Investigation and Structural Studies of the Carbohydrates metabolized by the Green Algae Urospora and Mougeotia.

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by

Martin Lindsay Megarry

Chemistry Department
Royal Holloway College
Englefield Green
Surrey.

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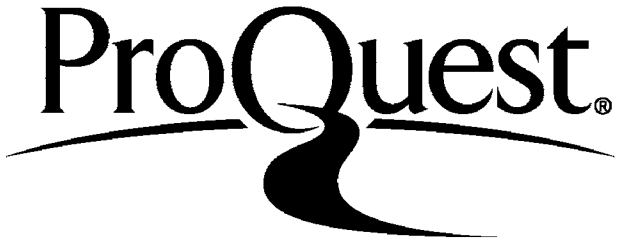


Contents

ABSTRACT

Part I Investigation of the Carbohydrates synthesized by the marine green alga Urospora penicilliformis.

The alga was sequentially extracted, and the following carbohydrates were isolated and characterised.

1) Low molecular weight carbohydrates: glyceric acid, myo-inositol, glucose, fructose, sucrose and a homologous series of malto-oligosaccharides.

2) The aqueous extracts yielded a mixture of polysaccharides

   a) Amylose was fractionated from the mixture as the starch-iodine complex, and characterised

   b) An α- 1,3-linked mannan was fractionated from the mixture by DE.52 cellulose, and characterised by classical techniques.

   c) The major polysaccharide, a sulphated glucuronoxylorhamnan, contains rhamnose, xylose and glucuronic acid in the approximate molar proportions 5:7:2, together with 17-22% of sulphate. Each constituent sugar was separated and characterised.

The polysaccharide was examined by desulphation, methylation, periodate oxidation and autohydrolysis techniques. The major structural features are 1,3-linked rhamnose usually sulphated at C-2 and sometimes also at C-4; 1,4 linked xylose together with some units linked additionally through C-2; and end group 1,4-linked glucuronic acid. In addition small amounts of 1,4-linked rhamnose, end-group xylose and 1,3-linked glucuronic acid and xylose, with lesser amounts of triply linked rhamnose, also occur.

3) Alkali extraction yielded a β-1,4-linked glucan, thought to be a degraded cellulose. The residue also contains a cellulose-type glucan.

Part II. Mougeotia.

This alga was also sequentially extracted, and the following carbohydrates isolated

a) Low molecular weight carbohydrates showed sucrose, glucose, fructose, myo-inositol, galactose and a homologous series of malto-oligosaccharides.

b) The aqueous extract showed a complex mixture containing principally uronic acid, glucose, galactose, mannose, xylose, and arabinose, with lesser amounts of rhamnose and fucose. The glucose appeared to be derived from contaminating cell wall debris.

Fractionation on DE52-cellulose yielded five fractions with increasing proportions of uronic acid but of similar carbohydrate composition. None of these fractions contained glucose.

Periodate oxidation followed by hydrolysis gave erythritol, glycerol, erthythronic acid, and glyceric acid, together with uncleaved sugars, suggesting a highly branched polysaccharide mixture with 1,4-linked, 1,3-linked (or branched) and end group residues.

Hydrolysis led to the separation of a β-D-glucuronosyl (1→3)-D-galactose, indicating that this is a structural feature of the macromolecule.

The presence of cellulose in the cell walls was inferred from a parallel study on a cell-debris rich extract.

c) Subsequent sequential extracts with acid and alkali showed a similar pattern of constituent sugar residues, with increasing proportions of glucose and xylose. The final residue again contains cellulose-type polysaccharides.

Appendix I

A preliminary study of the freshwater green alga Microspora sp. indicated that sucrose (major), glucose, fructose, galactose and myoinosital constituted the low molecular weight carbohydrates. A complex mixture of polysaccharides containing galactose, glucose, mannose, xylose, rhamnose and fucose were also extracted. These defied fractionation on DE-cellulose.

PREFACE

The work described in this thesis was carried out principally on Urospora penicilliformis. Since this alga is highly seasonal in its nature, and since also it never grew in large amounts, it was necessary to work on a second alga, Mougeotia, when the supplies of Urospora material were unavailable.

I am most grateful both to the Governors of Royal Holloway College and of Christ's Hospital for the award of scholarships, and to Dr E Percival and Professor E J Bourne for financial assistance from funds at their disposal.

It is a pleasure to acknowledge the help that I have had from many quarters within the College and from my family. I would wish to acknowledge especially the assistance of my supervisor, Dr E Percival, and of my typist, Mrs Kearsey, together with the willing co-operation of the technical and secretarial staff of this department without whose courtesy and help the work would have been both more arduous and less pleasant.

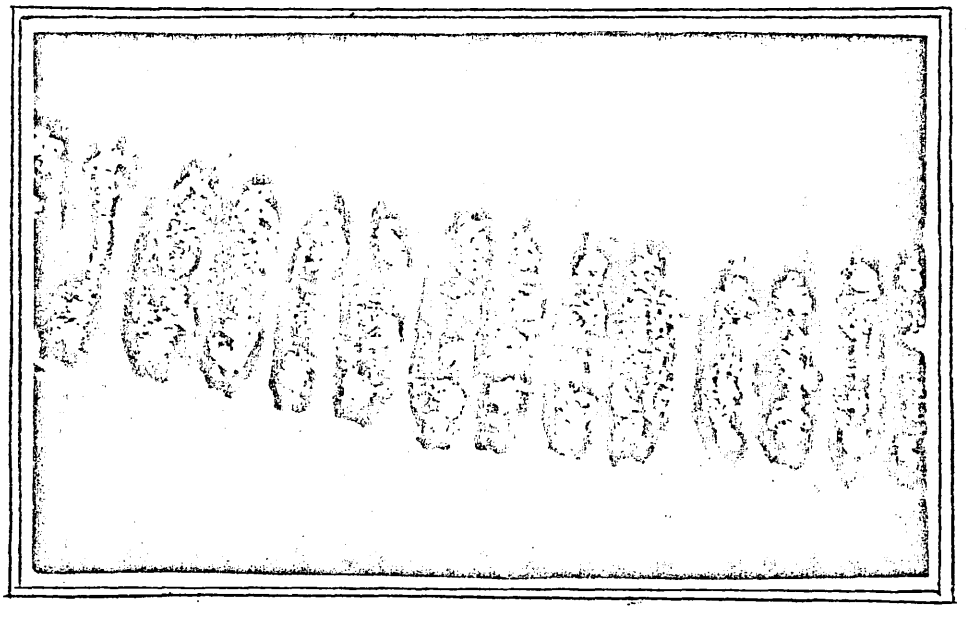
M. L. Megarry.

M.L.Megarry
17.4.1973.

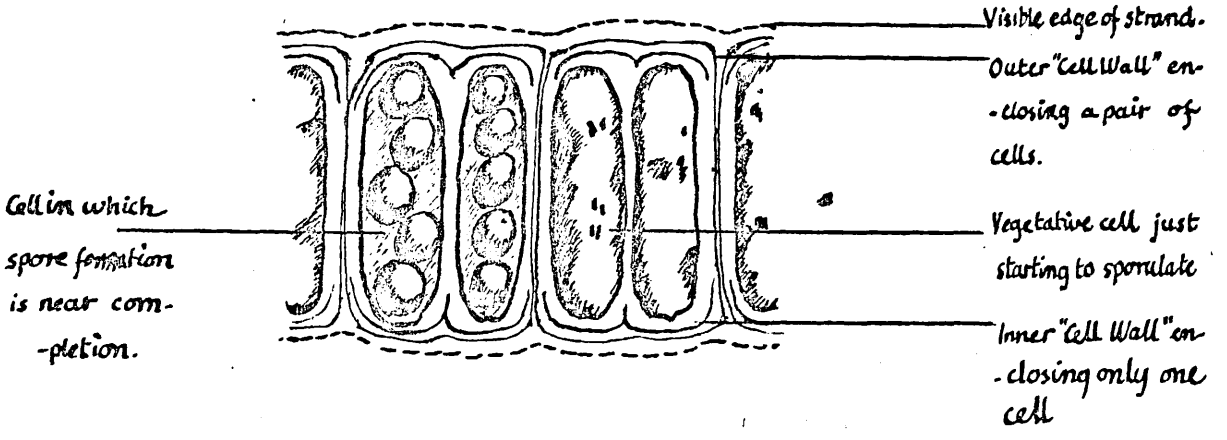
Frontispiece

Urospora penicilliformis filament X 1000

In Indian ink suspension



The vegetative filaments are just starting to sporulate. The total thickness of the mucilage is not visible, but the cell walls can be seen:-



INTRODUCTION

The term algae covers the very wide range of typically aquatic, morphologically simple organisms that make up the greater part of the earth's photosynthesizing biomass. They range in size from simple unicells less than a micron across, to large seaweeds over a hundred metres long. Although usually marine or fresh water organisms, algae have been found growing in hot springs (70°C), ice and snow, and on dry ground; in symbiotic association with fungi they form lichens.

The classification of the algae[1] is accordingly complex and subject to frequent, mainly minor, changes. Among the features used for this purpose are the presence or absence of cell components such as nuclei, membranes, flagellae, and cell walls, together with their structure; the methods of movement and reproduction; the pigment balance; and the chemical nature of the food reserve materials, mucilages and cell walls present.

The more prominent algal divisions are the Cyanophyta (blue-green algae), Rhodophyta (red algae), Phaeophyta (brown algae), Bacillariophyta (diatoms) and Chlorophyta (green algae), some of which are of economic importance either directly as sources of raw materials or food, or indirectly as manures.

The carbohydrates metabolized by the algae can be divided roughly into five groups, not all of which are necessarily present in each species:-

1) Low molecular weight materials which occur dissolved in the cell sap.

2) Food reserve polysaccharides, often deposited as granules in the cytoplasm.

3) Mucilages, found on the cell surface. These serve as a protective coating, so that epiphytes and parasites do not easily secure a hold on the cell surface. The mucilage is replaced from within the cell as it is dissolved off the surface.

4) A matrix of polysaccharide material encrusting

5) the "cellulosic" fibrils, the two together constituting the cell wall.

While the type of polysaccharide remains constant for a given group of algae, there is evidence for seasonal, environmental and physiological variations in the amount and fine structure of a particular polysaccharide. Thus, for example, laminaran is more abundant in spring and summer than in winter, while the proportions of laminaran and fucoidan appear to vary with the degree of exposure to air between tides, the latter being more abundant in weeds higher up the shoreline than in those further down.[2]

Major Taxonomic Divisons of the Algae

Cyanophyta

The Cyanophyta are largely freshwater plants with some marine genera, though the range of habitat also includes hot springs, snow and ice, lichens, and dry ground. Some genera share with bacteria an ability to fix nitrogen.[3]

The division is devoid of nuclear membranes, endoplasmic reticulum and Golgi body. The food reserve material is an amylopectin-type glucan.[4] Unlike the mucilage of other algae, the mucilage of *Microcystis flos-aquae* is impermeable to electrolyte.[5]

Rhodophyta

The Rhodophyta consist mainly of red seaweeds[1] which are normally attached to sublittoral rocks, though a number of littoral, planktonic and freshwater species are known.

The major low molecular weight materials are floridoside[6] isofloridoside,[7] 3-O-floridoside α-D-mannopyranoside,[8] and 2-O-D glyceric acid α-D-mannopyranoside,[9] while the main food reserve material is an amylopectin-like material known as floridean starch, which occurs as granules deposited in the cytoplasm.[10] A xylan containing varying proportions of β-1,3-and β-1,4-linked xylose residues in random order has also been isolated from some seaweeds[11] and possibly fulfils the same function.[12]

Sulphated polysaccharides are synthesized and/[a] constitute family of galactans consisting of alternately α-1,3- and β-1,4-linked galactose residues. These vary in the proportions of D- and L-galactose and 3,6 anhydrogalactose; in the extent of sulphation and methylation of the individual units,[13] and in the presence, in some cases, of small amounts of xylose, glucuronic acid, and pyruvic acid.[14] Trivial names such as agar, carrageenan, furcellaran, porphyran[12] and aeodan[15] have been given to extracts from various algae. Some of these can be fractionated into a number of components, for example agar into agarose and agaropectin, and carrageenan into K-carrageenan and a number of other molecular species differing principally in the content and site of the half-ester sulphate.[16,12] Some of these are of considerable commercial and scientific value, this, in the case of the agars for example, being due to their excellent gelling properties and their resistance to bacterial attack.

The structural carbohydrate of the cell wall appears to be cellulose,[17] except in Porphyra spp., where it is at least partially replaced by a β-1,4-linked mannan.[18]

Phaeophyta

The Phaeophyta or brown algae are almost exclusively marine, and normally grow as a somewhat differentiated thallus attached by a rhizoid or holdfast to littoral or sub-littoral rocks, although unattached and planktonic forms are found.

D-mannitol,[19] together with lesser amounts of 1-O-D-mannitol β-D-glucopyranoside and 1,6-O-D-mannitol di-(β-D-glucopyranoside)[20] form the major low molecular weight materials metabolized by this division.

A linear glucan, laminaran, forms the major food reserve material. The glucose residues are mainly β-1,3-linked, though a small variable number are β-1,6-linked.[21] In Laminaria spp. about half of the laminaran chains are terminated at the reducing end by D-mannitol,[22] but this is not the case with the Bifucaria, Himanthalia and Padina spp.[23]

A sulphated family of xylogalactoglucuronofucans is also metabolized, which ranges from a glucuronic acid rich, fucose and sulphate poor, polysaccharide to a practically pure fucan sulphate (fucoidan)[23] which acts as a food-reserve or mucilage material. In all of them the fucose is mainly 1,2-linked with half-ester sulphate on C-3 and C-4, the galactose being terminal.[23b] The glucuronic acid and xylose present are either 1,4-linked or terminal.[23a] A suggested metabolic pathway[24] is from the fucose-poor to the fucose-rich polysaccharide.

However the major polysaccharide of economic importance in this division is alginic acid, which is widely used, for example, as a food additive. This is a family of polyuronides which contain varying

proportions of β-1,4-linked mannuronic[25] and guluronic acid[26] residues.

The structural polysaccharide is again cellulose, which rarely attains 10% of the dry weight of the plant.[19]

Bacillariophyta

The Bacillariophyta or diatoms are a large class of small unicellular or filamentous algae which secrete silica cell walls. Deposits of these are of commercial value and have been used as sources of 'Celite' and related materials.

One of the few species investigated chemically is Phaeodactylum tricornutum[28] which may later prove to be typical of the division. The main low molecular weight carbohydrates are glucose, laminitol and other inositols, while the food reserve material is a laminaran-type β-1,3-linked glucan. The sulphated mucilage appears to have a 1,3-linked D-mannose backbone occasionally sulphated, to which are attached branches capable of yielding D-glucuronosyl-(1→ 3)-D-mannopyranosyl(1→ 2)-D-mannopyranose on mild hydrolysis, though the actual linkage to the backbone is not known.

Chlorophyta

The Chlorophyta are distinguished from other algal divisions, and resemble higher plants, in metabolizing a starch comprising amylose and amylopectin, which are of lower molecular weight and deposited in smaller granules than the starches of higher plants,[29] and in the possession of Chlorophyll b. While the distribution of the division is largely fresh-water, marine and terrestrial genera are known.[1]

The Chlorophyceae number about eight thousand species distributed in about twelve orders, of which only three, the Cladophorales, Siphonales and Ulvales have been investigated in chemical detail.

The main low molecular weight carbohydrates found are sucrose, glucose and fructose, though inositols and other carbohydrates occur,[30] sometimes abundantly.

Apart from starch, other food reserves have been found in the Dasycladeaceae (Siphonales) characterized as fructans of the levan (2,6'-linked) or inulin (2,1'-linked) type.[31] This uncertainty has been resolved in the case of Acetabularia crenulata where the fructan has been shown to be of the inulin type.[32]

The sulphated mucilages metabolized by the Chlorophyceae can be divided into three types. The first is typified by Cladophora rupestris[33] and Chaetomorpha spp.[34] (Cladophorales)[/Polysaccharides of a similar type has been found in Caulerpa[35] and Codium[36a] (Siphonales)] and contains D-galactose, D-xylose and L-arabinose, together with about 18% half-ester sulphate and lesser amounts of other sugars. The macromolecule consists of blocks of α-1,4-linked arabinose units sulphated occasionally at C-3. The galactose in the molecule is 1,3-linked pyranose and 1,6-linked furanose linked and is also occasionally sulphated. The xylose, when it occurs, is 1,4-linked or end-group. Small amounts of rhamnose (1,3-linked), mannose and glucose have also been reported. The second type has been found in Acetabularia crenulata[32] and contains from 6-27% half-ester sulphate. The galactose in the macromolecule is 1,3-linked or end group, variably sulphated at C-4 and C-6, and some at least is mutually linked. L-rhamnose (1,2-linked or end group) and D-glucuronic acid (end-group), linked to either rhamnose or galactose are present, with lesser amounts of 4-O-methyl-D-galactose and D-xylose.

The third type, typified by the mucilage of Ulva lactuca[37] and Enteromorpha spp.[38] (Ulvales), and Acrosiphonia arcta[39] (Cladophorales) is a glucuronorhamnoxylan carrying from 6-20% sulphate. A more detailed

description of these extracts will be found in the introduction to Part I.

The main structural polysaccharide of the Chlorophyceae is cellulose, the fibrils of which are often thickly overlaid with a polysaccharide containing galactose, arabinose, xylose and glucuronic acid.[12] In addition a β-1,4-linked mannan has been found in the Siphonales[40,36b] which has crystalline and amorphous regions similar to those of cellulose. Two families of the Siphonales (the Caulerpaceae and Codiaceae[40,41]) also deposit a linear β-1,3-linked xylan.

Techniques available for Purification and Structural Investigation of Carbohydrates in Algae

It is essential that the alga under investigation should be as free from other algae and debris as possible. This is a reasonably simple operation in the case of the large brown seaweeds, but with filamentous green algae it is often a difficult and tedious task, in which hand-picking, flotation, differential sedimentation and sieving methods are used. Furthermore these methods may fail to separate algae of similar form but different genera, and so the stand of algae used must be predominantly of one genus for the structural work to be meaningful.

Extraction, purification and separation

The extraction techniques employed on a particular alga varies from one genus to another according to the specific problems presented by each one. However the basis of most methods is a sequential extraction by solvents of increasing solvolytic strength, a process combining the minimum of degradation of the derived materials with the most efficient means for their extraction. Some filamentous algae, for example, after freeze-drying, are initially extracted with chloroform, which removes lipids, glycolipids

and most pigments, and are then extracted with cold followed by hot ethanol (80%). This removes the bulk of the amino acids, inorganic salts and low molecular weight carbohydrates present, and is followed by a cold water extraction, during which the soluble polysaccharides and any remaining low molecular weight materials are removed. The residual mucilage and food reserve polysaccharides are largely extracted with hot water, after which the residue may be bleached with hot dilute sodium chlorite, a treatment which facilitates further extraction. The bleached material is further extracted either with dilute cold or hot acid, or with cold dilute followed by concentrated alkali, or by a combination of these.

The resulting series of extracts falls into two groups. The first, containing the low molecular weight carbohydrate material, may be freed from chloroform soluble material by partition between chloroform and water and from salts and amino acids by precipitation in hot ethanol and removal of the remaining dissolved ionic material by ion exchange resins. The second group, containing the polysaccharides, may occasionally contain fine cell debris, removable by prolonged centrifugation at high speeds followed by filtration through fine filters, and usually contains inorganic salts, removable by dialysis. The variable amount of protein which may be present can be removed either by precipitation between an organic and an aqueous phase;[42a] or at an appropriate pH, for example with trichloracetic acid;[43] or it can be removed by digestion with a proteolytic enzyme. Where these techniques fail to completely remove the protein contaminant, it is possible that the polysaccharide and the residual protein are covalently linked.

The residual salt-free protein-reduced polysaccharide material can often be separated into several components by fractional precipitation, by

salt and complex formation, and by column chromatography. Fractional precipitation by, for example, ethanol, will often demonstrate heterogeneity even though the separation into individual species is usually poor.[44]

Metallic salts have been used to precipitate one component out of a mixture in solution - thus K- carrageenan is removed from solution of other galactans by 0.25% potassium chloride,[45] while alginic acid can be freed from contaminating glucuronoxylofucans by its precipitation with 2% calcium chloride,[46] its further fractionation with potassium chloride or manganous sulphate yielding two fractions, one rich in mannuronic acid and the other in guluronic acid.[42b,47]

A number of metal complexing agents have been employed to achieve a separation[42d] of which cupric salts[42c,48] often in the form of Fehlings solution[49] are probably the most widely used. With this reagent both β-1,4-linked mannans[50] and a β-1,3-linked xylan[51] have been separated. Quarternary ammonium ions are also widely used as complexing agents, both on their own[42e] or in conjunction with borate[52] to separate charged from neutral polysaccharides.

Column chromatography is a powerful method for the fractionation of polysaccharides, the two main variants of which separate the components either according to their charge or to their molecular size. The first is exemplified by anionic exchange resins and celluloses such as Whatman's D.E.52 cellulose.[53] The loaded column is often eluted in a gradient or stepwise process with increasing salt concentrations, and the method is capable of very efficient separations according to increasing molecular charge. The second is exemplified by a wide range of cross-linked dextrans

such as the Sephadex series, which separate the polysaccharides according to decreasing molecular size by a gel-filtration or exclusion effect.

