</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>4.4</td>
<td>1.4 (G)</td>
<td>6.70 (G)</td>
</tr>
<tr>
<td>Glucuronic acid %</td>
<td>20.8</td>
<td>19.3 (G)</td>
<td>17.2 (G)</td>
</tr>
<tr>
<td>Sulphate %</td>
<td>17.5 (G)</td>
<td>7.8 (G)</td>
<td>11.45 (G)</td>
</tr>
<tr>
<td>Ash %</td>
<td>19.0 (G)</td>
<td>10.0 (G)</td>
<td>11.0 (G)</td>
</tr>
<tr>
<td>$\left[\alpha\right]_D$ in water</td>
<td>$-47^\circ$ (G)</td>
<td>$-31^\circ$ (G)</td>
<td>$-49^\circ$ (G)</td>
</tr>
<tr>
<td>% Starch (c)</td>
<td>ca. 2.0 (G)</td>
<td>9.0 (G)</td>
<td>3.5 (G)</td>
</tr>
<tr>
<td>1,3'-linked xylose and rhamnose</td>
<td>x</td>
<td>x (G)</td>
<td>x (G)</td>
</tr>
<tr>
<td>1,4'-linked xylose and rhamnose</td>
<td>x</td>
<td>x (G)</td>
<td>x (G)</td>
</tr>
<tr>
<td>4-O-glucuronosyl rhamnose</td>
<td>x</td>
<td>x (G)</td>
<td>x (G)</td>
</tr>
<tr>
<td>Iodine stain</td>
<td>purple</td>
<td>purple</td>
<td>purple</td>
</tr>
</tbody>
</table>

(a) *Brading 1954; Wold 1961.*

(b) *O'Donnell and Percival 1959.*

(c) *Possibly influenced by seasonal variation.*
Thus each weed metabolises a starch polymer similar in many respects to land plant starches, and also appears to contain a small proportion of a laminarin-type glucan which is mainly immune to oxidation by periodate. The main monosaccharide residues in each case are glucose, xylose and rhamnose, along with ca. 20% glucuronic acid, the latter being attached to the C₄ position of rhamnose and apparently occupying an end-group position, since each of the water-soluble polysaccharides on acidic hydrolysis yield 4-0-glucuronosyl-L-rhamnose. Furthermore, the polysaccharides contain a comparable proportion of sulphate ester groups some of which in *E. compressa* are now known to be linked to position C₂ of rhamnose; there are indications that rhamnose also carries sulphate in *A. centralis* (O'Donnell and Percival 1959). Again, it seems likely that, since none of these weeds has a hexose as a major sugar unit, the majority of sulphate residues are attached to secondary hydroxyl groups and will probably undergo desulphation in a similar manner to those of *E. compressa* polysaccharide.

Examination of the oxopolysaccharides from each polymer indicates the presence of many 1,3'-linked residues, since a fairly high proportion of xylose and rhamnose units remain unattacked. Furthermore, methylation of *U. lactuca* and *A. centralis* showed the presence of both 1,4'-linked xylose and rhamnose, as did methylation of mixed *Enteromorpha* spp. by Lowe (1956).
The rotation of each water-soluble extract is similar, the difference probably being due to the varying starch content of the material, and it seems likely that the starch-free polymers also have similar rotations.

E. compressa and U. lactuca have $[\alpha]_D -87^\circ$ and $-71^\circ$ after removal of the starch-type glucan.

Attempts to effect a fractionation of the xylose-rhamnose-glucuronic acid complex from A. centralis, E. compressa and U. lactuca have failed, thus emphasizing that each comprises a single heteropolysaccharide consisting of these units.

With the accumulation of information on the constitution of the polysaccharides metabolised by Ulva lactuca, Acrosiphonia centralis and Enteromorpha compressa it becomes more and more evident that the carbohydrate synthesis of these three species of green algae, if not identical, is indeed very similar and the present studies on E. compressa have served to emphasize these similarities. With the advent of desulphation of these polysaccharides considerable progress in their structural elucidation should now be possible. Methylation studies on the original and desulphated material and comparison of the hydrolysis products should provide definite proof of the site of the sulphate ester groups and additional information on the types of linkage and shape of the molecule.
BIBLIOGRAPHY