The homogeneity of the purified polysaccharides can be tested by use of the ultracentrifuge, which separates molecules according to their size and density; by zone electrophoresis, which separates according to the molecular degree of ionization; and gel-electrophoresis,[54] which separates according to both the charge-density and the size of the molecule. With differential staining techniques this latter method can be made to detect if any residual protein in the extract has the same electrophoretic mobility as the polysaccharide, in which event a covalent link between the two is likely.

Analysis and Characterization of Polysaccharides

The polysaccharide to be investigated can vary from a pure homopolymer whose analysis is relatively simple, to a complex heteropolysaccharide with variable amounts of several kinds of sugar residue, uronic acid, and protein. The correspondingly more difficult analysis of such a polysaccharide may be further complicated by anhydride formation and substitution of the sugar residues with, for example, methyl groups, pyruvic acid and half ester sulphate.

Carbohydrate Content

The carbohydrate content may be assayed by methods such as the phenol-sulphuric acid [55] or the cysteine-sulphuric acid[56a] methods. Since different carbohydrates have different specific absorptions with these reagents, accurate results can only be obtained by reading against a standard graph of a synthetic mixture of sugars which are in the same

proportions as those found in the polysaccharide. These proportions may be found from the alditol trimethylsilyl (T.M.S.) derivatives of the reduced hydrolysate analyzed by Gas liquid chromatography (G.L.C.), or by elution and assay of the bands on a developed paper chromatogram; and may be checked by assays specific for one group of sugars, such as the Dische cysteine-sulphuric acid assay for 6-deoxy sugars,[56b] or the carbazole-sulphuric acid assay for uronic acids,[56c,57] in which the colour formation appears to be caused by condensation of the furfuraldehyde derivative derived from the carbohydrate with the chromogen.[56d,58]

Uronic acid may otherwise be estimated by decarboxylation[59a] or by complexing with cetyl pyridinium cations. This latter method may be extended to cover the estimation of sulphate,[60] which can also be estimated by precipitation with 4-chloro-4'-aminodiphenyl and determination of the residual absorbance.[61]

Any contaminating protein can be estimated by converting the %N to protein (100% protein = 16%N)[62] or by the Lowry modification of the Folin method.[63]

Hydrolysis and Chromatographic Analysis

The initial analysis of the constituents of a polysaccharide is normally carried out by hydrolysis with either 90% formic acid or N-sulphuric acid, under which conditions the pyranoside linkages are usually completely hydrolyzed. However uronic acid containing polysaccharides form an exception to this rule through the resistance of the uronosyl linkage to hydrolysis, and so yield aldoses, oligouronic acids and very small amounts of free uronic acid. In contrast to these, polysaccharides with furanoside residues hydrolyze very rapidly with some degradation, and are therefore hydrolyzed under milder conditions.[59b]

The derived concentrated hydrolysate is examined by paper chromatography in a number of acidic, neutral and basic solvents, the developed constituents being located with one of a number of reagents. The colour of the spot with certain of these indicates the class of sugar, for example aniline oxalate gives pink and brown colours with pentoses and hexoses respectively, while the ketose-specific reagent urea hydrochloride gives a brilliant blue colour with alduloses. Certain enzyme systems, such as glucose oxidase, have also been used in conjunction with a chromogen as specific locating agents for a particular sugar.

The chromatograms are normally run with the appropriate standards, and by comparison of the mobility and colour of the unknowns with these their probable identity may be found. Certain sugars however, such as glucose and mannose, resolve poorly in most solvents, and so a large excess of one may disguise the presence of the other. This difficulty may be overcome by running a chromatogram in a borate containing solvent, since mannose complexes more effectively with the borate than does the glucose, and so a good separation is obtained.

The presence of any uronic and aldobiuronic acids suggested by paper chromatography can be checked by electrophoresis in a neutral buffer. Pyridine:acetic acid buffer is nearly ideal, since it does not complex with the sugars, and, owing to its volatility, may be readily removed from the chromatogram. In this solvent the charged molecules move with a characteristic mobility, leaving the neutral sugars on the base-line. These latter may also be analyzed electrophoretically in the presence of molybdate or borate buffer[64,56e] with which they form charged complexes.

The original hydrolysate and the alditols derived by reduction with sodium borohydride can be characterized as their T.M.S. derivatives by

G.L.C. The resulting pattern of peaks both forms a good guide to the carbohydrates present and/also confirms the earlier paper chromatographic work.

Characterization of the Individual Sugars

Where sufficient material is available, and after the removal of any acidic sugars by ethanolic precipitation of their barium salts followed by deionization of the supernatant solution, it is usual for the remaining neutral sugars in the hydrolysate to be separated on thick paper or by column chromatography. These may crystallize, or can be converted into crystalline derivatives, which then may be characterized by their melting point and optical rotation, and possibly by infrared and X-ray powder analysis as well.

The mixture of the oligo- and mono-uronic acids precipitated as their barium salts may meanwhile be separated by paper and electrophoretic chromatography, and the mono-uronic acids isolated and characterized as the free acid, as the lactone, and as the free sugar derived by hydrolysis of the reduced methylglycoside methyl ester. These methods are also applicable to the analysis of the constituents of the oligo-uronic acids.

Linkage Analysis

Once the constituent sugars and their substituents have been characterized and their relative proportions found, it is then necessary to find out how and in what order these are linked together, a problem often solved by the use of the complementary techniques of methylation, periodate oxidation, partial hydrolysis and desulphation.

I. Methylation

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The polysaccharide may be methylated by a variety of methods. If uronic acid residues are present, complete methylation is difficult

and these residues in the partially methylated polymer are usually then reduced to the parent sugar by lithium aluminium hydride[65a] in an appropriate solvent. After remethylation the fully methylated polymer is hydrolyzed to its constituent sugars. Since all the free hydroxyls in the original polysaccharide were methylated, it follows that the free hydroxyls in the hydrolysate must have been linked either to another sugar residue or substituent such as sulphate, or to have been occupied in internal pyranoside, furanoside or anhydride formation.

Characterization of the Methylated sugars.

The identity of the individual components of the hydrolysate may be indicated by the colour and mobility of the spots given on paper chromatography. If there is sufficient material available these may then be separated and characterized as crystalline materials or as their corresponding derivatives.

Where material is limited their identity may be confirmed by analysis of their methyl glycosides on G.L.C., each glycoside usually giving two or more peaks with characteristic retention times. However, since several components in a mixture can result in a very complex spectrum and monomethyl and unmethylated glycosides cannot be recovered from a stationary phase such as butan-/diol succinate, the complementary method of the G.L.C. of the alditol acetates derived from the reduced acetylated hydrolysate is needed. In this method each partially methylated sugar derivative gives one peak only, and the mono-O-methyl and unmethylated acetylated alditols have reasonable retention times.

Mass spectroscopy

The recent advent of mass-spectroscopy (M.S.) has provided a method whereby the probable structure of the methylated material identified by paper and gas-liquid chromatography can be confirmed. The smallness of the sample needed for mass-spectroscopy has meant that this method may be used in conjunction with G.L.C., the peaks being analysed as they are eluted off the column. Probably the most valuable application of this has been to the analysis of alditol acetates analysed,[66] the mass-spectrometry of trimethylsilylated or acetylated methyl glycosides being complicated by the complexity of the G.L.C. spectrum.[67,66]

In this technique the molecule may be ionized either by direct electron bombardment, or by "chemical ionization". In the former method[68a] the molecule m is ionized by the ejection of an electron: $m + e + h\nu \longrightarrow m^+ + 2e$, a high energy process which results in the complete and extensive fragmentation of the molecular ion, while in the latter, where the sample is diluted with a large excess of a suitable carrier gas such as methane,[68b] the latter is ionized:-

$$CH_4 + e + h\nu \longrightarrow CH_4^{+\cdot} + 2e.$$

which then reacts with more methane:-

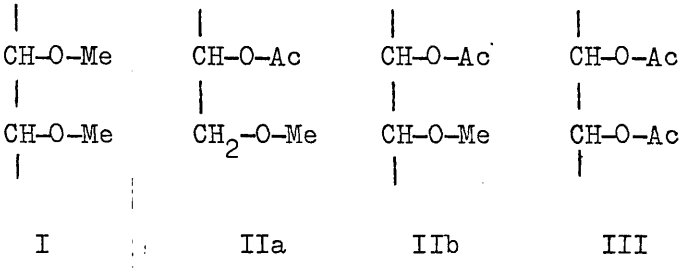
$$CH_4^{+\cdot} + CH_4 \longrightarrow CH_5^+ + CH_3^{\cdot}$$

These products may react with more methane, or the CH_5^+ ion may ionize the sample instead by transferring a proton:-

$$M + CH_5^+ \longrightarrow MH^+ + CH_4$$

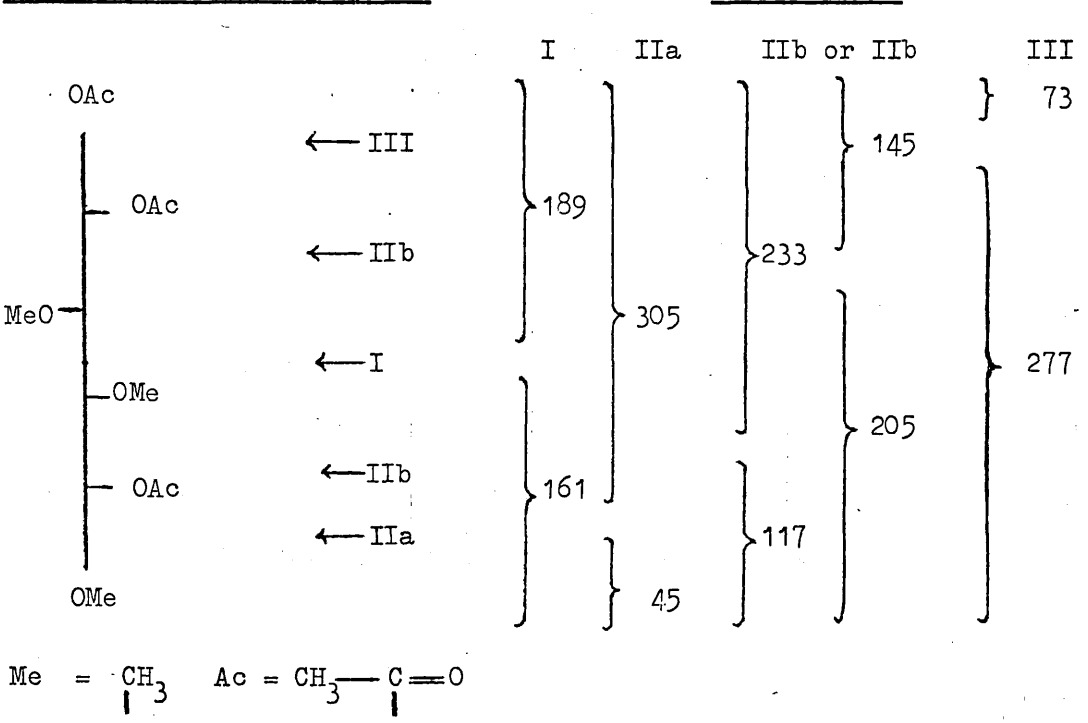
a lower energy process which results in less fragmentation of the "molecular" ion.

It has been found that ions with even numbers of electrons and therefore with odd masses are more stable than those with an odd number of electrons, and that primary ions will tend to shed their larger substituents to give smaller secondary ions.[69] With alditol acetates, in which the following groupings occur



it has been found that these will be preferentially cleaved in the order I > IIa ≫ IIb > III. In the fragmentation of 3,4,6-tri-O-methyl glucitol the following breakdown into primary ions may occur (Figure I):-

Figure I. Possible primary ions from 3,4,6-tri-O-methyl glucitol



Me = CH_3 Ac = $CH_3-C(=O)-$

Quoted figures are the mass/charge (m/e) ratios, which, since the latter is usually unitary, is equivalent to the mass of the ion.

However, the major ion produced, otherwise known as the base peak, is not derived from the primary ions as shown above, but is derived instead by fission of an acetonium ion, $CH_3-C^+=O$ from the molecular ion to give a peak of m/e = 43. Apart from this, though, the most abundant ions expected would be those derived from fission type I (m/e 161, 189) with a smaller number from type IIa (m/e 45, 305). This analysis is in fact borne out by the observed spectrum which contains the following peaks with an abundance greater than 10% of the base peak m/e 43; m/e 45 ($CH_3 - O^+ = CH_2$,) 161, and 189 as primary fragments, with m/e 87, 99, 101 and 129 derived from these and the other primary ion m/e 305 by the fission off of molecules of acetic acid (m.w. = 60) and ketene (42) or formaldehyde (30) although other alditol acetates may also eliminate methanol (32).

Since the pattern of breakdown at a given energy level is dependent only on the pattern of substitution and not on the configuration of the molecule, it follows that a 2,4-di-O-methyl hexitol acetate will be distinguished from one that is 3,4-methylated, but conversely that hexitols derived from identically substituted mannose and glucose will not be distinguished, as similarly neither will the 2,4-dimethyl hexitol derived from, for example, a 1,3,6-linked glucopyranose residue be separated from that derived from a 1,2,6-linked galactofuranose. In this case, however, the problem may be solved by reducing the original methylated sugars with sodium borodeuteride, a procedure which increases the mass of the fragments derived from the reducing end of the molecule by two.[70]

II Periodate oxidation and Smith degradation

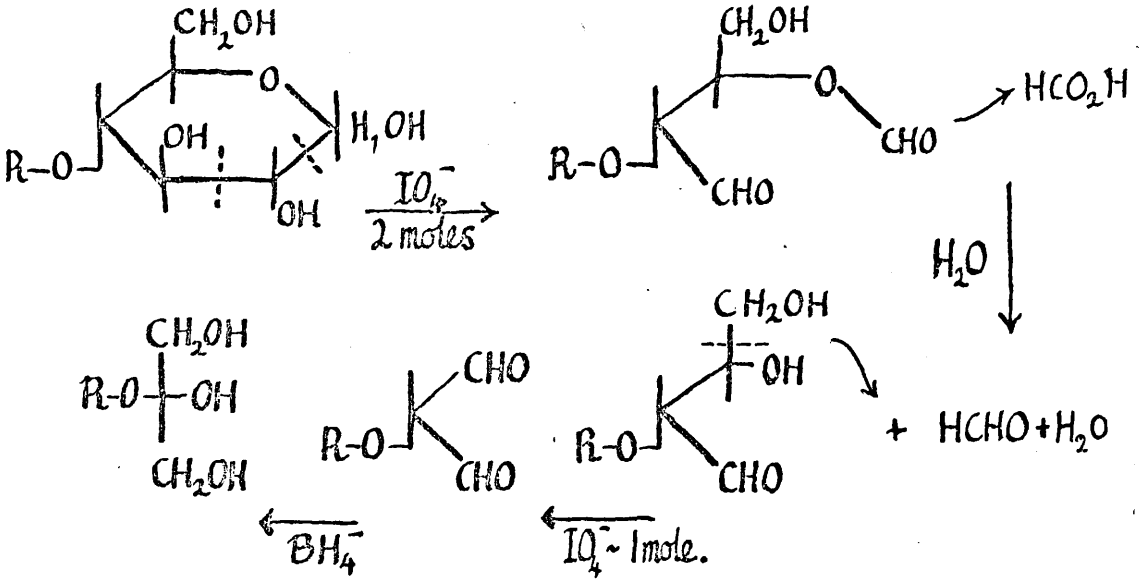
Dilute periodate solutions will cleave the carbon-carbon bond of glycol groups, with the result that in a polysaccharide those sugar residues which are either end group or 1,2- 1,4- or 1,6-linked will be cleaved, and yield characteristic fragments on hydrolysis. Since the carbonyl groups in the polyaldehyde are very unstable, it has become the usual practice to reduce the oxidized polysaccharide to the polyalcohol.[71]

A certain amount of structural information may be gained while the progress of the oxidation is being monitored. This arises from the fact that cis-glycols are cleaved rather more readily than trans-glycols[72] especially at reduced temperatures,[73] with the result that 1,4-linked rhamnose or mannose is cleaved far more readily than, for example, 1,4-linked xylose or glucose. The difference in periodate reduction at 2°C and at 20°C may therefore be used to find the approximate relative abundance of these residues in the macromolecule.

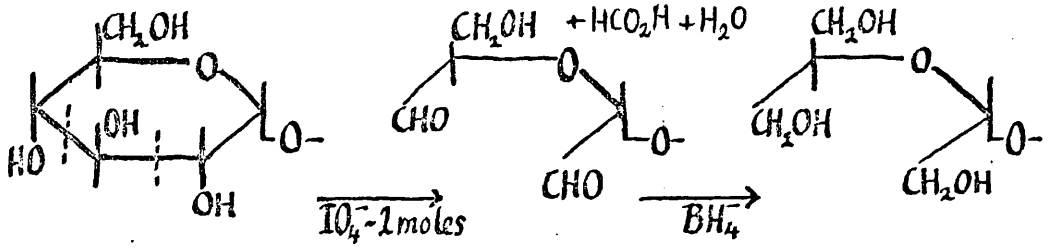
The reducing end group, providing that it is free and that it is not 2-linked, will release formaldehyde on oxidation (Figure IIa) and the assay of this will give the number average molecular weight. The degree of branching of the molecule may also be found by comparing this value for the average chain length found from the titration of the formic acid released from the non-reducing end groups (Figure IIb). However these results may be invalidated by the presence of non-reducing end-group pentofuranoside residues which do not release formic acid on oxidation (Figure IIc), and by 1,6-linked residues, which do.

Figure II. Cleavage of end-group sugar residues by periodate

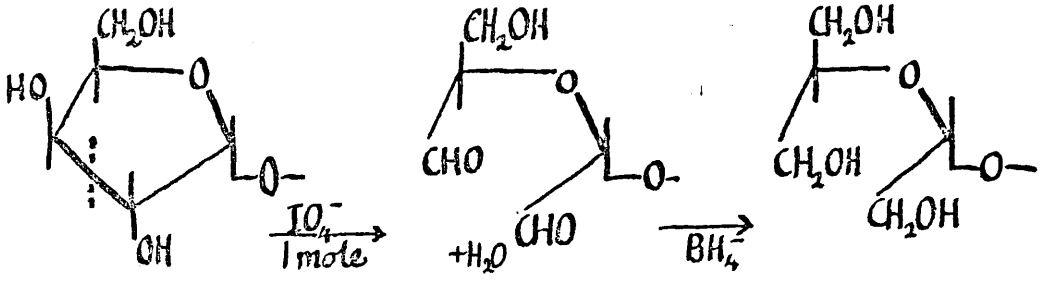
(a) Reducing end-group



(b) Non-reducing pyranosidic end-group



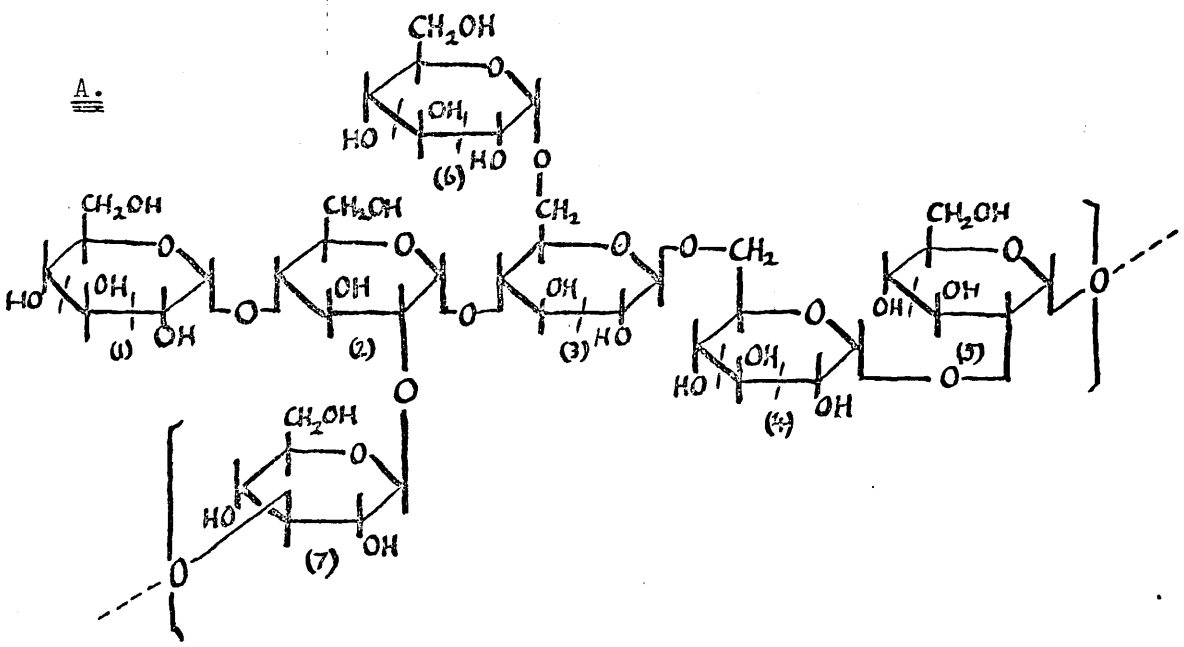
(c) Non-reducing end-group pentafuranoside



Although total hydrolysis of the derived polyalcohol and analysis of the resulting sugars will confirm the evidence of linkage given by methylation, it will not show the relative positions of the various residues within the polysaccharide. This problem may be partly overcome by a very mild hydrolysis of the polyalcohol, in which the highly acid-labile acetal links are cleaved but the glycosidic linkages are left intact, as shown in Figure III.

Figure III. Periodate oxidation and Smith degradation of a hypothetical D-glucan

A.



(1) 8 moles IO_4^-

(2) BH_4^-

3 moles HCO_2H released

B. [overleaf.]

Methylation of A: results:-

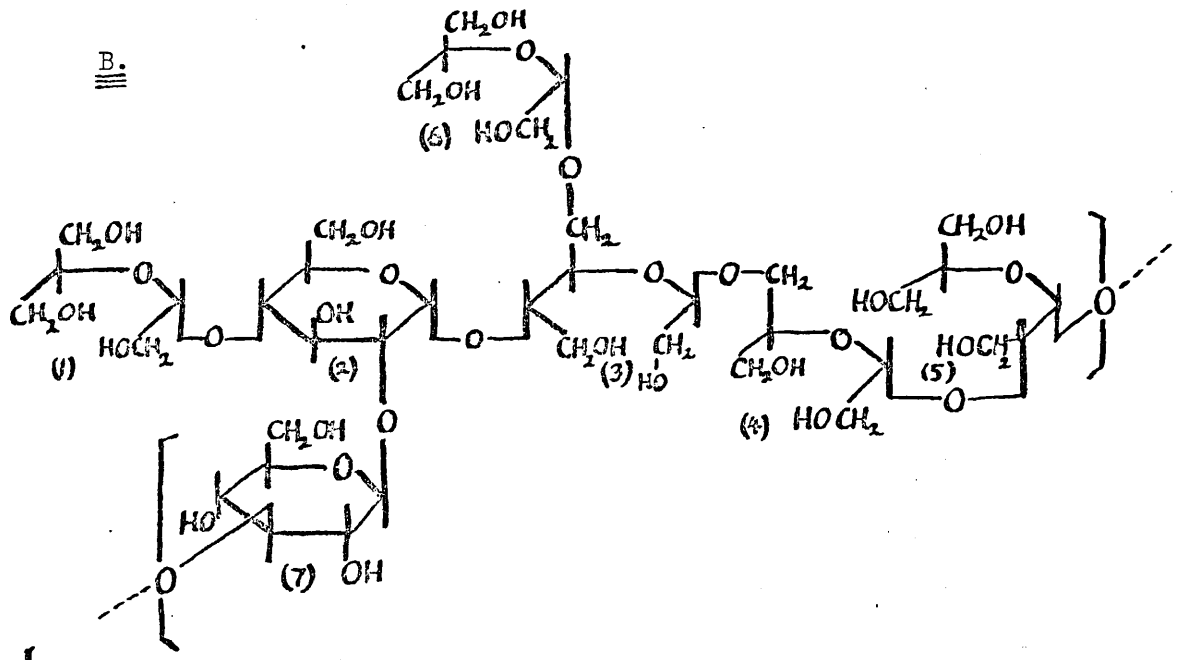
(1) 2 moles of end-group glucose.

(2) mole each of 1,3-; 1,6-; and 1,2-linked glucose.

(3) mole each of 1,4,6- and 1,2,4-linked glucose.

Continued from previous page

B.

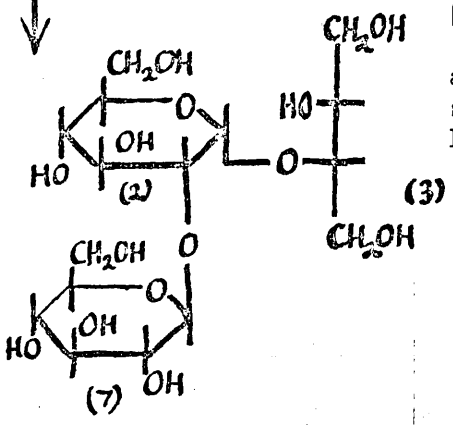


Mild Hydrolysis.
[0.01 M-H_2SO_4,
~20°C, 24hr.]

Total hydrolysis of B: result:-

(1)	Two moles of glucose	- 2 & 7
(2)	One mole of glyceraldehyde	- 5
(3)	Four moles of glycolaldehyde	- 1,3,4,6
(4)	One mole of erythritol	- 3
(5)	Four moles of glycerol	- 1,4,5,6

C.

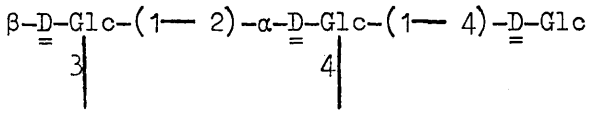


Result

β-D-glucopyranosyl (1 → 2) α-D-glucopyranosyl (1 → 2) erythritol, together with glycol aldehyde, glyceraldehyde and glycerol in the same proprotions as those derived from full hydrolysis.

Since two residues (2 & 7) are immune to periodate, these must be 1,3- or triply linked, and so confirms the presence of the 1,3- and 1,2,4-linked residues shown by methylation. The isolation of the disaccharide C shows

that these residues must have been mutually linked in the order shown to a 1,4-linked residue, which must therefore be the 1,4,6-linked residue shown by methylation, and which indicates the presence of the sub-structure



in the repeating unit of this polysaccharide. While the above formula (A) is in agreement with both the methylation and the periodate oxidation studies, other formulae can be drawn which fit the facts equally well. These formulae arise both from the variations given by the various anomeric linkages, and from the fact that either residue 4 or 5 or both can be placed between units 6 and 3 or 1 and 2 as well as between 3 and 7.

Complex heteropolymers may not, however, degrade completely to small fragments and oligosaccharides, but in addition yield a resistant "core" material after Smith degradation. This degradation will have exposed fresh glycol groupings however, and further methylation and periodate oxidation studies of this resistant core will yield additional information about the inner parts of the molecule,[74] and so this analytical cycle may be repeated. Further examples of the applications of periodate oxidation will be given later in this present work.

III. Partial Hydrolysis and analysis of the resulting oligosaccharides

When the polysaccharide is hydrolyzed by a reagent such as dilute acid a number of oligosaccharides result which embody linkages present in the original macromolecule. Not all of these linkages are equally resistant to hydrolysis, and therefore the hydrolysis is not a completely

random process; it has been found, for example, that for D-glucopyrans α-(1 → 4)-linkages are hydrolyzed four times faster than α-(1 → 6)-linkages,[75] while (1 → 3) linkages are broken even more rapidly. Certain linkages may also be cleaved by specific hydrolases and these enzymes may also be used to partially hydrolyze a polysaccharide, thus α - amylase will only hydrolyze α-(1 → 4)-D-glucopyranose linkages while β-glucuronidase will only cleave the β-linkages of D-glucuronopyranose residues.

The resulting oligosaccharides may be separated and partially characterized by the techniques already outlined. An ingenious extension of these techniques has been the recent application of mass-spectrometry to fully methylated di- and tri-saccharide alditols which has enabled these oligosaccharides to be readily characterized.[70] Their degree of polymerization (D.P.) may be found by the difference in absorbtion given on phenol-sulphuric acid assay of the reduced and unreduced oligosaccharides,[76] while the chromatographic comparison of the hydrolysates of these two materials provides evidence of the nature of the reducing end residue.

Several methods can be used for the determination of the configuration of the anomeric linkage. These include optical rotation, enzymic hydrolysis, and nuclear magnetic resonance (N.M.R.) spectroscopy, of which the most widely used is optical rotation. Unfortunately the result cannot always be interpreted unequivocally, especially for the larger oligosaccharides, since the position[77] and size[78] of each substituent affects the optical rotation.

Although the appropriate enzyme is not always available to confirm the assignment of configuration, the nuclear magnetic resonance spectrum of the reduced oligosaccharide may often be used instead.[79] Owing to the fact that C-1 is attached to two oxygen atoms rather than one, the anomeric proton resonance is separated from the signals given by the other ring protons. The exact position of the resonance varies from about 4.6 p.p.m. for an equatorial proton to about 5.5 p.p.m. for one that is axial. This signal is in turn split by coupling with the proton on C-2, the coupling constant varying from about 3.4 cps for a small angle (about 50-60°) for the intersection of the C-1–H-1 and the C-2–H-2 bonds, to about 5.7 cps for a large dihedral angle (about 150°). This information, coupled with a knowledge of the sugar residue involved, will give the configuration of the anomeric proton and greatly restrict the number of possible ring conformations. For a 1,4-linked glucose residue, the probable values are as shown in Figure IV.

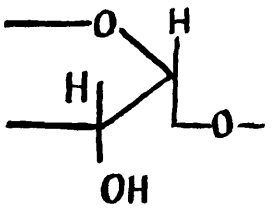
This method has also been applied to the establishment of the configuration of the anomeric carbon in other oligosaccharides[80] and to the conformation of the sugar residues of polysaccharides in solution.[79a] The usual solvent for running N.M.R. spectra is deuterium oxide, which replaces water, as it does not give an N.M.R. signal, and so will not interfere with the spectrum of the carbohydrate being examined.

Mild hydrolysis may yield an apparently homologous series of oligosaccharides, in which case a plot of the degree of polymerization (D.P.) against log (1/Rf-1), where Rf is the paper chromatographic mobility with reference to the solvent front, will yield a straight line[81] in all the usual solvents.

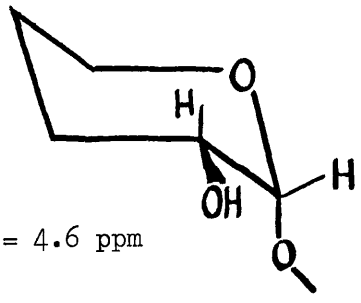
Figure IV. Probable N.M.R. signals for an α and β 1 → 4 link with glucose in the C1 or 1C conformation

α

Haworth



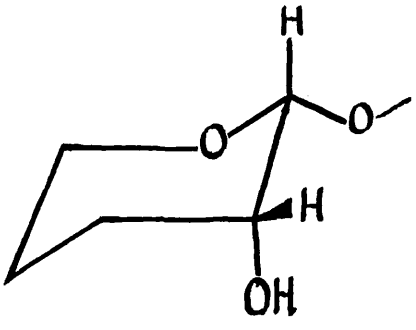
C1



τ_{H-1} = 4.6 ppm

J_{H1H2} = 3.4 cps

1C

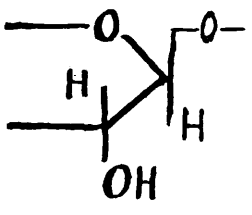


τ_{H-1} = 5.5 ppm

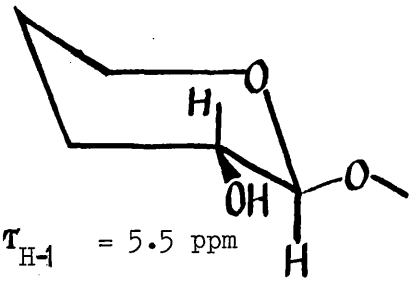
J_{H-1H-2} = 3.4 cps

β

Haworth



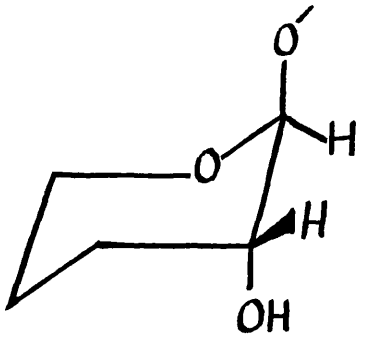
C1



τ_{H-1} = 5.5 ppm

J_{H-1H-2} = 5.7 cps

1C



τ_{H-1} = 4.6 ppm

J_{H-1H-2} = 5.7 cps

Found for maltitol τ_{H-1} = 4.62 ppm J_{H-1H-2} = 3.4 cps

Found for cellobiitol τ_{H-1} = 5.50 ppm J_{H-1H-2} = 5.7 cps

From the above maltitol must be α linked and cellobiitol β linked, and both exist in the C1 conformation.

On the application of these mild hydrolysis methods to the polysaccharide with the repeating unit (A) illustrated in Figure III, it should be possible to determine both the relative position of the respective units to each other, and also the configuration of some, though probably not all, of the glycosidic linkages joining them.

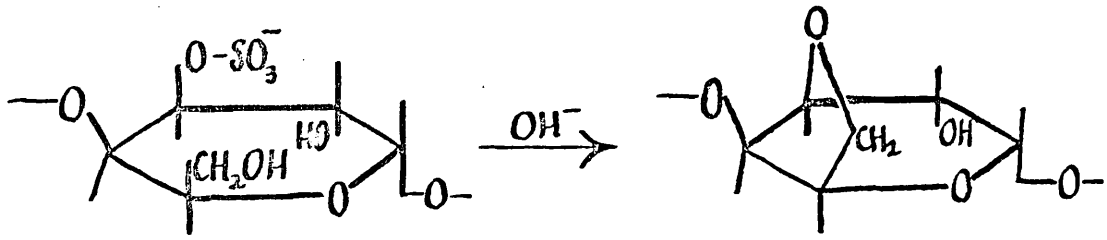
IV Desulphation of sulphated polysaccharides

The presence of half ester sulphate attached to the sugar rings complicates the linkage analysis of sulphated polysaccharides. Unfortunately the resistance to acidic hydrolysis of the $C-O-SO_3^-$ bond is of the same order as that for a glycosidic linkage, although the strength of the linkage varies somewhat with the type of substitution. In general it has been found that primary half ester sulphates ($-CH_2-O-SO_3^-$) are less readily hydrolyzed than those which are secondary ($>CH-O-SO_3^-$), the latter however being rather more resistant when axial than when equatorial.[82]

Sulphate may be sometimes nearly completely removed by a trans-esterification reaction with a reagent such as 0.09M methanolic hydrogen chloride at room temperature[83] without the macromolecule being excessively degraded. In these cases the position of the sulphate may be deduced by comparing the results obtained by periodate and methylation analyses of both the fully sulphated and the partially desulphated polymer.

If the sulphate occupies a primary hydroxyl or an axial position, mild hydrolysis will often yield sulphated mono- and oligo-saccharides which may be characterized. Galactose 6- and 4-sulphates, for example, were separated from the partial hydrolysate of the water soluble polysaccharide from Codium fragile.[84]

Some sulphate may be removed from the reduced polysaccharide by an alkaline reagent such as N-sodium hydroxide, but this will only occur if an anhydride or an epoxide ring can be formed.[85] Thus with galactose, for example, the 3,6-anhydro ring can only be formed if either C-6 or C-3 carries a free hydroxyl group, for example, as in 1,4-linked L-galactose-3-sulphate residues:-

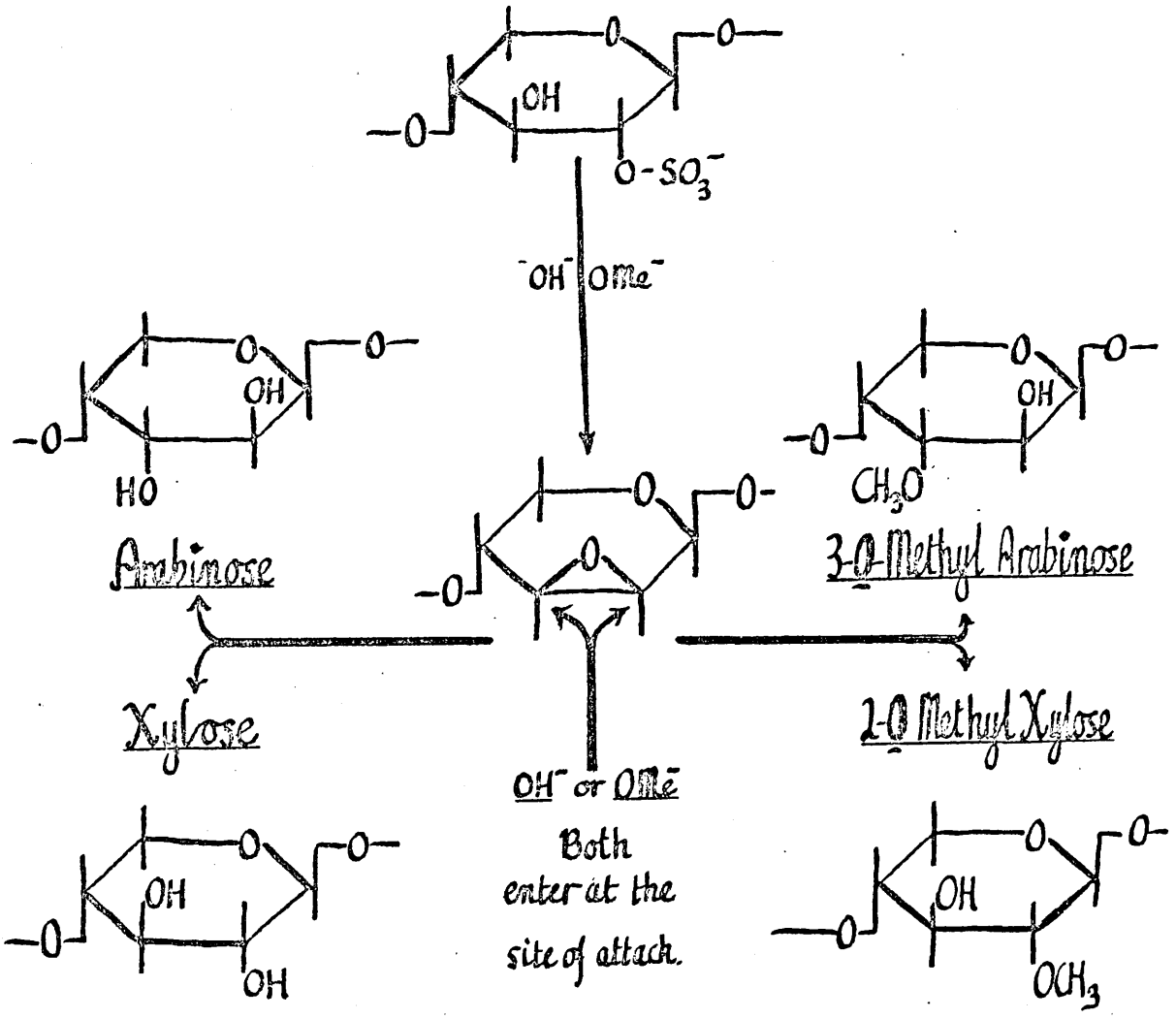


$+ SO_4^{=} + H_2O$

L-Galactose 3-sulphate — 3,6-anhydro-L-galactose

With 1,4-linked xylose 2-sulphate on the other hand, where the adjacent trans-hydroxyl is free, inversion and epoxide formation with the hydroxyl on C-3 takes place. Subsequent opening of this epoxide ring results in the formation of xylose and arabinose (Figure V). This will not distinguish between sulphate originally on C-2 from that originally on C-3, but desulphation and epoxide ring opening with sodium methoxide will result in the formation of a pair of monomethyl pentoses which are characteristic of the position of the original sulphate.[73]

Figure V. Desulphation of 1,4-linked xylosyl-2-sulphate with sodium hydroxide or methoxide



Both sugars also given by sulphate on C-3

Sulphate on C-3 would yield 3-O-methyl xylose and 2-O-methyl arabinose, since the epoxide would be below and not above the plane of the ring.

Infrared analysis of galactan sulphates showed several bands for sulphate.[86] The S=O stretching frequency occurred at 1240 cm^{-1} and was given by all sulphates, while the C-O-S frequency occurred at 800 to 860 cm^{-1}

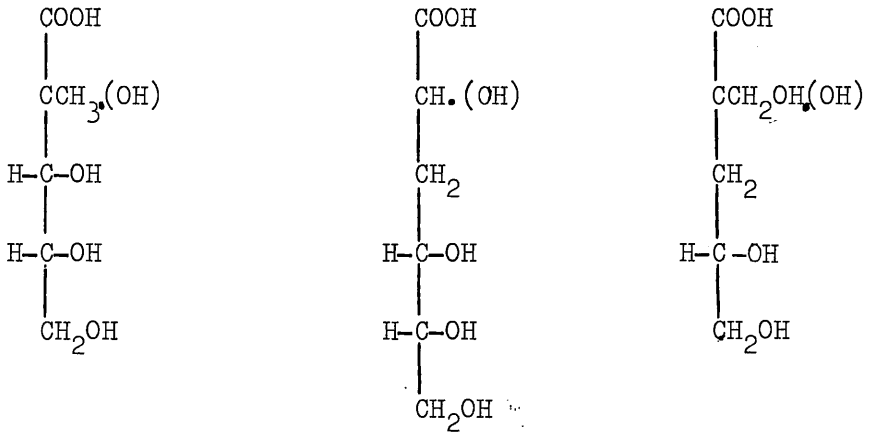
depending on the position of substitution. It was found that primary sulphates absorb at 820 cm^{-1} while equatorial and axial secondary sulphates gave absorptions at 830 and at 850 cm^{-1} respectively. If it is assumed that these results hold true for other sugar residues and furthermore that the sugar rings exist in their expected (most stable) conformation within the macromolecule, then the infrared spectra of other sulphated polysaccharides will provide a useful method for deducing the position of their sulphate groups.