(2) Anderson & Crowder, (1950), J.A.C.S., 52, 3711.
(3) Anderson & Wylam, (1950), CHEM. & IND., 141.
(5) Anderson, Hirst & Manners, (1957), CHEM. & IND.
(9) Araki, (1944), J. CHEM. SOC. JAPAN, 65, 725.
(10) Araki, (1944a), J. CHEM. SOC. JAPAN, 65, 533; 627.
(11) Araki & Hirase, (1953), BULL. CHEM. SOC. JAPAN, 26, 463.
(21) Barry, (1939), SCI. PROC. ROY. DUBLIN SOC., 28, 59.
(22) Barry, (1942), J.C.S., 578.
(23) Barry & Dillon, (1945), PROC. ROY. IRISH ACAD., 50B, 349.
(28) Bauer, (1894), J. PRAKT. CHEM., 30, 367.
(31) Bishop, (1953), CANAD. J. CHEM., 31, 134.
(33) Bonner & Koehler, (1948), J.A.C.S., 70, 315.
(34) Bottle, Gilbert, Greenwood & Saad, (1953), CHEM. & IND., 541.
(35) Bourne, Haworth, Macey & Peat, (1943), J.C.S., 924.
(40) Broatch, (1956), PH.D. THESIS, EDINBURGH.
(42) Butler & Cretcher, (1929), J.A.C.S., 51, 1519.
(44) Challinor, Haworth & Hirst, (1931), J.C.S., 258.
(48) Colin & Richard, (1930), BULL. SOC. CHIM. BICL., 12, 1392.
(49) Colin & Augier, (1933), COMPT. REND., 197, 423.
(73) Fleming, Hirst & Manners, (1956), J.C.S., 2831.
(74) Fleury & Lange, (1933), J. Pharm. Chim., 17, 196.
(76) Freudenberg & Raschig, (1929), BER., 62, 673.
(77) Frush & Isbell, (1956), J.A.C.S., 78, 2844.
(78) Gee, (1940), TRANS. FARADAY SOC., 36, 1164.
(80) Gilbert, Stärke, (1958), 5, 95.
(82) Greenwood, (1956), ADV. CARB. CHEM., 383.
(85) Haggland, Lindberg & McPherson, (1956), ACTA CHEM. SCAND.,
(86) Hands & Peat, (1939), NATURE, 142, 797.
(89) Head & Hughes, (1952), J.C.S., 2046.
(90) Higginbotham, (1949), SHIRLEY INST. MEM., 23, 171.
(92) Hirase, (1957), BULL. CHEM. SOC. JAPAN, 30, 68.
(93) Hirst, Jones & Jones, (1939), NATURE, 143, 857.
(94) Hirst, Jones & Roudier, (1948), J.C.S., 1779.
(95) Hirst, Hough & Jones, (1949), J.C.S., 928.
(96) Hirst, (1958), PROC. CHEM. SOC., 177.
(98) Hodge, Karjala & Ribbert, (1951), J.A.C.S., 73, 3312.
(99) Hostettler & Deuel, (1951), HELV. CHIM. ACTA, 34, 2240.
(100) Hough, Jones & Wadman, (1952), J.C.S., 3323.
(113) Liddle & Manners, (1957), *J.C.S.*, 3432.
(115a) Lowe, (1961), Private Communication.
(122) Manners & Wright, (1960), unpublished work.
(127) Monier-Williams, (1921), J.C.S., 303.
(129) Mori & Tsuchiya, (1941), J. AGR. CHEM. SOC. JAPAN, 14, 585.
(130) Mori, (1953), ADV. CARB. CHEM., 8, 315.
(131) Muskat, (1934), J.A.C.S., 56, 693.
(134) Neuberg & Ohle, (1921), BIOCHEM. Z., 125, 311.
(136) Nicolai & Preston, (1952), PROC. ROY. SOC. B, 140, 244.
(138) Nissenbaum & Hassid, (1951), J. BIOL. CHEM., 190, 673.
(150) Peat & Turvey, (1959), NATURE, 179, 262.
(154) Percival E.G.V., (1944), NATURE, 154, 673.
(155) Percival E.G.V. & Ross, (1949), J.C.S., 3041.
(155a) Percival E.G.V., (1949), QUART. REVS., 3, 369.
(156) Percival E.G.V. & Chanda, (1950), NATURE, 166, 787.
(158) Percival E.G.V. & Ross, (1951), J.C.S., 720.
(159) Percival E.G.V., (1950), MURJ., 154, 673.
(160) Percival E.G.V. & Boss, (1949), 3041.
(161) Percival E.G.V. & Boss, (1950), HAT, 1KB., 166, 787.
(162) Percival E.G.V. & Ross, (1950a), J.C.S., 717.
(163) Percival E.G.V. & Ross, (1951), J.A.C.S., 72, 593.
(164) Percival E.G.V. & Ross, (1950a), J.C.S., 717.
(165) Percival E.G.V. & Ross, (1951), J.A.C.S., 72, 593.
(166) Percival E.G.V. & Ross, (1950a), J.C.S., 717.
(167) Percival E.G.V. & Ross, (1951), J.A.C.S., 72, 593.
(168) Percival E.G.V. & Ross, (1950a), J.C.S., 717.
(169) Percival E.G.V. & Ross, (1951), J.A.C.S., 72, 593.
(170) Percival E.G.V. & Ross, (1950a), J.C.S., 717.
(179) Spoehr, (1947), ARCH. BIOCHEM., 14, 153.
(180) Spolter & Marx, (1960), BIOCHIM. BIPHYS. ACTA., 28, 123.
(182) Steiner & Guthrie, (1944), IND. ENG. CHEM. (ANAL. ED.), 16, 736.
(183) Stephen, (1951), J.C.S., 646.
(184) Stumpf, (1948), J.B.C., 176, 240.
(185) Swenson, McCready & Maclay, (1946), IND. ENG. CHEM. (ANAL. ED.), 290.
(187) Thompson, Wolfrom & Quinn, (1953), J.A.C.S., 75, 3003.
(188) Tipson, Christman & Levene, (1939), J. BIOL. CHEM., 138, 609.
(190) Vincent, (1960), CHEM. & IND., 38, 1109.
(195) Williams, (1957) PH.D. THESIS, EDINBURGH.
(196) Wilson, (1959), ANAL. CHEM., 21, 1199.
(197) Wise, (1945), IND. ENG. CHEM. ANAL. ED., 17, 63.
(198) Wise, (1946), IND. ENG. CHEM. (ANAL. ED.), 290.
(199) Wold, (1961), PRIVATE COMMUNICATION.

(204) Wolstenholme & O'Connor, (1958), Ciba FOUNDATIONS SYMPOSIUM, "CHEMISTRY AND BIOLOGY OF MUCOPOLYSACCHARIDES", CHURCHILL, LONDON.

(205) Wright, (1960), PH. D. THESIS, EDINBURGH.
ACKNOWLEDGMENTS

The author wishes to record his thanks to Professor E.L. Hirst, F.R.S., for his interest in this work. He also wishes to express his sincere gratitude to Dr. E.E. Percival under whose supervision the experimental work was carried out.

In conclusion, the author is indebted to the Department of Scientific and Industrial Research whose financial assistance made this research programme possible.