V. Other methods of linkage analysis

While infrared analysis has also proved of value in comparing whether or not one particular polysaccharide is similar to a standard polysaccharide,[87] its value as a method for the diagnosis of a particular linkage in the macromolecule is unfortunately extremely limited. Enzymes are of very much greater value since they will hydrolyze only specific links, and have been widely used to elucidate the fine structures of some abundant polysaccharides, particularly the amylopectins.[88]

In the absence of oxygen, mild alkali will degrade 2 $\longrightarrow$ 1, 1 $\longrightarrow$ 3, and 1 $\longrightarrow$ 4 linked[89] polymers from the reducing end to saccharinic, meta-saccharinic and iso-saccharinic acids respectively (Figure VI), a method which was used to prove the structure of laminaran.[90] The degradation of these linkages in the molecule can however be modified or prevented altogether by the presence of branch-points. Thus the presence of a branch at C-2 of a 1,4-linked polysaccharide will prevent any further degradation, whereas if it is branched at C-3 the degradation sequence continues up the branch and blocks the main chain and so prevents further

Figure VI. Saccharinic acids derived from glucans



Gluco-saccharinic acid (from 1,2-links)

Gluco-meta saccharinic acid (from 1,3-links)

Gluco-isosaccharinic acid (from 1,4-links)

attack. Other branches on alkali labile linkages are peeled off and blocked by a residue such as meta-saccharinic acid, and the degradation then continues unimpeded up the main chain.

Macromolecular Studies

Molecular Size and Shape

The average molecular weight of a polysaccharide may be found by a variety of chemical and physical means. Thus, for example, the number average molecular weight of a particular polysaccharide may be derived from the average degree of polymerization (D.P.) found from periodate oxidation and formaldehyde release (see page 18) or from the reducing power by multiplication of the D.P. with the average molecular weight of the

anhydro units present; the contribution of any substituents is then added to give the molecular weight of the entire molecule. The method is however rather sensitive both to impurities and to the presence of blocking groups such as mannitol at the reducing end of the molecule.

Another method for obtaining M_n depends on the difference in osmotic pressure between the pure solvent and the solution of the polysaccharide. In a closed system the process of isothermal distillation takes place,[91] in which the solvent distils from the pure solvent to the solution at a rate proportional to the number of solute molecules per unit volume of solution.

Several other physical methods are also used to find the molecular weight. These all depend on both the molecular size and shape, as well as on a variable number of other parameters. It is therefore necessary to calibrate each method with standard polysaccharides of known molecular size chosen so as to resemble the 'unknown' polysaccharide as closely as possible in their physical and chemical characteristics.

An example of such a method is gel filtration, which is widely used for determination of the molecular weight, since there exists an inverse linear relationship between the logarithm of the molecular weight and the time of elution;[92] this, however, is only true if the molecules being separated have similar shapes.

Another widely used method is that of ultracentrifugation,[93a,94] in which the molecules sediment in a gravitational field (usually 59,780 g - the rate is expressed in Svedburg units, which have dimensions cm $sec^{-1}g^{-1}$). A more accurate value may be obtained from the sedimentation equilibrium in which the sample is run in a lower gravitational field until equilibrium is attained between the rate of sedimentation and the rate of back diffusion, a procedure which though lengthy, eliminates the necessity

to find the coefficient of the frictional resistance of the solution.

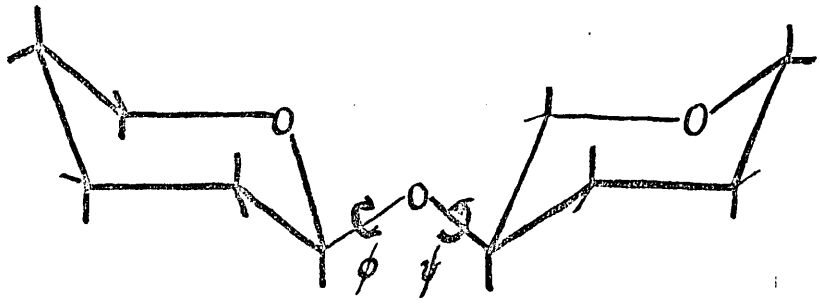
The simplest method for the determination of the molecular weight is probably the measurement of the intrinsic viscosity,[93b] but the drawback of this method is that it is strongly influenced by both the shape of the macromolecule and its degree of solvation. It has been found, for example, that the intrinsic viscosity increases as $M^{1.8}$ for a rod shaped molecule, although for a random coil it only varies as $M^{0.5}$-$M^{1.0}$, according to the degree of solvation. This method in conjunction with sedimentation techniques has proved of value in the elucidation of the shape of a molecule in solution.[95]

When light strikes a molecule in solution it will be scattered both according to the difference in refractive index of the polymer and the solvent and according to the polymer's molecular size and polarizability.[93c,96] Methods based on this effect will yield a weight average molecular weight (M_w) and the difference between this and M_n is a measure of polydispersity of the macromolecule.

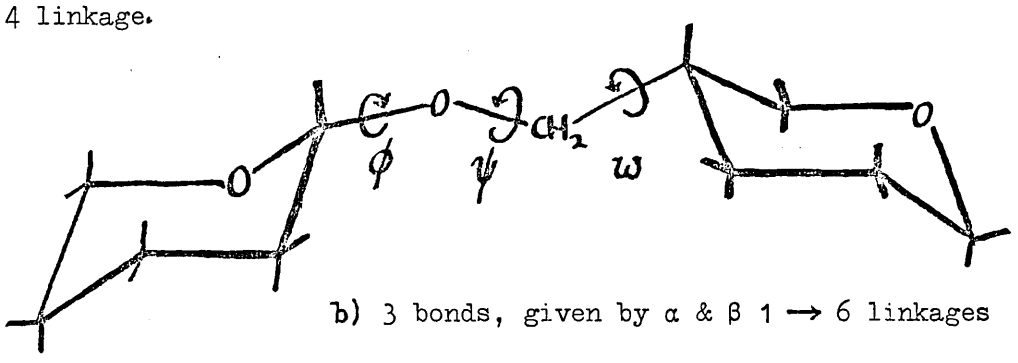
Molecular Conformation

The conformation of some polysaccharides with regular repeating units has been examined by computational analysis. Since the specific residues of each polysaccharide will all normally exist in either a 1C or a C1 conformation, it follows that variations in the shape of the molecule may only be brought about by the rotation of the two or three bonds (Figure VII) involved in each glycosidic linkage, whose flexibility in turn is partly determined by the steric hindrance of those equatorial substituents on that part of the carbohydrate ring nearest to the glycosidic linkage.

Figure VII



a) Two bonds, as in an
α1⟶4 linkage.



b) 3 bonds, given by α & β 1 ⟶ 6 linkages

From these analyses it has been found that there are four types (A ⟶ D) of polysaccharide structure, and that these four types show a distinct correlation with the biological function of the polysaccharide.[97]

Type A polysaccharides are usually structural in nature, and form extended ribbon-like helices in which the projected length of each sugar residue on the helix axis is close to its true length and is associated with a "ring twist" of close on 180°. This type of structure is obtained with, for example, the β 1 ⟶ 4 linked structural glucan, cellulose.[98]

Type B polysaccharides form a flexible helix, and are usually associated with, for example, α 1,4- and β 1,3-linked food reserve or mucilage glucans and galactans. They are characterized by a "ring twist" of 0° in most instances, and a projected length on the helix axis for

each residue that is considerably shorter than its 'true' length.[97] It has been found that this structure is widespread in some carrageenans, and in several other polysaccharides,[99,100] and these polysaccharides often form a three dimensional network of interlocking double helices.

Type C polysaccharides have a "ring twist" of about 60° and would be given by most 1,2-linked polymers. The structure of such macromolecules is however very compressed and rigid, and this probably accounts for the great scarcity of this type of polysaccharide. The only comparatively abundant 1,2-linked polysaccharide recorded in Nature is an α 1,2-linked mannan, which adopts, a 'B type' structure and has a "ring twist" of about 180°.

The last type of structure, type D, gives rise to polysaccharides with a highly flexible and extended conformation. This arises as a direct result of the rather flexible 1 → 6 linkages which comprise the majority of the linkages present in such polysaccharides. This flexibility arises in turn both from the fact that the glycosidic linkage is long, there being three bonds involved instead of just the two bonds in the other linkages (Figure VII), and also from the fact that there is an extra bond around which the linked residues may rotate.

GENERAL METHODS

I. Physical Techniques

a) Evaporations were carried out under reduced pressure between 35 and 45°C.

b) The water used in all work was either distilled or distilled and deionized.

c) Dialysis of solutions was performed either in Visking Cellophane tubes against water, with toluene added where necessary to prevent microbial growth; or in a 100 ml Dow Hollow Fibre beaker with water passing through the cellophane fibres.

d) Dry solvents. Methanol and Ethanol were dried with magnesium/iodine, distilled off the solid residue, and stored over molecular sieves (Type 3A or 4A).

Dimethyl sulphoxide and tetrahydrofuran were distilled over calcium hydride before use. The dry dimethyl sulphoxide was stored over molecular sieve type 4A for later use.

e) Specific rotations were measured in aqueous solution in a Perkin-Elmer 141 Polarimeter using the Sodium D lines.

f) Melting points were determined with either a Gallenkamp three place micromelting point instrument, or with the Kofler microscope-stage apparatus, and are uncorrected.

g) Ethanolic precipitation. "Precipitated in ethanol" means that the material concerned was precipitated out of solution by 100% ethanol, the final concentration of ethanol in the supernatant being about 95% unless otherwise stated.

"Precipitated in 80% ethanol" – the % here refers to the final, and not the initial, concentration of ethanol in the supernatant solution.

II Hydrolysis and Methanolysis

a) 90% Formic acid method. 1-2 ml of the acid was added to a tube or small flask containing the material (5-50 mg) and a small lump of solid carbon dioxide. After evaporation of the cardice the vessel was stoppered and heated at 100°C for 5-15 hr, after which it was opened, the solution diluted tenfold with water, and the heating continued for a further 2 hr. The resulting hydrolysate was then freed from the acid by coevaporation of the latter with methanol.

b) N-Sulphuric acid method. The acid and the sample (not more than 10% of the total weight of the solution) were heated for 2-6 hr at 100°C and then cooled. The acid was then removed by the addition of barium carbonate.

c) 72% sulphuric acid method.[101] To the sample (5-50 mg) and a small lump of solid carbon dioxide in a small flask there was added 1-2 ml of the ice cold acid after which the flask was stoppered and set aside for 24 hr. After cautiously, and with constant cooling, diluting the acid tenfold with water, the flask was heated for 2-4 hr at 100°C and then cooled; after which the acid was largely removed by the addition of barium hydroxide, and the neutralization completed with solid barium carbonate.

d) 2% Oxalic acid method. The polysaccharide was dissolved in 2.25% oxalic acid solution and refluxed for 4 hr at 100°C, after which the solution was cooled and neutralized with calcium carbonate.

e) Methanolysis. The polysaccharide (about 100 mg) was refluxed for 6 hr with 10-50 ml of 4% methanolic hydrogen chloride. The hydrogen chloride was then neutralized with silver carbonate, the solution filtered, and the residual silver salts thoroughly washed with dry methanol and the combined filtrate and washings were evaporated to a syrup.

III. Paper chromatography (P.C.) and Electrophoresis

a) Paper and thin layer chromatography

1) Supporting materials. Whatman Chromatography Paper grade No. 1 was used for routine analytical work. Preparative separations were carried out on water-washed sheets, the grades used being 3 MM (load 150 mg per 18 x 22½" sheet) or No. 17 (up to 1 g per 18 x 22½" sheet).

Pre-coated silica gel plates were used for thin layer work with solvent G.

2) Solvent systems (v/v)

A. 18.3.1.4. Ethylacetate : Acetic acid : Formic acid : Water.

B. 40.11.19 n-Butanol; Ethanol: Water.

C. 6.4.3 Pyridine : n-Butanol : Water.

D. 90.9.1 Methyl ethyl ketone : Water : Ammonia (S.G 0.880).

E. 5.5.1.3 Ethylacetate; Pyridine: Acetic acid: Water.

F. 5.3.1.3 n-Butanol: Pyridine: Benzene: Water (upper phase).

G.
(a) 9:1 Benzene : Ethanol
(b) 20:3 Benzene : Ethanol

3). Locating reagents

i) Silver nitrate, sodium hydroxide, sodium thiosulphate.[102] Dip:- for polyhydric carbohydrates and alcohols.

ii) Aniline oxalate-saturated solution in 1:1 ethanol:water solution[103]. Spray: then the papers are heated at 105°C for 5-10 min for reducing carbohydrates.

iii) p-Anisidine hydrochloride. 0.5 g in 10 ml ethanol to which is added 40 ml n-butanol.[104] Spray: treat as in 2) - for reducing carbohydrates.

iv) Urea hydrochloride. 10 g urea in 200 ml of EtOH to which is added 8 ml concentrated hydrochloric acid and 32 ml of water.[105] Spray: treat as in 2) - for ketoses.

v) Orcinol. 0.5% orcinol in 17:3 water saturated n-butanol: trichloroacetic acid.[106] Spray: treat as in 2) - for ketoses.

vi) Ninhydrin. 2% in ethanol. Dip, then heat at 70°C for 5 min - for amino acids.

vii) Glucose oxidase. 2-3 mg glucose oxidase + 2-3 mg horseradish peroxidase + 10 mg chromogen in 10 ml of 0.1M phosphate buffer pH 6.9.[107] Dip: allow to dry at room temperature - specific for D-glucose.

viii) Galactose oxidase. 2-3 mg galactose oxidase instead of glucose oxidase but then as for 7) - Specific for D-galactose.

It has been found that reagents ii) - v) may be used as dips when diluted 2-3 times with acetone.

4) Reference values

$$\text{Rf value} = \frac{\text{Distance travelled by compound x}}{\text{Distance travelled by solvent front}}$$

$$\text{Rg value} = \frac{\text{Distance travelled by compound x}}{\text{Distance travelled by reference compound g (normally glucose)}}$$

5. Location of carbohydrates on thick preparative papers

i) 3 MM papers

Side and centre strips were cut and developed with reagent (1). Those parts of the paper opposite to the carbohydrate spots found in the side strips were then eluted by washing with water and the resulting solution was freed from debris by filtering through Millipore

(pore size 0.45μ), and concentrated to dryness.

ii) No. 17 papers

The paper is too thick and brittle to permit the location of carbohydrates by the development of side strips. Accordingly the damp paper was spread on a clean surface, and a clean sheet of No.1 paper exactly aligned on the top. This 'sandwich' was then run over with a small hard object such as a pen top so that the No.1 paper became damp with solvent. Since the carbohydrates present were also carried with the solvent, they could be detected on the No.1 paper with reagent (i). The corresponding areas of the No.17 paper were then eluted with water and the carbohydrate recovered as described above for the 3 MM paper.

b) Electrophoresis

A Shandon L24 High Voltage Electrophoresis apparatus was used at 2-3 Kv for 2-4 hours with a pyridine/acetic acid buffer at pH 6.7. The buffer was prepared by the addition of 600 ml of a 5% solution (v/v) of acetic acid to 2 litres of pyridine.+ Carbohydrates were located with reagents i) and ii) and amino acids by vi). The mobility of an acidic carbohydrate X was defined by:-

$$M_{Glc\ UA}{}^{X} = \frac{\text{Distance travelled by compound X from a neutral sugar cospotted with it}}{\text{Distance travelled by glucuronic acid from the same sugar cospotted with it}}$$

It was necessary to include the neutral sugar since although the neutral sugars were inert towards this buffer, all the spots on an electrophoretogram were subject to endosmosis.

+ This solution was diluted with 1 volume of water before use.

IV. Assays and Analyses

a) Carbohydrate Content was assayed by the phenol-sulphuric acid method of Dubois et al[108] and the values obtained compared either with a standard composed of an appropriate synthetic mixture of sugars, or with glucose if such a standard was not available.

b) 6-deoxy hexoses were assayed by the cysteine method of Dische[109] against an appropriate standard. In this method 1 ml of the sample solution containing 10-100 μg of 6-deoxy hexose was carefully layered onto 5 ml of an ice cold 6:1 sulphuric acid : water mixture, after which the mixture was first gently and then vigorously shaken. After cooling to room temperature, the solution was then heated for 3 minutes at 100°C and cooled again. 0.1 ml of a 3% solution of L-cysteine hydrochloride monohydrate was then added and the tube again shaken and heated, this time for 10 min. After cooling, the optical density was read at 400 nm against a blank containing water instead of the original sample solution.

c) Uronic acid spot-test.[110] The compound (less than 2 mg) was added to 1-2 ml of concentrated hydrochloric acid containing 1-2 mg of naphthoresorcinol, and the solution boiled for 3-4 minutes. After cooling, the solution was extracted with cyclohexane or ether, and, if the test was positive, a purplish colour was extracted into the upper organic layer. Two controls, one containing glucose and the other glucuronic acid, were run at the same time.

d) Uronic acid was assayed either by the carbazole-sulphuric acid method with appropriate standards[111] or by precipitation of the uronic acid containing polysaccharide with cetyl pyridinium chloride.[112]

e) Sulphate was either assayed by the cetyl pyridinium chloride method at pH 2, where the uronic acid ionization was effectively suppressed;[112] or after precipitation of the sulphate left after oxidation of the polymer with concentrated nitric acid (and boiling off the latter) with 4-chloro-4'aminodiphenylhydrogen chloride,[113] by measurement of the residual absorbance at 254 nm.

f) Molar proportions of sugars. These were calculated from the peak areas of the TMS derivatives of the derived alcohols, using standard graphs of known weights of sugars reduced to their respective alcohols. The proportion of uronic acid was found by method d) and the proportion of 6-deoxy hexose could be checked by method b).

g) The protein content was calculated by conversion of the nitrogen content (100% protein = %N x 6.25 [114]), the %N being determined by the Alfred Bernhardt microanalytical laboratories in W. Germany.

h) Degree of polymerization (D.P.) This was found by the method of Peat[115] as modified by Timmell[116] who replaced the anthrone reagent of the former by the phenol reagent of Dubois.[108]

Two sets of tubes were set up. The first contained 0.5 ml of 2% sodium borohydride solution, and the second contained the same volume of this solution which had been inactivated by acid. To each 0.5 ml of the oligosaccharide solution was added, and the tubes covered and set aside for 6 hr. The carbohydrate content of each was then determined (GM IVa) against a blank in which the oligosaccharide solution had been replaced with water. The degree of polymerization for a homo-oligosaccharide was given by the relation

$$\frac{\text{Absorbance of the unreduced solution}}{\text{Difference in absorbance between the unreduced and reduced solutions}}$$

The D.P. of hetero-oligosaccharides may be found similarly after allowing for the differences in absorption given by the individual sugar residues.

V. Ion exchange cellulose Column Chromatography

Material Diethylaminoethyl (D.E.A.E.) cellulose, grade D.E.52 as supplied by W.R. Balston & Co.,[117] which comes in the pre-swollen state and as such contains 70-75% of water.

Preparation Before use the cellulose was degassed for 20 min. under reduced pressure in 0.5M hydrochloric acid. The acid was then neutralized with strong potassium hydroxide solution, and the supernatant solution decanted off. The cellulose was packed as a thick slurry into a column, (200 g of damp exchanger was found to be sufficient to fill a column of internal diameter 3.7 cm to a depth of about 45 cm) and washed with molar potassium chloride and then with water until the eluate was free from chloride ions. The homogeneity of the packing was checked by layering a weak solution of Malachite Green onto the surface, and observing the movement of the colour as the column was eluted with water.

Operation The prepared column was layered with the sample containing not more than 0.5 g of carbohydrate in not more than 1.0 g total material per 200 g of the damp exchanger and eluted with water to remove any neutral carbohydrate.

For a pilot run the column was next eluted with an appropriate gradient, normally 0 → 1 molar potassium chloride solution over 2 litres, and the eluate (10 ml fractions) assayed for carbohydrate (G.M.IVa).

When the appropriate conditions were found preparative separations were carried out by washing the column with batches of potassium chloride

solution in order of increasing strength, the strength of which was the same as that at which the major peaks were eluted. It was found that 0.3M KCl will remove most charged, unsulphated polysaccharides while the sulphated polysaccharides can be removed by subsequent elution with 1.0M KCl.

VI. Gas liquid Chromatography and Mass Spectrometry

a) Gas liquid Chromatography (G.L.C.)

(1) Instrumentation

Either a Pye-Argon Gas Chromatograph with 4 ft long columns using dry argon as the carrier gas, and fitted with an argon ionization detector,

Or a Pye 104 Gas Chromatograph with 6ft or 9 ft long columns using dry nitrogen as the carrier gas, and fitted with a flame ionization detector.

2) Columns

For trimethyl silyl ethers of sugars and alditols

3) S.E. 30 3% on Chromosorb W.

5) Apiezon K.(ApK) 7.5% on Chromosorb W.

For methylated methyl glycosides

1) Butane 1,4-diol succinate polyester, 15% on Celite.

2) Polyphenyl ether (P.P.E.)- m-bis(m-phenoxyphenoxy)benzene 10% on Celite.

11) Polyethylene glycol adipate 10% on Celite.

For methylated alditol acetates

12) ECNSS-M 3% on Chromosorb W.

13) OV 225 3% on Gas Chrom Q.

The liquid phase was coated onto the acid and alkali washed support material which had been precoated either with dichloro dimethyl silane or with hexamethyl disilazane. The columns were run at a gas flow rate and at a temperature adjusted so as to give the best results for the mixture being separated.

b) Gas liquid chromatography with mass spectrometry

1) Instrumentation

The G.L.C. apparatus was a Perkin-Elmer F11 Gas Chromatograph run with 6 ft columns with column (12). Later, column (13) was used instead since the former tended to bleed, and so to severely damage the mass spectrometer. The effluent from the column was split so that part went to the flame ionization detector of the G.L.C. and the remainder was fed through a glass line 10 C^{o} warmer than the column into the ion source of the Hitachi Perkin-Elmer R.M.S.4 mass spectrometer, and from thence past the total ion monitor to the analyzer and detector.

2) Recording

The G.L.C. trace was recorded with the trace from the total ion monitor by the pen Hitachi Perkin-Elmer 196 recorder, while the mass spectra were printed on Kodak Linagraph Direct Print paper 1895 by a S.E. U.V. Recorder 3005.

3) Samples

The samples analyzed were methylated alditol acetates, with the conditions and system modified to give best results possible. It was found that metal columns catalytically destroyed the alditol acetates and so these were replaced by glass columns.

VII General reactions

a) Trimethyl silyl derivatives[118]

The dry material (5-15 mg) was dissolved in dry pyridine and 0.1 ml trimethyl chlorosilane followed by 0.2 ml of hexamethyl disilazane added. After shaking for several minutes (for quantitative work it is necessary to heat at 100°C for 10 minutes) the mixture was centrifuged to remove ammonium chloride and evaporated to dryness. The residue was dissolved in n-hexane and analyzed by G.L.C. on columns 3 or 5, the retention times T being calculated relative to that of Me_3Si derivative of xylitol.

b) Methylation

1) The polysaccharide (5 → 35 mg) was normally methylated according to the method of Hakomori[119] as modified by Bjorndal and Lindberg,[120] with the dimethylsulphinyl carbanion being prepared as Sandford and Conrad[121] describe. The methylated product was hydrolyzed with 90% formic acid (G.M. IIa).

2) A modified[122a] Kuhn[122b] method was also used for some polysaccharides and for oligosaccharides, in which 0.5 - 2.0 mg of material was dissolved in 0.2 ml of dimethyl formamide and 0.2 ml of methyl iodide with 0.2 g of silver oxide added. The whole was left to stand in the dark for 3 hr at 0-4°C before being shaken for a further 15-24 hr in the dark.

Full methylation was checked either by thin layer chromatography in solvent (G) (GM IIIa) and location of the polysaccharide spot by exposure to an iodine saturated atmosphere, or by infrared analysis.

The hydroxyl absorption at 3,400 cm^{-1} (broad, strong, and resulting from intermolecular -OH bonding) normally disappears and is replaced by a sharp medium-strength absorption owing to C-H on methoxy groupings at 2860 cm^{-1}.

However water in the sample will also give an absorption at 3400 cm^{-1} and the band at 2860 cm^{-1} may not always appear. If the observed absorption is owing to water, its presence may be confirmed by a strong absorption at 1660 cm^{-1}, the other hydroxyl groupings giving an absorption at 1200 - 1000 cm^{-1}. Absorptions in this region which are derived from -OH groups also disappear on full methylation.

c) Methyl glycosidation: esterification of uronic acid

The dry hydrolysate (from either methylated or unmethylated polysaccharides) was refluxed for at least 4 hr over Amberlite IR120 H^+ resin previously dried by washing several times with - and storage in - dry methanol.[123]

Methylated glycosides were then analyzed by G.L.C. on columns 1, 2 or 11 and their retention times calculated relative to that of methyl 2,3,4,6-tetra-O-methyl β-D-glucopyranoside (T.M.G. 1st peak).

Methyl uronosyl methyl glycosides derived from an unmethylated acidic polysaccharide were reduced to the corresponding sugar glycoside by borohydride (see below - d)).

d) Reduction of uronic ester glycosides to sugar glycosides and sugars to sugar alcohols

The material to be reduced was dissolved in water (1-2 ml) and sodium or potassium borohydride was then added (20-30 mg). After 4-16 hr the solution was tested for alkalinity: if it was still alkaline the

reduction was complete since not all the borohydride had been consumed. If it was not still alkaline the reduction procedure was repeated. The cations were then removed with I.R.120H^+, and the residual boric acid removed by co-evaporation with methanol. The reduced ester glycosides were then hydrolyzed with 90% formic acid to yield the free sugars (G.M. IIa).

e) Preparation and analysis of methylated alditol acetates.

The alditols resulting from the reduction of the hydrolysate of a methylated polysaccharide were acetylated by a 1:1 pyridine: acetic anhydride mixture for 10 min at 100°C, and the residual reagent then removed by co-evaporation with water or methanol.[124] The resulting syrupy residue was then dissolved in chloroform and analyzed by G.L.C. or G.L.C./M.S. on columns 12 and 13, the retention times T being quoted with respect to 1,5-diacetyl 2,3,4,6-tetra-O-methyl glucitol.

f) Removal of glucose from a solution

Since glucose oxidase oxidizes glucose to gluconic acid,[107] solutions contaminated with glucose were freed from this sugar by incubation with the enzyme in 0.1M phosphate buffer (pH 6.7) at 37°C for 24 hr. The enzyme was then destroyed by heating and the gluconic acid and buffer ions removed by the mixed bed resin "Biodeminrolit" (carbonate form) after which the solution was filtered and concentrated to dryness.

g) Removal of a large amounts of ionic material

The removal of large amounts of ionic material from solutions containing low molecular weight carbohydrates was achieved by sequential passage through a column of IR120 H^+ (strong acid) resin followed by a column of IR 45B OH^- (weak base) resin.

h) Periodate Oxidation [0.03M solution, stabilized with n-propanol (10%)]

The sample and periodate solutions were mixed in equal volumes. The reaction was followed by the change in absorption (223 nm).[125] When the reaction was complete, the solution was reduced (2% $NaBH_4$ + 0.5% HBO_3 soln - see GM VIIa).

PART I

INVESTIGATION OF THE CARBOHYDRATES SYNTHESIZED BY UROSPORA REF. PENICILLIFORMIS

Introduction

The genus Urospora (once known as Hormiscia or Conferva) has a controversial classificatory history. It was originally placed in the Cladophoraceae, with the closely related genera Spongomorpha and Acrosiphonia being classed together in a sub-section of the type genus Cladophora.[126] These were later placed in a genus of their own (Spongomorpha) and a possible division of this into the two present genera was suggested.[127] Unlike the rest of the Cladophoraceae so far then studied, these three genera possess a unicellular "Codiolum" stage in their life history[128a] and lack cellulose I in their cell walls[128b] and on the basis of these and other criteria were placed in a new family, the Acrosiphoneaceae,[128a] which was later separated from the Cladophorales by being raised to ordinal rank.[129] The discovery of a "Codiolum" like stage in the life-cycle of a culture of Cladophora rupestris[130] has thrown doubt on the validity of this distinction. However, that a fundamental division does in fact exist is suggested by the different constitutions of the sulphated mucilages metabolized by the Cladophora and Acrosiphonia sections of the old Cladophorales, since the former synthesises an arabinogalactan (p. 6.) while the latter synthesizes a complex glucuronoxylorhamnan[131] which closely resembles that extracted from two species of the Ulvales, Enteromorpha compressa and Ulva lactuca. Preliminary studies on Urospora have shown the presence of a sulphated polysaccharide resembling that from Ulva and Acrosiphonia.

The mucilages from the three algae Acrosiphonia arcta,[131] Ulva lactua[132-5] and Enteromorpha compressa[136] have been shown to be built on a similar pattern. The polysaccharides all have negative rotations, that from Ulva lactuca having a rotation of -74°. It was found that the constituents

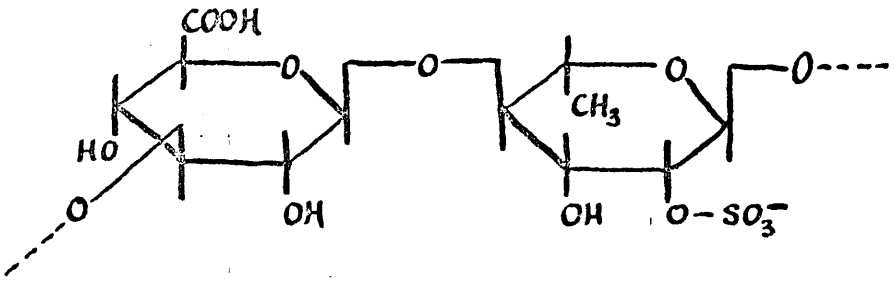
of this polysaccharide, D-glucose: D-glucuronic acid: D-xylose: L-rhamnose were in the molar ratio 2:4:7:10-14 (the figure for rhamnose increases as the time of hydrolysis is increased from 6→16 hr[133]) together with 17% sulphate. The negative rotation suggests that the linkages are predominantly β but it must be remembered that rhamnose is in the L form and therefore would give a low positive rotation for a β-linkage. On fractionation of the polysaccharide on D.E.A.E.-Cellulose three similar fractions were eluted in order of increasing molecular size, thus showing that the polysaccharide is polydisperse.

The resistance of a small amount of sulphate to acid hydrolysis suggested that some of the glucose was sulphated at C-6, while alkaline desulphation with sodium methoxide showed that about 15% of the xylose was mono-sulphated at C-2. An infrared spectrum of the polysaccharide showed an absorption at 850 cm^{-1}, which has been shown to be owing to the presence of axial sulphate in galactose sulphates. Assuming that these results are valid for rhamnose, and that this sugar is in its more stable 1C conformation, the sulphate therefore can only be located at C-2.[133]

The evidence showed that the rhamnose and most of the xylose were largely 1,4- or triply linked, although some of the latter was 1,3-linked, and rather more formed end-group residues. Methylation of the reduced polysaccharide in which the glucuronosyl residues were converted to glucose showed end-group, 1,3-linked and a little 1,4-linked glucose.[132,134]

These results were borne out by partial hydrolysis studies, which yielded large amounts of 4-O-β-D-glucopyruronosyl-L-rhamnose, which probably occurs as its 2-sulphate (Fig. VIII) in the polysaccharide:-

Fig. VIII



Small amounts of both 1,3- and 1,4-linked glucuronosylxylose were found together with a tetrasaccharide tentatively described as Glucuronosyl (1 → 4) Rhamnosyl (1 → 3 or 4) Glucuronosyl (1 → 3) Xylose, while from a partial hydrolysate of a polysaccharide in which the glucuronic acid had been reduced to glucose a trisaccharide, Rhamnosyl-(1 → 4) Xylosyl (1 → 3) Glucose was characterized.

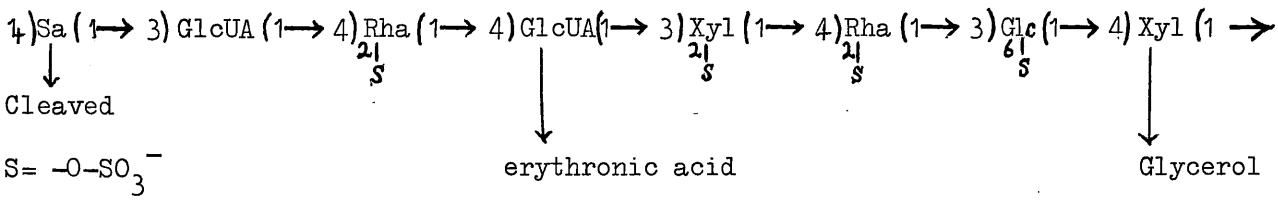
Periodate oxidation of the polysaccharide reduced 0.20 mole per anhydro unit, the end-group and the few internal 1,4-linked residues being cleaved. On oxidation of a partially desulphated polysaccharide at 2°C, however, under which conditions cis-diols are cleaved more rapidly than trans-diols, a reduction of 0.40 moles of periodate occured thus confirming that the rhamnose, the only sugar present with cis-diols, is largely 1,4-linked[133] and showing that sulphate has been removed from C-2 or C-3 of the rhamnose units.

The original polysaccharide after oxidation, and the reduction of the resulting oxopolysaccharide followed by mild hydrolysis (Smith degradation) yielded a number of fragments, together with a hepta-saccharide which accounted for most of the total material (61%). These fragments, and the structural units which gave rise to them, are tabulated below, the cleaved sugar giving rise to the fragment named being underlined:

Origin	Fragment
(1) C-1 + C-2 of 1,4-linked and end-group residues.	Glycollic aldehyde
(2) 4 Sa 1→4 Xyl p 1 →	Glycerol
(3) 4 Rha, Xyl, Glc 1 →4 Glc p 1 →	Erythritol
(4) 3 Glc 1 →4 Glc p 1 →	2-O-D-glucosyl erythritol
(5) 3 Xyl p / 4 Xyl p 2-SO_4) 1 →4 Xyl p 1	2-O-D-xylosyl glycerol
(6) 3 Xyl p / 4 Xyl p 2-SO_4) 1 →4 Glc p 1	2-O-D-xylosyl erythritol

Sa = Rhamnose or Xylose or Glucose

(7) The heptasaccharide on hydrolysis gave glycerol, which was derived from xylose on the reducing end, with rhamnose, xylose, glucose and 4-O-β-D-glucopyranuronosyl L-rhamnose, together with some erythronic acid. This last would be derived from a 1,4-linked glucuronosyl residue, the glycoside of the cleaved acid being stabilized by the erythruronic moiety so that it was not broken during the mild hydrolysis. The heptasaccharide carried about 25% sulphate, and could in the original polysaccharide have the structure:-



4 Sa 1 = 1,4-linked sugar i.e. Rha, Glc or Xyl.

Combining all the information together, the polysaccharide could consist of units such as that shown in Fig. IX.

Figure IX Possible structure of the repeating unit of Ulva mucilage

This structure accommodates most of the evidence, but is not the only structure possible.

Oligosaccharides

Tetrasaccharide

GlcUA(1-4) Rha(1-4/3) GlcUA(1 → 3) Xyl p

Disaccharide

4-O-β-D-GlcUA L-Rha.

Disaccharide

GlcUA (1 → 3) Xyl.

Trisaccharide

Rha (1 — 4) Xyl (1 — 3) GlcUA

Smith degradation

4 Rha (1 → 4) Xyl (1 → 3) GlcUA (1 → 4) Rha (1 → 4) GlcUA (1 → 3) Xyl (1 → 4) Rha (1 → 3) Glc (1 → 4) Xyl (→ repeat

? Glycerol — Erythronic acid — s — 2 — 6 — s — Glycerol — or 3 Glc 1, 4 Glc 1, 4 Xyl 1, s etc.)

Heptasaccharide

Although rhamnose is cleaved, the fragment $CH_3(CHOH)_2CH_2OH$ has not been isolated so far.

Although the polysaccharide from Enteromorpha compressa and Acrosiphonia arcta have been less extensively investigated, the overall pattern seems very similar to that of Ulva, except that the Enteromorpha mucilage reduces rather more periodate both before (0.38 mole) and after (0.68 mole) desulphation, indicating that more rhamnose is attacked, which may be explained by the greater proportion of rhamnose to sulphate in this alga.[133] In Acrosiphonia this periodate reduction is even greater, probably because of the much lower sulphate content of the polysaccharide (6%), even though the molar proportion of rhamnose is lower than that of Ulva and Enteromorpha. Since a considerable proportion of this rhamnose is linked to glucuronic acid, the polysaccharide on hydrolysis gives large amounts of 4-O-β-D-glucopyuronosyl L-rhamnose. Methylation has shown the presence of 1,4-linked glucose, some of which is 6-linked, possibly to sulphate, by analogy with Ulva.

While evidence at present suggests that the constitution of this type of polysaccharide is largely independent of environmental and physiological factors[133] and is therefore dependent only on the genetic constitution of the particular species, it appears that these polysaccharides form a distinct family, resembling in this the galactan sulphates of the Rhodophyta. Analysis of the hydrolysate of the mucilage from both Enteromorpha intestinalis and E. torta showed the same sugars as those found in Ulva, which therefore suggests that the polysaccharide from both algae belong to the same family.

The genus Urospora grows characteristically as tufts of green, unbranched and mucilaginous filaments in or just below the intertidal zone. It ranges in form and habitat from the large arctic U. wormskjoldii (≡ U. collabens, and rare in Britain[126]) which hangs in clumps from the walls of fjords, whose cells, normally up to 4 mm across, may grow to the size of small peas in culture,[137] and so cause the alga to resemble a green pearl necklace, to the pollution resistant Urospora penicilliformis[138] which can also withstand three weeks desiccation[128c] whose filaments seldom attain a diameter of 70 μ .

The two common British species are U. bangiodes and U. penicilliformis. The green filaments of the former grow attached to rocks at the extreme low water mark, and are normally 7 $\longrightarrow$ 15 cm long by 50 $\longrightarrow$ 450μ broad, the constituent cells of which vary from 50 $\longrightarrow$ 230μ in length; while those of the latter are content with any reasonably solid substratum, such as stones, other algae, and the bottom of ships.[126, 139] The filaments of this alga are usually smaller in all their parts than those of U. bangiodes, ranging from 1-8 cm length and 10-70μ in breadth, the constituent cells of which are normally 0.5 -5.0 times shorter than broad.

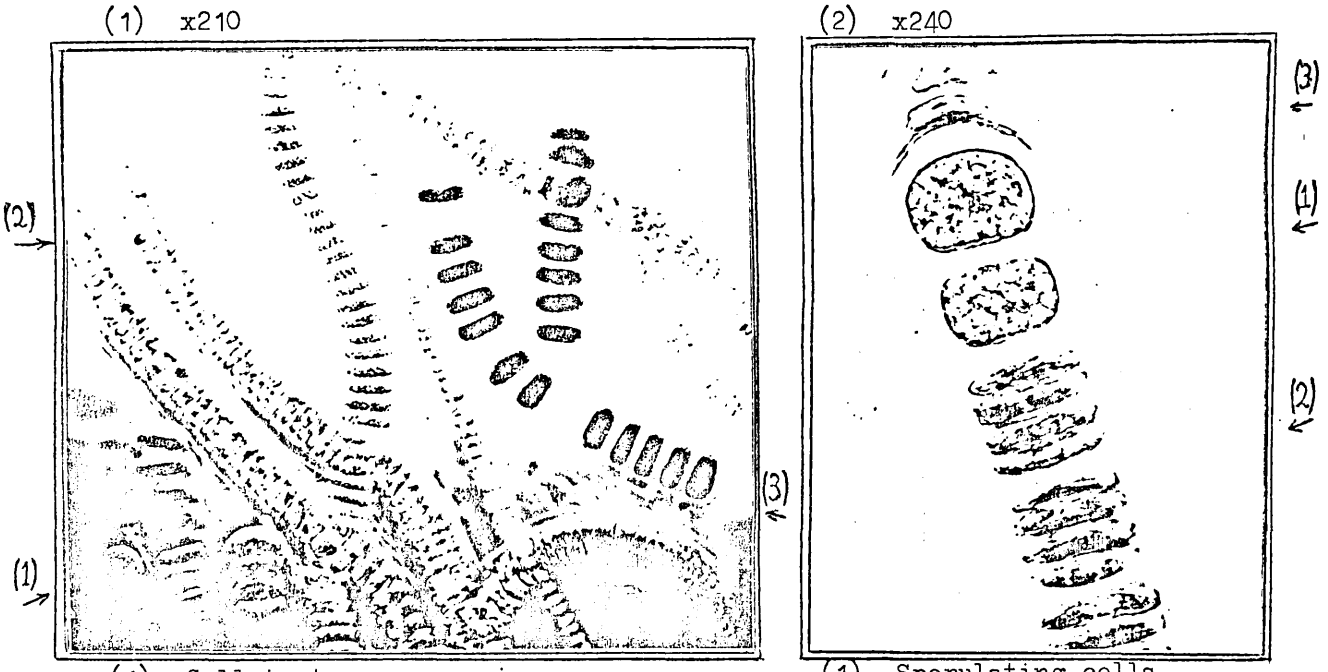
In both algae reproduction tends to start at the tip of the filament and work down its length, with the production and liberation of large numbers of spores.[126]

The species under investigation was harvested around the edge of rocks near the spring low water mark in inlets near Farr Bay, Sutherland in N.W. Scotland in late March or early April of 1969 - 1972 (Figure X). Each plant was usually less than 8 cm long, and varied in diameter from 45 - 60μ, of which at least a quarter was external mucilage. The constituent cells were about 10μ apart, and arranged in pairs of pairs, suggesting a rapid

cyclical division similar to the 4-day cycle shown for U. wormskjodii.[140] During April the plants become yellower from the tips of the filaments and later release large numbers of spores.

Although the habitat of this alga strongly suggested U. bangiodes, the dimensions and general appearance of the filaments suggest that the alga is probably Urospora penicilliformis (Roth) Areschoug, an identification tentatively confirmed by Dr. J.H. Evans, of the Botany Department of this College.

Figure X. Vegetative and sporulating filaments of Urospora



(1) Cell in transverse view.
(2) Vegetative filament.
(3) Darker sporulating filament.

(1) Sporulating cells.
(2) Vegetative cells in pairs of pairs suggesting rapid division.
(3) Damaged area.

The thickness of the mucilage is particularly evident in (2), where the edge lies from 0.3 to 0.6 cell diameters from the edge of the cells. In this filament the end cells are sporulating, in response to the damage which it received during growth.

Urospora Experimental Work

I. Extraction Procedures

a) Preliminary Studies

Experiment I. The extraction of Urospora collected on 7th April 1969

The alga was collected by Dr. Young from rocky inlets near Farr Bay N.W. Scotland. About 70 g damp weight (~10 g dry weight) was then plunged into ethanol (100%, final concentration about 60%) and stored. After removal of the ethanolic solution the alga was extracted with cold water overnight and then with boiling water for 6hr.

The residual material was extensively contaminated with sand and organic debris. The sand was largely removed by differential sedimentation and most of the organic contaminants were removed by hand. The remaining Urospora (about 0.9 g) was then twice extracted with boiling water for 6 hr. This aqueous extract after centrifugation, concentration and freeze-drying yielded a fawn solid [550 mg; 132 mg carbohydrate]. It was dissolved in water, exhaustively dialyzed and, after concentration, freeze-dried to a white solid (B) [210 mg; 86 mg carbohydrate].

The residual alga (about 0.3g) was treated for 24 hr at 20°C with an aqueous 0.5% sodium hypochlorite solution at a pH adjusted to 5 with acetic acid. After centrifugation, extensive dialysis and freeze-drying, the chlorite solution yielded a white solid [130 mg, 30 mg carbohydrate].

The bleached residual solid was extracted for 24 hr at 20°C under coal gas in a closed system with 4% sodium hydroxide. After centrifugation, dialysis and freeze-drying the supernatant solution gave a white solid [45 mg, 7.5 mg carbohydrate]. The residual material was then treated with

12% potassium hydroxide solution, (20°C, 24 hr, again under coal gas) and the alkaline solution after centrifugation, neutralization (with 3N-hydrochloric acid and constant cooling) dialysis and freeze-drying, gave a white solid [60 mg, 8 mg carbohydrate].

The insoluble residue was finally hydrolyzed by cold 70% sulphuric acid (G.M. IIc). The hydrolysate yielded mainly glucose and a black insoluble residue that was devoid of carbohydrate.

Experiment II. Variations in the extraction procedure of Expt. I during the extraction of the Urospora collected on 4th April 1970).

(i) Ethanolic extraction: the removal of Low Molecular Weights carbohydrates

The alga (about 4 g) was extracted exhaustively with hot 80% ethanol. The ethanolic solution was concentrated to a small volume, and the resulting greenish-black oily sludge (A) partitioned between chloroform and water (see Expt. V). The residual alga was then freed from organic debris by hand, a process which also resulted in the removal of most of the sand, and air-dried (yield about 4 g).

(ii) Aqueous extraction

The partially purified alga was exhaustively extracted with cold (four times, 12 hr each) and then with boiling water (five times, 8 hr each). The supernatant solutions of the former on dialysis in a closed system yielded a dialysate/B_1 which contained carbohydrate and was therefore concentrated and freeze-dried [282 mg, 164 mg carbohydrate]. The dialyzed solutions of both extracts on freeze-drying yielded off-white solids - the yields were 430 mg, 246 mg carbohydrate for the cold water extract, and 862 mg, 445 mg carbohydrate, for the hot.

(iii) Acidic extraction

The residual alga was suspended in 0.01M hydrochloric acid solution adjusted to pH 2 by the addition of more acid, and thrice extracted for 6 hr at 70°C. After centrifugation and neutralization with potassium bicarbonate, the supernatant solutions were concentrated, dialyzed and freeze-dried to a white solid [37 mg, 20 mg carbohydrate] while the residue was bleached as before [p56, yield 112 mg, 5 mg carbohydrate].

(iv) Alkaline extraction

The residue was extracted as described for Expt. I (p56), with two exceptions,:- (1) each extraction was continued until the amount of carbohydrate extracted was negligible (3x) and (2) an 18% sodium hydroxide solution replaced the 12% potassium hydroxide solution used previously. The recovered yield for the 4% alkali extract was 229 mg, 45 mg carbohydrate; of which 16 mg was a precipitate formed on dialysis, while that for the 18% alkali extract was 88 mg, 27 mg carbohydrate. The figure also includes the 52 mg, [18 mg carbohydrate] of precipitate which was deposited during dialysis.

(v) Examination of the insoluble residue (22 mg)

Hydrolysis of the residue with 72% sulphuric acid yielded 7 mg of a material devoid of carbohydrate.

b) Final Extraction Procedures

Experiment III. Extraction of Urospora collected in 1971

The Urospora, together with much adhering sand (ca. 2 Kg), containing an estimated 40 g freeze-dried weight of alga, was collected from Farr Bay, and immediately plunged into cold ethanol. Microscopic examination showed

about 5% (by wt.) contamination with the filamentous alga Ulothrix ref. flacca, and also showed that the Urospora itself was made up of about 40% of apparently sporulating golden filaments, the remainder being the normal vegetative strands.

(i) Ethanolic Extraction

The alga was largely freed from sand by washing in ethanol before being extracted with boiling 80% ethanol until nearly colourless (5x). The ethanolic solutions (A) were filtered, combined and set aside. Attempted purification of the residual air-dried alga (66 g) from large organic debris by sieving and from sand by differential sedimentation in water was only partially successful. The derived material was therefore suspended in water, freeze-dried, and ground to a powder in liquid nitrogen, and again suspended in water. It was observed that on decantation of the suspension from one vessel to another the Urospora particles entrapped a large number of air bubbles and floated to the surface, while the sand and organic debris sedimented out. The suspension was therefore repeatedly decanted from one vessel to another and the resultant froth collected. The sediment (32 g dry wt) was discarded. Microscopic examination showed that the algae in the derived foam were comprised of Urospora/Ulothrix in at least 99% abundance.

The greenish-yellow Urospora foam was treated with toluene:butanol(n-) (1:1 v/v), stirred and set aside for three days. The upper organic layer was separated and combined with the ethanolic extracts (A). The lower colourless aqueous suspension of Urospora was freed from adhering organic solvent by evaporation, and the Urospora which had now sedimented was removed by centrifugation. The resulting supernatant solution was regarded as the first aqueous extract.

(ii) Cold and boiling water extractions

The Urospora was thrice extracted with cold water and thrice with boiling water (1000 ml each). After each extraction the mixture was centrifuged and the supernatant solutions were concentrated to small volume and poured into cold 80% ethanol. The resulting precipitates were removed by centrifugation and the ethanolic supernatants combined with (A).

The precipitates were resuspended in water and, after removal of the residual ethanol by evaporation, were freeze-dried to white solids (yield: cold water extract (B)-4g, hot water extract(C)- 2.25g). The residual material was not extracted further.

Experiment IV. The extraction of the Urospora collected in April, 1972.

The Urospora was again collected at low tide around 6 p.m. on the 2nd/3rd of April 1972, after a week of fine weather. It was immediately freed from most of the contaminating sand by differential sedimentation in sea water, after which it was drained (560 g approx) and plunged into ethanol (final concentration 50-60%).

On microscopic examination the Urospora was found to be almost completely in the vegetative state. The Ulothrix contamination was found to be about 1% by wt., and the alga, as in previous years, appeared to be devoid of epiphytes.

(i) Ethanolic extraction and further purification

The alga was extracted five times with cold 80% ethanol. The supernatant solutions (A) were removed by filtration, and partitioned between toluene and water (see flow chart p63) The residue was air dried

(yield 63g) and after grinding in liquid nitrogen was purified by foam flotation as described in Expt. III (i), although the foam in this instance was broken by the addition of ethanol. The derived suspension was concentrated, poured into 80% ethanol, and allowed to stand. The liquid was then removed by filtration and combined with the ethanolic solution (A) while the alga and any precipitated polysaccharide was air-dried to an off-white fibrous powder [30.2 g, 10.6 g carbohydrate].

(ii) Aqueous extraction

The air dried powder (30.0 g) was dispersed in 600 ml of water, and quickly formed a thick gelatinous paste, the water becoming noticeably warmer.

This paste was diluted and extracted with hot water as shown in Table I.

Table I. Hot water extraction of residual Urospora

Extract	Conditions			Weight	Carbohydrate weight
1	80°C	1 hr	1.5 l	6.6 g	4.68 g
2	100°C	1.5 hr	1.5 l	4.0 g	1.42 g
3	"	2.5 hr	1.2 l		0.32 g
4	"	42 hr	0.9 l	3.97 g	1.62 g
5	"	4.5 hr	0.8 l	1.73 g	0.22 g
6	"	17 hr	0.7 l		0.22 g
7	"	8 hr	1.8 l	0.64 g	0.08 g
				10.3 g	3.88 g

(iii) Acidic extraction

The residue (11 g., 1.7 g carbohydrate) was extracted four times with 0.01M hydrochloric acid (550 ml) as described in experiment II (iii) and the resulting solutions freeze-dried to white solids (total yield 0.63 g, 0.275 g carbohydrate).

(iv) Chlorite treatment and alkali extraction

The residue was suspended in 550 ml of water acidulated with 1 ml of glacial acetic acid, and bleached with sodium hypochlorite (1 g) for 2 hr at 70°C. After centrifugation the supernatant solution was discarded, and the residue extracted with 13% sodium hydroxide solution as shown in the flow chart (p.63).

(v) Examination of the residual material (192 mg)

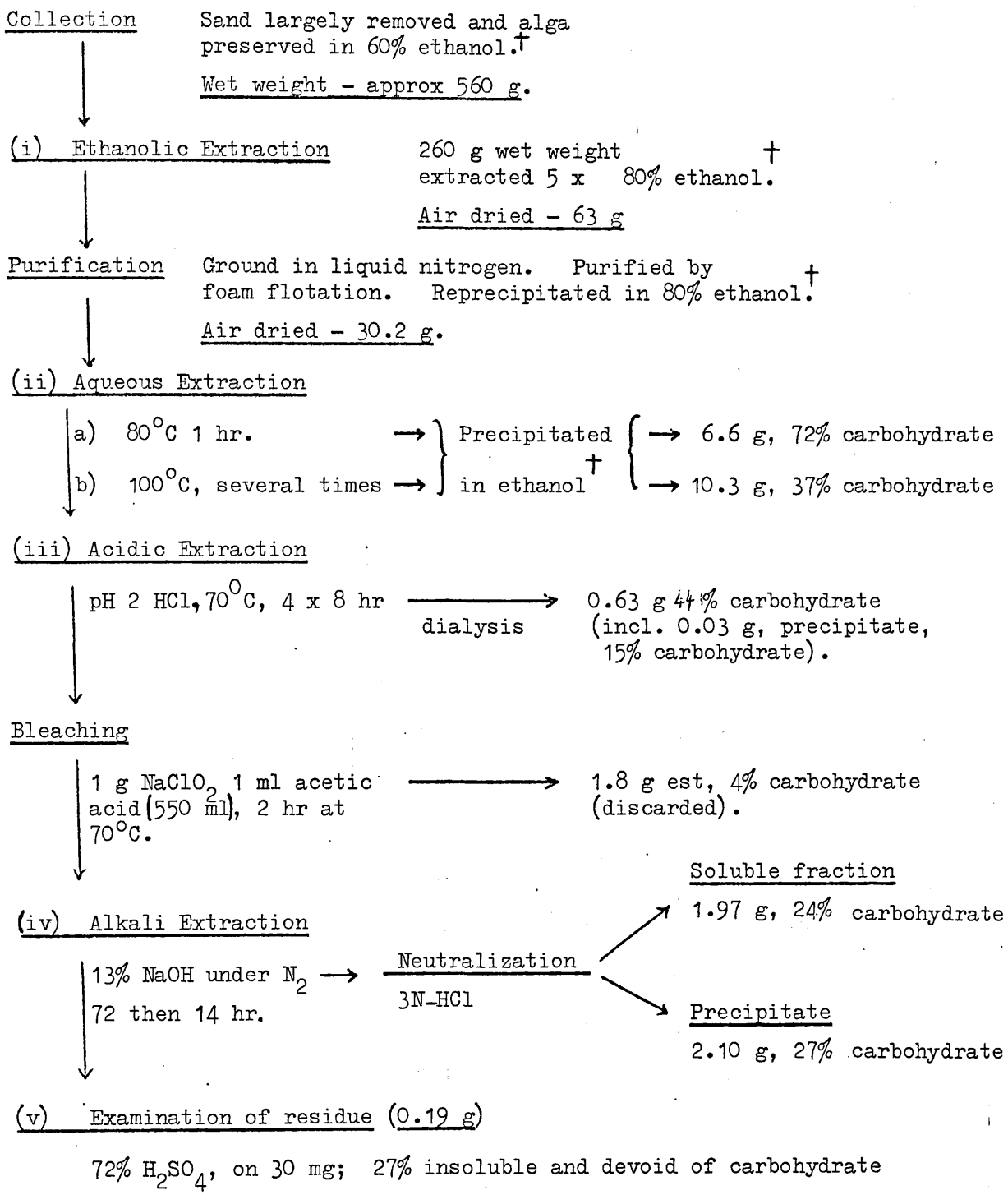
An aliquot (30 mg) was hydrolyzed with cold 72% sulphuric acid (G.M. IIC) which left 8.1 mg of a residue devoid of carbohydrate.

Experiment V. Purification of the ethanolic extracts

(i) Extract derived from Expt. II (i) [1970]

The oily sludge (A) (p.57) derived from the ethanolic extraction on partition between chloroform and water yielded a lower green glyco-lipid containing layer of chloroform, and an upper colourless aqueous layer highly contaminated with salts. This latter after washing with chloroform was dried. The solid residue was dissolved in water (10 ml) and the resulting solution poured into ethanol (400 ml). The largely inorganic precipitate, after filtration from the supernatant solution A_2, was dissolved in the minimum volume of water. This solution on standing deposited several batches of crystals which were removed and freed from the adhering mother liquor by recrystallization (twice) when they were largely free from carbohydrates.

Flow Chart I. Extraction procedure of Experiment IV summarized



† Ethanolic solutions

Combined.
Partitioned between toluene and water with 5% n-BuOH to break the emulsion.

Aqueous layer 13.4 g
(est. 6.0 g salts, 0.86 g carbohydrate)

Toluene layer 7.6 g

After evaporation of the ethanol from the filtrate A_2, the residual aqueous solution was combined with the mother liquors left after the removal of the several batches of inorganic crystals, and freed from the remaining salts by deionization with Biodeminrolit (carbonate form). It was then freeze-dried to a clear pale yellow glass [(i) - yield about 80 mg, 72 mg carbohydrate].

(ii) Extract derived from Expt. III (i)

The ethanolic solution A (p.59) was concentrated and partitioned between toluene (250 ml) and water (250 ml) with 5% n-butanol added to break the resultant emulsion. Both layers were evaporated to dryness and freeze-dried. The upper toluene layer yielded a green-black tar (7.0 g) which was discarded, while the lower aqueous layer yielded a brown semi-crystalline mass (17.0 g) which was dissolved in water (500 ml) and deionized by passage through resin columns (GM VII g). The resulting deionized solution on concentration and freeze-drying yielded a crisp pale yellow glass [(ii) 1.2 g, 1.08 g carbohydrate].

II. Low molecular weight materials

Paper chromatograms of the following unknowns, together with controls consisting of authentic sugars and sugar alcohols, were run in solvents A, B and C. The developed spots were then located with reagents (i), (ii), (iv), (v) and (vii-glucose oxidase) (GM III a - 2 and 3).

Experiment VI. Initial investigation of the low molecular weight materials (i) and (ii) from Expt V. (p. 62).

The results from paper chromatography (P.C.) of both (i) and (ii) gave spots with the mobilities of a homologous series of malto-oligosaccharide series, together with glucose and spots with the mobility of fructose, sucrose, and myoinositol. A small amount of a material, with a mobility similar to that of glycerol in solvent A, was also found.

An aliquot of each material was then hydrolyzed (GM II a) and the resulting hydrolysate examined by P.C. When these chromatograms were compared with those from the unhydrolyzed material it was found that not only had the oligosaccharide spots disappeared, but also that the glucose spot was greatly enhanced.

Since both materials appeared to be identical on paper chromatographic analysis, they were combined.

Experiment VII. Characterization of the low molecular weight carbohydrates

The combined material was fractionated on three sheets of No.17 paper (GM IIIa - 5 (ii)). A portion of each of the derived fractions was converted into its Me_3Si derivative (GM VIIa) and analyzed by G.L.C. on column 5 (GM VIa).

Fraction 1

After reseparation on a 3 MM paper (GM IIIa - 5 (i)) a non-reducing syrup was obtained. Electrophoresis at pH 6.7 (GM III b) and P.C. (solvent A) gave single spots with mobilities identical with those of glyceric acid run as a control (R_{glc} 3.56 (Rhamnose = 2.34) and M_{GlcUA} 1.48 - see Appendix, Tables I and II).

Fraction 2

A reducing syrup, which on P.C. analysis gave a major spot with the mobility of fructose together with a lesser spot with the mobility of glucose, was obtained. This on analysis by G.L.C. gave peaks with the same retention times as those given by the Me_3Si derivatives of fructose and glucose (See Appendix, Table III). Incubation of the residual material with glucose oxidase (GM VII f) followed by G.L.C. analysis showed only those peaks with the retention times of fructose, those for glucose having disappeared.

Fraction 3

A reducing syrup which gave a single spot with the mobility of glucose on P.C. analysis was confirmed as D-glucose by reagent (vii)-glucose oxidase). G.L.C. analysis of an aliquot showed peaks with the same retention times as those for glucose, but these were absent from an aliquot which had been previously incubated with glucose oxidase (GM VII f).

Fraction 4

A non-reducing syrup was obtained which on P.C. analysis gave a single spot with the mobility of sucrose and the colour reactions of a ketose with locating reagents (iv) and (v). G.L.C. analysis of an aliquot gave a single peak with the same retention time as the Me_3Si derivative of sucrose on both columns 3 and 5, while the hydrolysis of another aliquot followed by P.C. analysis showed only glucose and fructose.

Fraction 5

A reducing syrup was obtained which P.C. analysis showed to contain two spots, one with the mobility of maltose (reducing) and the other with the mobility of myo-inositol (non-reducing). Hydrolysis of an aliquot followed by P.C. and G.L.C. analysis showed glucose, which was confirmed

as D-glucose by glucose oxidase [reagent (vii)]. The remaining glucose in the hydrolysate was then removed with glucose oxidase (GM VII f) and the residue analyzed by G.L.C. on both columns 3 and 5. In both cases a peak with the same retention time as myo-inositol was obtained.

Fraction 6

P.C. analysis of the large amount of reducing syrup obtained showed that this consisted almost entirely of a malto-oligosaccharide series (see Expt. VIII).

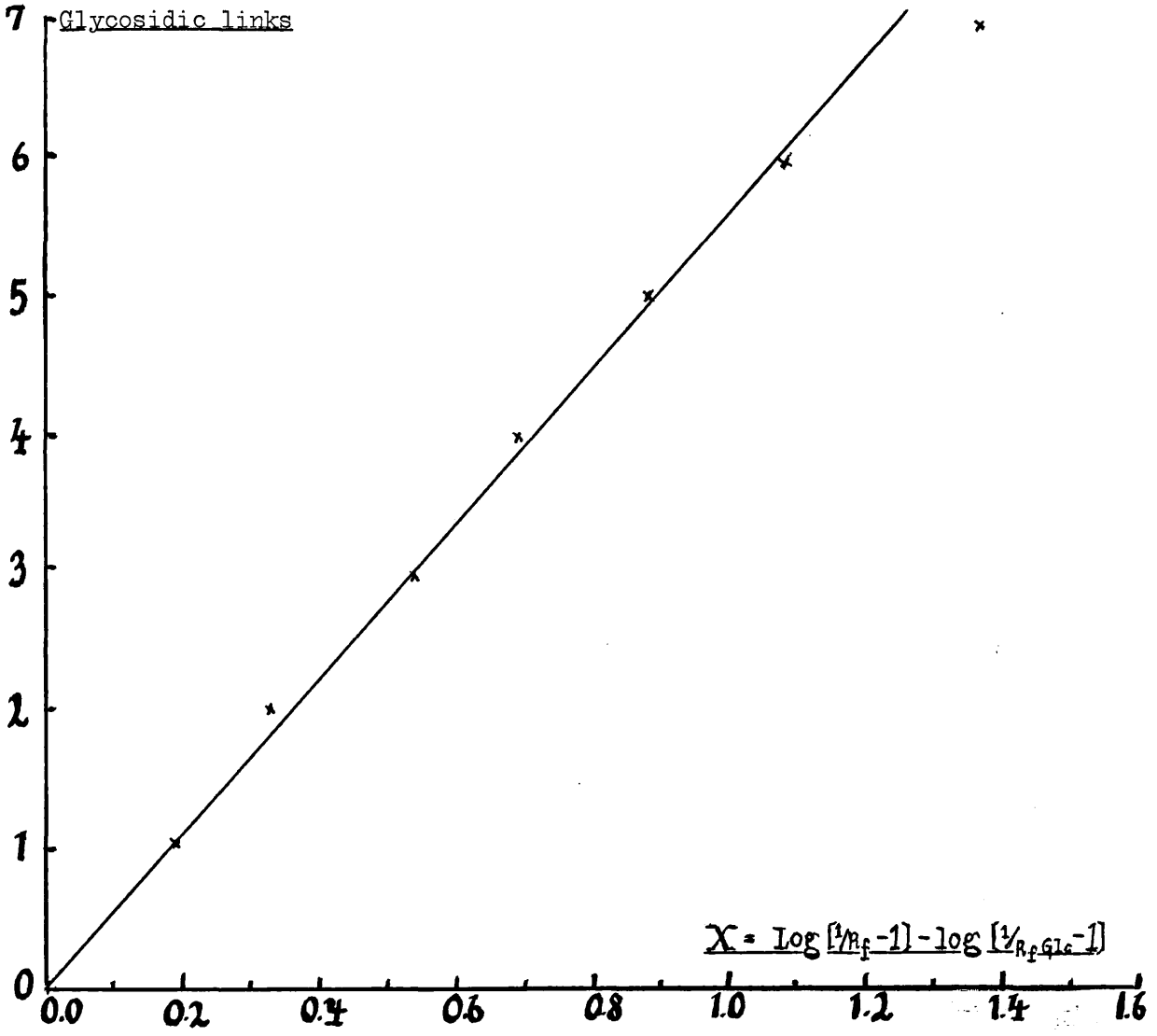
Experiment VIII. Examination of the malto-oligosaccharide solid B_1 derived from Experiment II (ii) (p. 57).

The solid B_1 on paper chromatographic analysis gave a series of spots with mobilities identical to those given by both a standard malto-oligosaccharide series and the oligosaccharides in fraction 6 (see above). The optical rotation, $[\alpha]_D^{23^\circ}$, was found to be 141° (c = 1.0 g/100 ml of water). A plot of the D.P-1 vs. $\log\left(\frac{1}{R_f}-1\right)-\log\left(\frac{1}{R_{f\,Glc}}-1\right)$ yielded a straight line for these oligosaccharides when they were run in solvent C, thus showing that they all belong to a true homologous series (Figure XI).[141]

A portion (25 mg) of the syrup was methylated (GM VII b 2) and hydrolyzed. P.C. analysis in solvents A, B and D with locating reagent (ii) showed the presence of 2,3,6-tri-O-methyl glucose and 2,3,4,6-tetra-O-methyl glucose. This was confirmed by G.L.C. analysis of the derived glycosides (GM VII c) on columns 1,2 and 11 (see Appendix Table V).

Another portion (50 mg) of the syrup was treated with salivary α-amylase at 37°C for 48 hr. The enzyme was then destroyed by boiling, the solution filtered and concentrated and the product analyzed by paper

Figure XI. Plot of No. of glycosidic links vs. χ.

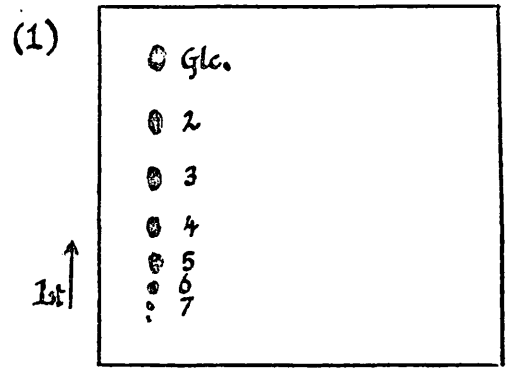


chromatography. This showed that the maltotriose, tetraose and pentaose had been attacked, giving a glucose spot and an increase in the intensity of maltose and the other malto-oligosaccharide spots.

An aliquot of syrupy B_1 was also spotted onto a sheet of No. 1 paper and developed in solvent C for four days (Figure XII(1)).

The paper was dried at 110°C for 4 hr, sprayed with sweet potato β-amylase (C. 50 units per ml in 0.01M sodium acetate buffer at pH 4.8) and incubated in a moist atmosphere at 37°C for 8 hr. The paper was then dried at 110°C to destroy the enzyme, and developed in solvent C in a direction at right angles to the original direction and the spots located with reagent 1. The result is shown diagrammatically in Fig. XII(3) and was identical to that obtained from a paper on which the standard series of malto-oligosaccharides had been spotted. Fig. XII(2) shows the result found from a paper which had not been sprayed with β-amylase.

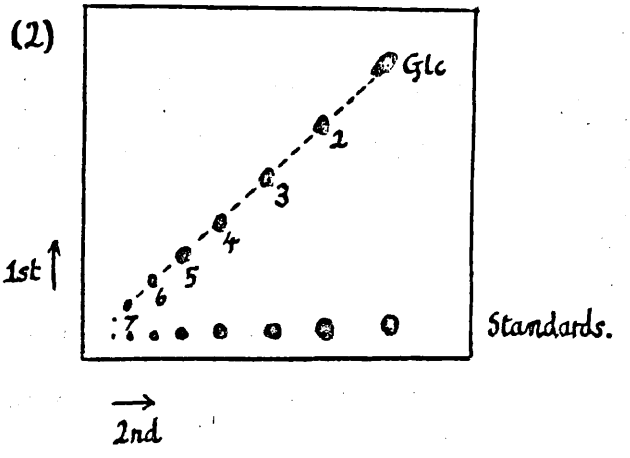
Figure XII. Two way chromatography of malto-oligosaccharides

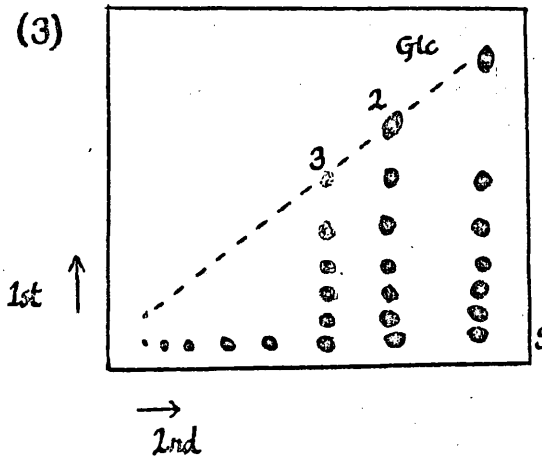


(1) Paper developed in one dimension:

Glc – Glucose
2,3,4 – No. of glucose units in the oligosaccharide.

(2) Paper developed in both dimensions without enzyme incubation in between. Standards added before 2nd run.





(3) Paper developed in both directions with enzyme incubation in between. The enzyme cleaves eventually to maltose and glucose, but maltotriose is cleaved rather more slowly than the higher oligomers. Standards added before 2nd run.

III. Fractionation and Examination of the Aqueous Extracts

a) Preliminary studies and fractionation methods

Experiment IX Preliminary examination of the aqueous extract

The hot water extract (p.56) was hydrolyzed (GM IIa and b) Paper chromatography in solvents A, B, and C followed by location with reagents (i), (ii) and (iv) showed spots whose mobility and relative abundance is shown in Table II (p. 96). An aliquot of one of the hydrolysates was then reduced (GM VIId) and converted into its Me_3Si derivative (GM VIIa) and analyzed by G.L.C. on column 3.

The naphthoresorcinol test (GM IVc) for uronic acid on the original polysaccharide was positive, and assay of both uronic acid and half ester sulphate (GM IVd and e) showed that they respectively constituted 19% and 22% of the polysaccharide based on its carbohydrate content, which was calculated from a graph of a synthetic mixture of rhamnose:xylose:glucuronic acid in the molar ratio of 7:4:2 (GM IVd and f).

The iodine test for amylose gave a faint positive blue colour, while the ferric chloride (1% in water) test for polyphenolic compounds was negative.

Similar results were obtained for all the other aqueous extracts obtained from samples harvested in subsequent years. All the chromatograms dipped in glucose oxidase [locating reagent (7)] of hydrolysates of these extracts gave a pink spot with the mobility of glucose.

Experiment X. Attempted fractionation methods

(1) With Fehlings solution[142]

No precipitate was formed on the addition of Fehlings solution to the polysaccharide solution, and so no fractionation into separate components was achieved.

(2) With Celite

Celite (ca. 25 g) was added to the polysaccharide solution [about 1% (carbohydrate content 40%) in 2% potassium chloride, 400 ml] and the resulting suspension stirred and then filtered at the pump. The Celite was washed with 20% potassium chloride, and then the contents transferred to another Buchner flask and the Celite again washed, this time with water until carbohydrate free. Both filtrates were separately dialyzed and analyzed by paper chromatography. The results are given on p.98. The recovery of carbohydrate from the Celite was about 1200 mg.

Experiment XI. Fractionation with D.E.52 Cellulose

(i) Gradient elution

A D.E. 52 column (45 x 3.75 cm, void volume 120 ml) was prepared (GM V). The fawn hot water extract [100 mg, 49 mg carbohydrate as glucose] from Expt. II (ii) was dissolved in ca. 10 ml of water and layered onto the cellulose surface. After the absorption of this material the column was eluted with distilled water. In the first 250 ml 36 mg [9 mg carbohydrate] were recovered, but although the column was eluted with a further 3l. of water no further carbohydrate material was recovered.

The column was then eluted over 1.4 litres by a 0 $\longrightarrow$ 1M potassium chloride gradient solution, and the eluant was collected in 10 ml fractions and assayed for carbohydrate (GM IVa) and protein. The protein assay was

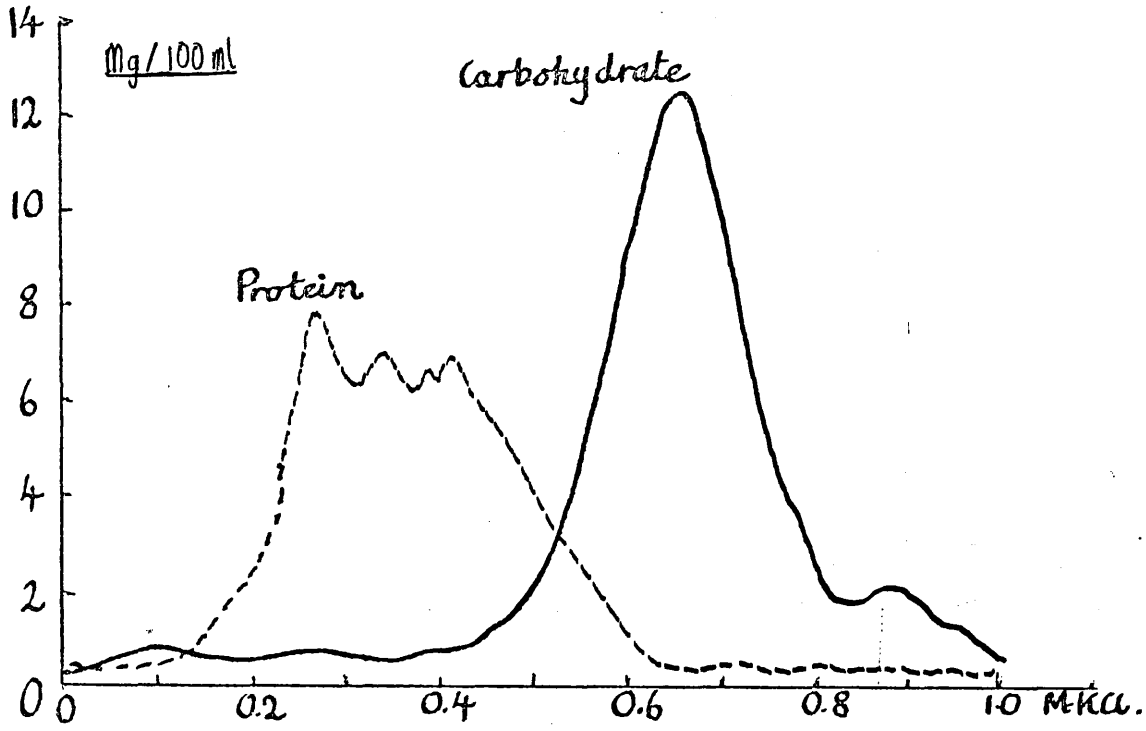
an absorbtiometric method based on the formula[143]:-

$$\text{protein mg/ml} = O.D_{280} \times 1.55 - O.D_{260} \times 0.76$$

where O.D = the optical density at the wavelength (in nm) indicated.

The results are given below: (Figure XIII).

Figure XIII Gradient fractionation of a hot water extract



Neutral	fraction 4.0 mg	[4 mg carbohydrate as glucose]
0. — 0.4M	fraction 25.4 mg	[6.4 mg carbohydrate as glucose]
0.4 — 0.8M	fraction 50 mg	[25.5 mg carbohydrate, 25% protein, 17% sulphate]
0.8 — 1.0M	fraction 8 mg	[7 mg carbohydrate, 21% sulphate]

Each fraction was hydrolyzed and examined by paper chromatography and electrophoresis (Table IV p.99).

(ii) Batch elutions

Following these results some of the remaining aqueous extracts were fractionated by stepwise increases in the concentration of potassium chloride in solution. The column was eluted first with distilled water,

and then successively, with 0.3, 0.8, 1.0 and 1.3M-KCl solutions, the latter two being combined.

An aliquot of each fraction was hydrolyzed and examined by electrophoresis and paper chromatography. This showed generally similar results to those obtained by the gradient elution technique, with the exception that the 0.8 M-KCl fraction appeared to be nearly devoid of protein. The recovery of carbohydrate was 86%.

b) Characterization of the constituent monosaccharides in the aqueous extracted polysaccharides.

Experiment XII Chromatographic analysis of the hydrolysate

Paper chromatographic analysis in solvents A, B and C with locating reagents (i), (ii) and (iv) gave spots with identical mobilities to those of rhamnose (major), xylose (major), mannose, glucose, galactose and glucuronic acid (both as the free acid and as its lactone) with the standard sugars run as controls. (see Appendix, Table I). Electrophoresis at pH 6.7 gave a spot with an identical mobility to that of glucuronic acid run as a control.

An aliquot of the hydrolysate was converted into its Me_3Si derivative, and analyzed by G.L.C. on columns 3 and 5 (for col 5 see Appendix, Table III). These gave peaks with identical retention times to those of rhamnose, xylose, mannose (minor) and glucose (minor). On column (5) a peak with the same retention time as authentic glucurone was also obtained.

Experiment XIII. Characterization of the individual monosaccharides.

(i) D-Galactose. Chromatography and location with reagent (viii) - D-galactose oxidase - showed a pink spot with the mobility of galactose.

(ii) D-Glucose. Chromatography and location with reagent (vii) - D-glucose oxidase - showed a pink spot with the mobility of glucose.

(iii) D-Mannose. Separation of a hydrolysate of the 0.3M KCl fraction (ca. 30 mg hydrolyzed) on 3MM paper yielded a sugar (ca. 20 mg) with the chromatographic mobility of mannose. To this syrup of $[\alpha]_D^{22}$ + 16.4° (C = 0.56) [cf. D-mannose equil $[\alpha]_D^{20}$ + 14.2][144] there was added ethanol (0.3 ml) and phenylhydrazine (20 µl).[145] The solution was then heated (50°C) until a white precipitate started to form (10 - 15 minutes). It was then stored overnight at 0°C. The mass of crystals were then washed with water, ethanol, and diethyl ether, and finally dried over phosphorus pentoxide under vacuum. Their m.p. and mixed m.p. with an authentic specimen of D-mannose phenylhydrazone (m.p. 188°C), prepared under similar conditions, was 188°C (decomposed).

(iv) D-Xylose. Separation of an Oxalic acid (2.25% - see GM IId) hydrolysate of the 0.8MKCl fraction (100 mg) gave ca. 23 mg of a syrup with the chromatographic mobility of xylose. It had $[\alpha]_D^{21°}$ + 20.2° (C 2.26) [cf. D-xylose equil $[\alpha]_D^{20}$ + 18.8°].[144] To this syrup there was added 1 ml of a 2:1 benzaldehyde: 2.5N-methanolic hydrogen chloride mixture.[146] At the end of four days long needle shaped crystals started to form, the crop being harvested at the end of seven days, washed with water and methanol, and then dried. The dry crystals were recrystallized from chloroform:petroleum ether b.p. 60-80° and had a m.p. and mixed m.p. with the authentic dibenzylidene dimethyl acetal of D-xylose (m.p. 208°C) of 206°C.

(v) D-Ribose. A spot with the mobility of ribose was obtained from a hydrolysate of the unfractionated material (ca. 250 mg). On separation of this hydrolysate on 3MM paper and eluting the band running between xylose and rhamnose a solution was obtained which on chromatographic analysis showed spots with the mobility ribose (++++ - ca. 50%) xylose (+++) and rhamnose (++).

This had $[\alpha]_D^{23^\circ}$ + 3.6° (C = 0.10). [cf. D-xylose + 18.8°, L-rhamnose + 8.2 → 8.9°, and D-ribose - 23.1° (all equilibrium values)].[144]

(vi) L-Rhamnose. Syrups with the chromatographic mobility of rhamnose were obtained from various hydrolysates reseparated on 3MM paper. One such syrup had $[\alpha]_D^{23^\circ}$ + 8.6° (C = 0.41) [cf. L-rhamnose equil + 8.2 → 8.9°].[144] All attempts to prepare the 2,4 dinitrophenylhydrazone[147] (on quantities of ⟨20 mg) were unsuccessful. Thin layer chromatography (Ethyl acetate solvent)[148] gave two spots, R_f 0.77 (+++++ 2,4 dinitrophenylhydrazine) and 0.2 (+ - Rhamnose 2,4 dinitrophenylhydrazone). Samples of this "rhamnose" were examined by electrophoresis with authentic rhamnose run as a control in borate buffer [M_{Glc} = 0.26, Boric acid (14.9 g) + Sodium hydroxide (8.0 g) + water (21.)][149] and in molybdate buffer [$M_{Glc'1}$ 0 — 0.73 (streak) di-sodium molybdate dihydrate 24.2 g/l adjusted to pH 5 with concentrated H_2SO_4 (ca. 5 ml/l)][150] The sample and the standard gave similar results.

To the remaining rhamnose (ca 5 mg) there was added ethanol (0.08 ml) and p-nitrophenylhydrazine (6 mg).[151] The mixture was heated at 100°C for 5–10 minutes, and then set aside at 0°C for 15 hr. The deposited crystals were washed with cold ethanol and then recrystallized from hot ethanol and dried over phosphorus pentoxide under vacuum. The derived p-nitrophenylhydrazone had a m.p. and mixed m.p., with the authentic L-rhamnose p-nitrophenylhydrazone (m.p. 186°) prepared under similar conditions, of 185.5°C. This phenylhydrazone was chromatographically pure (T.L.C. ethyl acetate; R_f 0.25 (streak) c.f. p-nitrophenylhydrazine R_f 0.85).

(vii) D-Glucuronic Acid. Several hydrolysates were combined on a 3MM paper and subjected to electrophoresis at pH 6.7 (GMIIIb). The band M_{GlcUA} = 1.00 was eluted and the derived solution filtered through Millipore (pore size 0.45 μ), evaporated to dryness, and triturated with galcial acetic

acid. Crystals (4-5 mg) then formed. These were separated and examined by X-ray powder crystallography, which showed that a substantial proportion of the crystals had the same crystal structure as authentic D-glucurone.

The crystals were then esterified, reduced and hydrolyzed. Chromatographic analysis of the hydrolysate gave a spot with the mobility and colour reactions of glucose; reagent (vii), D-glucose oxidase, giving a pink spot.

c) Examination of Glucose (Amylose) and Mannose-rich Materials

Experiment XIV

(1) The Neutral and 0.3M fractions

Both fractions appeared to be very similar on chromatographic analysis, and together formed between 5-10% of the aqueous extract. They did not give a precipitate with Fehlings solution.

(2) The Neutral fraction

The proportion of hexitol:6-deoxyhexitol:pentitol was found by gas liquid chromatography of the Me_3Si derivatives of the reduced hydrolysate (p.101).

Paper chromatographic analysis showed that mannose was by far the most abundant sugar present, with much less glucose and xylose, and trace amounts of galactose and rhamnose.

The polysaccharide was devoid of uronic acid and sulphate, and had $[\alpha]_D^{21}$ + 92° (C = 1.0; cf. Methyl α-D-mannopyranoside + 79°).

(3) Soluble Residue left after autohydrolysis of the aqueous extract (from p.85).

Hydrolysis and paper chromatographic analysis showed that this residue had a similar composition to the neutral material described above (2). It gave a blue colour with the I_2/KI reagent.

Periodate oxidation

On oxidation of the residue 100 mg with periodate (GM VIIh) 0.23 moles of periodate per anhydro unit were reduced. The derived polyalcohol was hydrolyzed and examined by paper chromatography [solvent A, reagent (i)].

Methylation

A portion of the residue (ca. 25 mg) was methylated (GM VII b), checked for full methylation (GM VIIb-infrared), and hydrolyzed. Chromatography [Solvent A, reagent (ii)] gave a major spot R_{TMG} 0·89. This hydrolysate was then reduced and converted to the alditol acetates, which were then analyzed by G.L.C./M.S. (GM VIb) on column 13 (page 102).

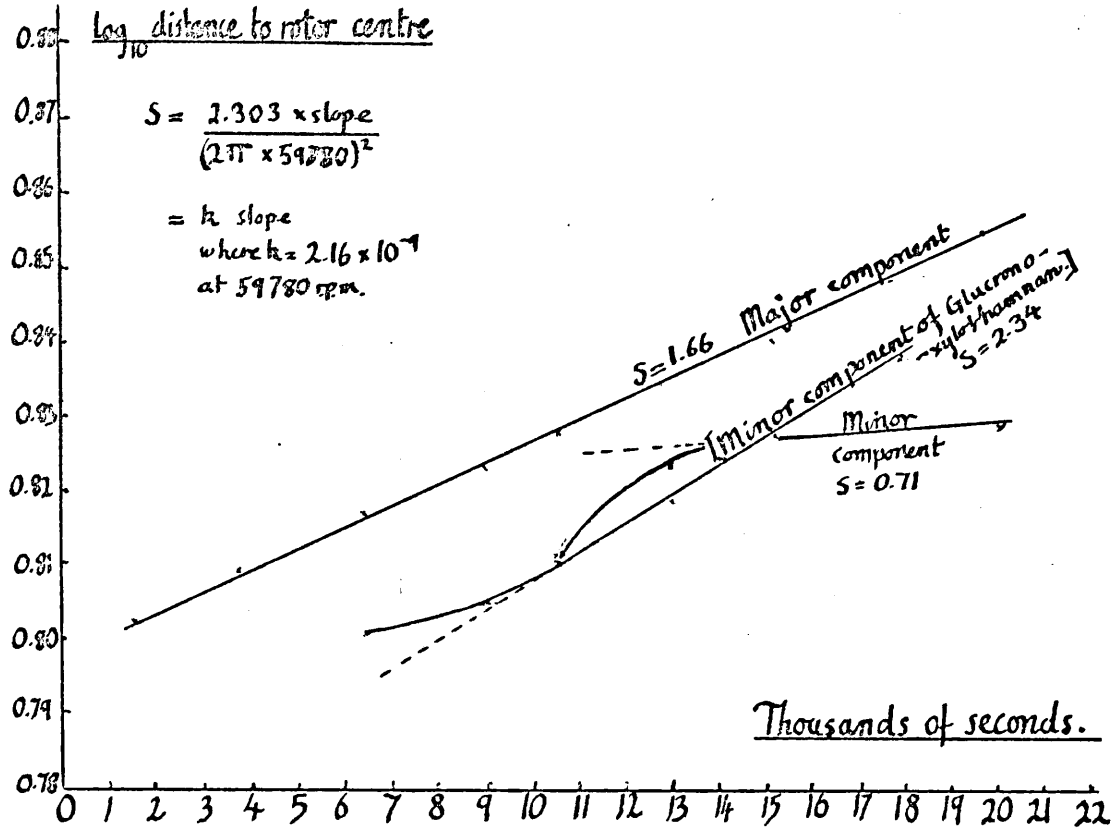
Digestion with α-amylase

An aliquot of residue (ca. 100 mg) was treated with salivary α-amylase for 24 hr at 30°C. The solution was then boiled and dialyzed. The dialysate gave a series of spots on chromatographic analysis suggesting a malto-oligosaccharide series. Hydrolysis of an aliquot and treatment with reagent (vii) confirmed D-glucose. The residual mannan in the dialysis sac had $[\alpha]_D^{28^\circ} = 50.0^\circ$.

Ultracentrifugation

The residue (10 mg) was dissolved in 0.2M-KCl solution (1 ml) and analyzed by ultracentrifugation (see p. 82). The results are shown on Fig. XIV p. 78. A plot of the time t against $\log_e x$ where x is the distance from the centre of the rotor (Fig. XIII) gave sedimentation coefficients (5.3°C, 0.2M KCl] of 1.66 S (major) and 0.71 S (minor). The calculated density for both the mannan and the amylose is ca. 1.539.

Figure XIV. Ultracentrifugational analysis of the residue



(4) Isolation of amylose from an aqueous extract (p.96).

A portion of the aqueous extract (300 mg, 24% carbohydrate) was then dissolved in 20% sodium chloride solution, and the amylose recovered by the iodine complex method.[152] On dialysis and freeze-drying the derived solution a brownish solid [30 mg - 19.4 mg carbohydrate] was obtained. This gave a blue colour with KI/I_2 solution. An aliquot (15 mg) was oxidized with periodate and reduced to the polyalcohol (GM VIIh). The derived solution of polyalcohol no longer gave a blue colour with KI/I_2 solution. Both the original solid and the derived polyalcohol were hydrolyzed and examined by paper chromatography (p.101). The positive carbohydrate test given by the polyalcohol with the phenol-sulphuric acid reagent was probably caused by the glycoaldehyde residues[153] present in the molecule.

d) Examination of the Glucuronoxylorhamnan

Experiment XV. Estimation of the proportions of the constituent sugars

(i) As the Me_3Si derivatives of the alditols derived from the 0.8M KCl fraction

(a) A preliminary estimation of the proportions of sugars by GLC as their Me_3Si derivatives on column 3 (GM IVf) showed rhamnose: xylose in the molar proportion 1.75:1.00, with about 17-19% uronic acid (GM IVd), although visual examination of a paper chromatogram of the hydrolysate suggested a molar ratio of 1:1 for rhamnose and xylose.

(b) (1) The polysaccharide (60 mg; 35-40 mg carbohydrate) was hydrolysed with 2.25% oxalic acid (GM IId.). The derived syrup was esterified and glycosided (GM VIIc) and when Fehling's test for reducing carbohydrate was negative, the product was reduced with borohydride and the derived sugar glycosides were hydrolyzed to the free sugars.

(2) A synthetic mixture containing xylose (22.2 mg), rhamnose (24 mg) and glucuronic acid (1.8 mg) was subjected to exactly the same treatment as the polysaccharide in (b).

(3) A synthetic mixture containing the same weights of rhamnose and xylose, but in which glucose (12.1 mg) replaced the glucuronic acid, was prepared.

Each of the products from (1), (2) and (3) were dissolved separately in water (10 ml). Equal volumes (3 ml) were removed from each solution. They were concentrated to dryness, and converted into their Me_3Si derivatives and analyzed by GLC (column 5). The sugars in the remainder of each solution were reduced to their respective alditols and the latter analyzed as their Me_3Si derivatives by GLC on column 5. The molar proportions of

the sugars in the different products were then deduced from the ratio of their peak areas (GM IVf) (Table VI p.104). Similar results (see Table VI) were obtained by GLC analysis on a Celite column coated with 10% polythene-glycol-20M and the peak areas measured by a Hewlett-Packard 3371B Integrator.

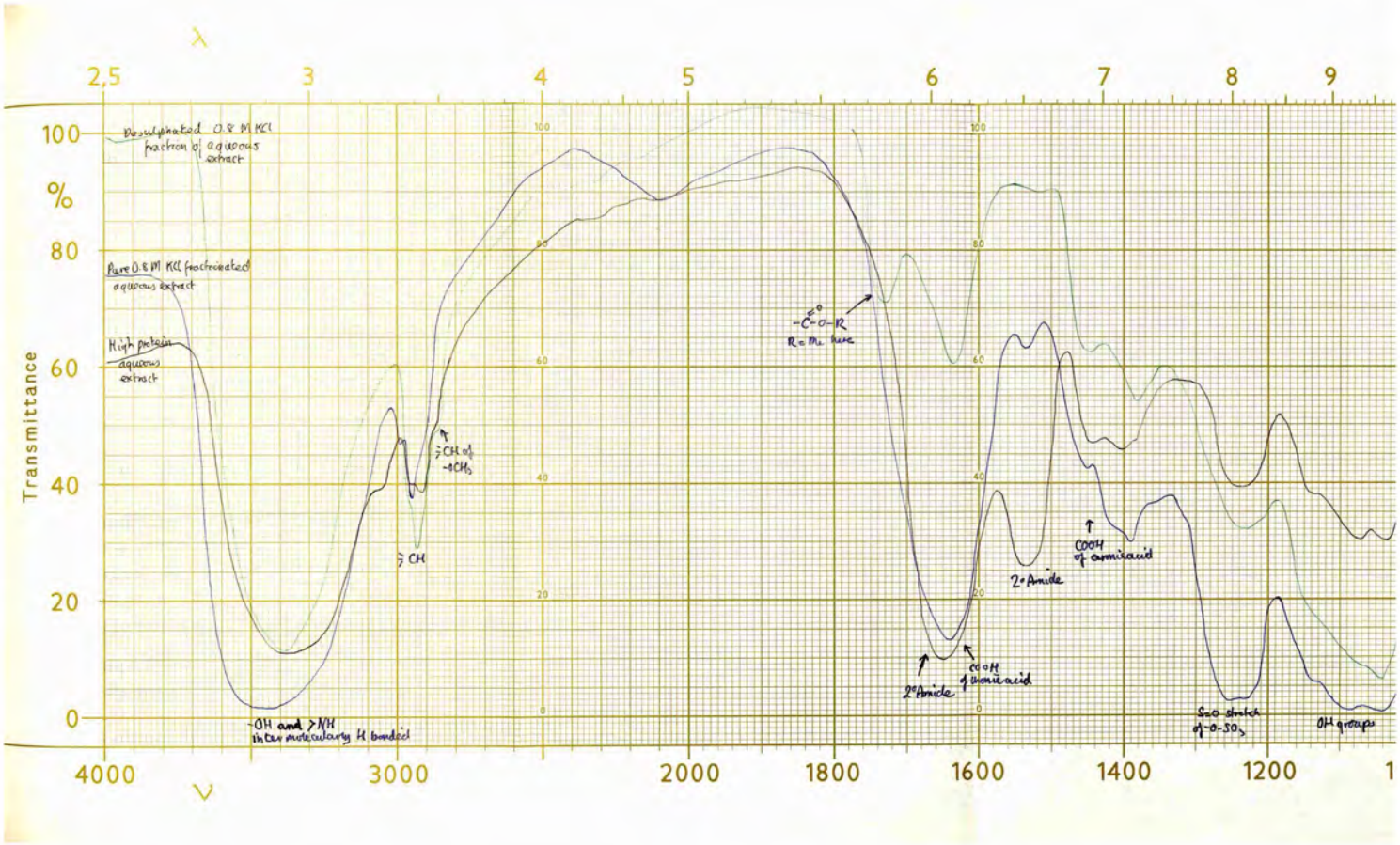
In view of these results the carbohydrate contents of all subsequent aqueous extracts were measured against a standard graph derived from a mixture of rhamnose, xylose and glucuronic acid in the molar ratio of 3:3:1.

(ii) By colorimetric determination of rhamnose and glucuronic acid

The proportion of rhamnose in the various fractions was assayed by the Dische method (GM IVb) and the uronic acid by the modified carbazole method (GM IVd). The proportion of xylose was calculated by difference and these results are also shown in Table VI (p.104).

Experiment XVI. Variation of the optical rotation of the Glucuronoxylorhamnan with Temperature

The 0.8M KCl fraction of the glucuronoxylorhamnan had $[\alpha]_D^{21^\circ}$ -89° (c, 1.0) going to -93° (c, 0.51) in different extracts. The extract with $[\alpha]_D^{24^\circ}$ -93° was heated to 75°C over 2.5 hr and the change in rotation plotted against time (Fig. XV curve I). On cooling overnight the rotation became $[\alpha]_D$ -102°. The heating was repeated, this time to 95° over 2.5 hr. The change in rotation was this time plotted throughout the heating (curve II) and cooling (curve III) cycle.


λ
2.5
3
4
5
6
7
8
9
100
%
80
60
40
20
0
Transmittance
Desulphated 0.8 M KCl fraction of aqueous extract
Pure 0.8 M KCl fractionated aqueous extract
High protein aqueous extract
-OH and >NH inter molecularly H bonded
>CH
>CH of -CH3
-C(=O)-O-R
R = Mucic acid
2° Amide
COOH of amino acid
2° Amide
COOH of amino acid
C=O stretch of -O-SO3
OH groups
4000
3000
2000
1800
1600
1400
1200
1
ν

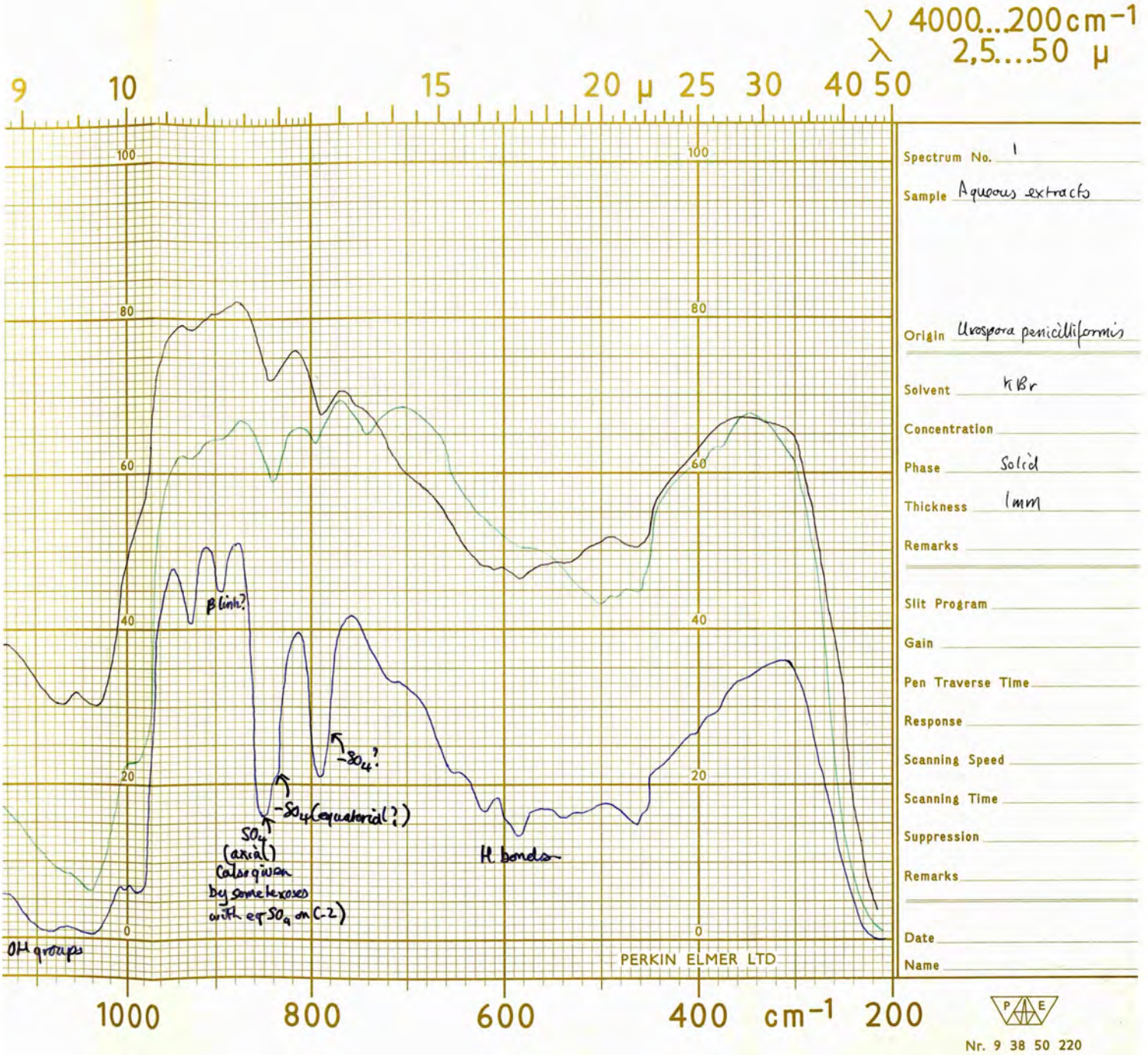
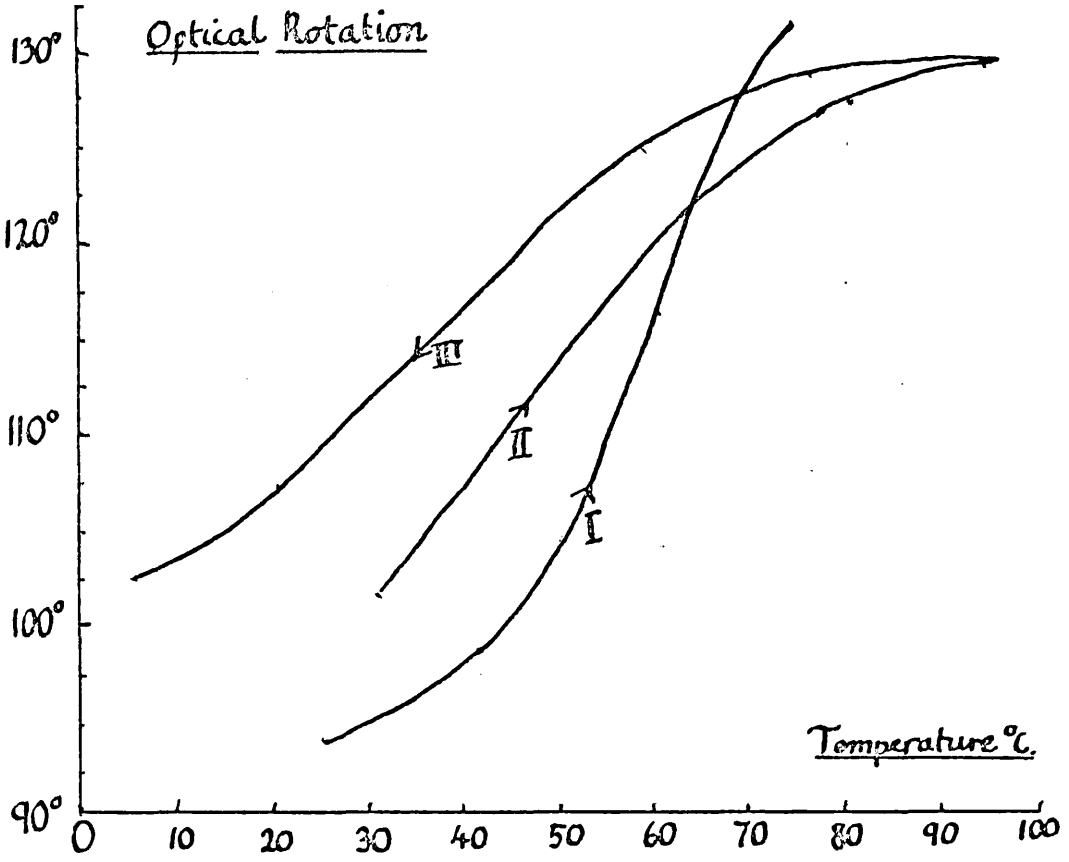

ν 4000...200 cm⁻¹
λ 2,5....50 μ
9 10 15 20 μ 25 30 40 50
100
80
60
40
20
0
β link?
-SO4 (equatorial?)
-SO4?
SO4 (axial) Characteristic by some hexoses with eq SO4 on C-2
H bonds
OH groups
PERKIN ELMER LTD
1000 800 600 400 cm⁻¹ 200
Spectrum No. 1
Sample Aqueous extracts
Origin Urospora penicilliformis
Solvent KBr
Concentration
Phase Solid
Thickness 1mm
Remarks
Slit Program
Gain
Pen Traverse Time
Response
Scanning Speed
Scanning Time
Suppression
Remarks
Date
Name
P E
Nr. 9 38 50 220

Figure XV. Dependance of the optical rotation of the 0.8M KCl fraction on temperature



Experiment XVII. Infrared examination of the Glucuronoxylorhamnan

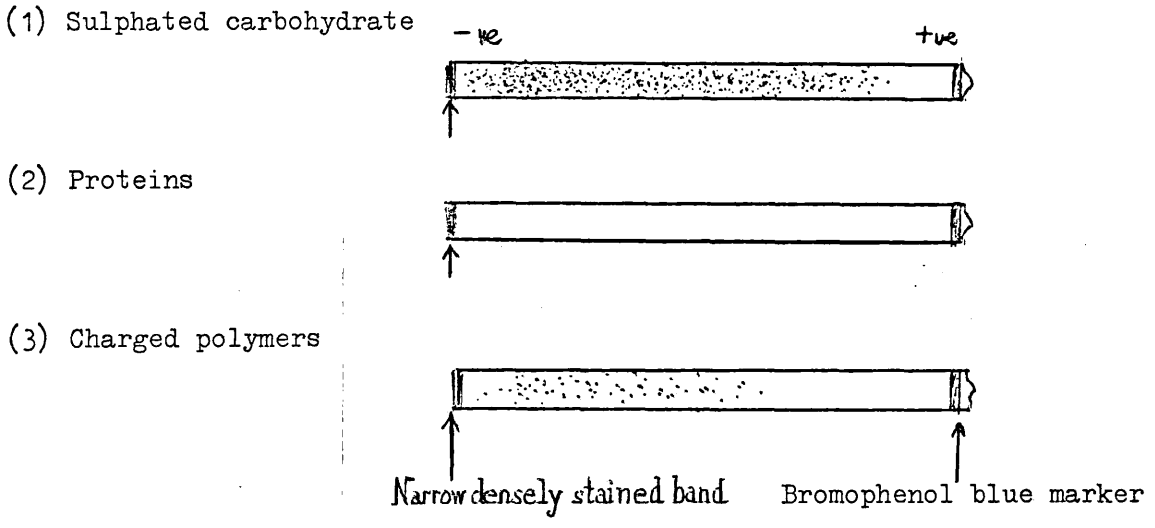
The 0.8M KCl fraction (negligible protein), the unfractionated extract (65-70% protein) and the partly desulphated 0.8M KCl fraction (p.87) were each dispersed in a KBr disc and examined with the Perkin Elmer S.P. 325 Infrared Spectograph. These spectra are given on p.81 (insert).

Experiment XVIII. Examination of the homogeneity of the extracts

(i) Gel electrophoresis

The 1.0M KCl fraction of the aqueous extract was subjected to analytical gel-electrophoresis in tris-glycine buffer (pH 8.5) using the Shandon Model SAE 2734 polyacrylamide gel electrophoresis apparatus.

The tubes (7 x 0.5 cm) of this apparatus were filled to a depth of 6.5 cm with a 7.5% acrylamide solution whose pore size of 15Å excludes proteins of a molecular weight in excess of 300,000.[154] After the solution had set the polysaccharide (1 mg in 0.2 ml 20% sucrose solution) was layered onto the surface of the polyacryalmide gel. A drop of bromophenol blue was added as a marker. After completion of the electrophoresis (shown by the bromophenol blue reaching the other end of the tube) the sulphated carbohydrate in one tube was located with toluidine blue,[155] the protein in another with amido black and all charged polymers in yet another with the photo-labile "stains-all" (Serva, Feinbiochemica Heidelberg). The results are shown below:-



(ii) Ultracentrifugation

The 1.0M KCl fraction was analyzed on the Beckman Spinco Model E ultracentrifuge at 58760 rpm using Schlieren optics to plot the rate of sedimentation with distance (Fig. XVI). The camera magnification factor is 2.224 .

The results are plotted as time t against $\log_e x$, where x is the distance from the centre of the rotor, in Fig. XVII

Fig. XVI. Results from a sedimentation velocity experiment on the ultracentrifuge

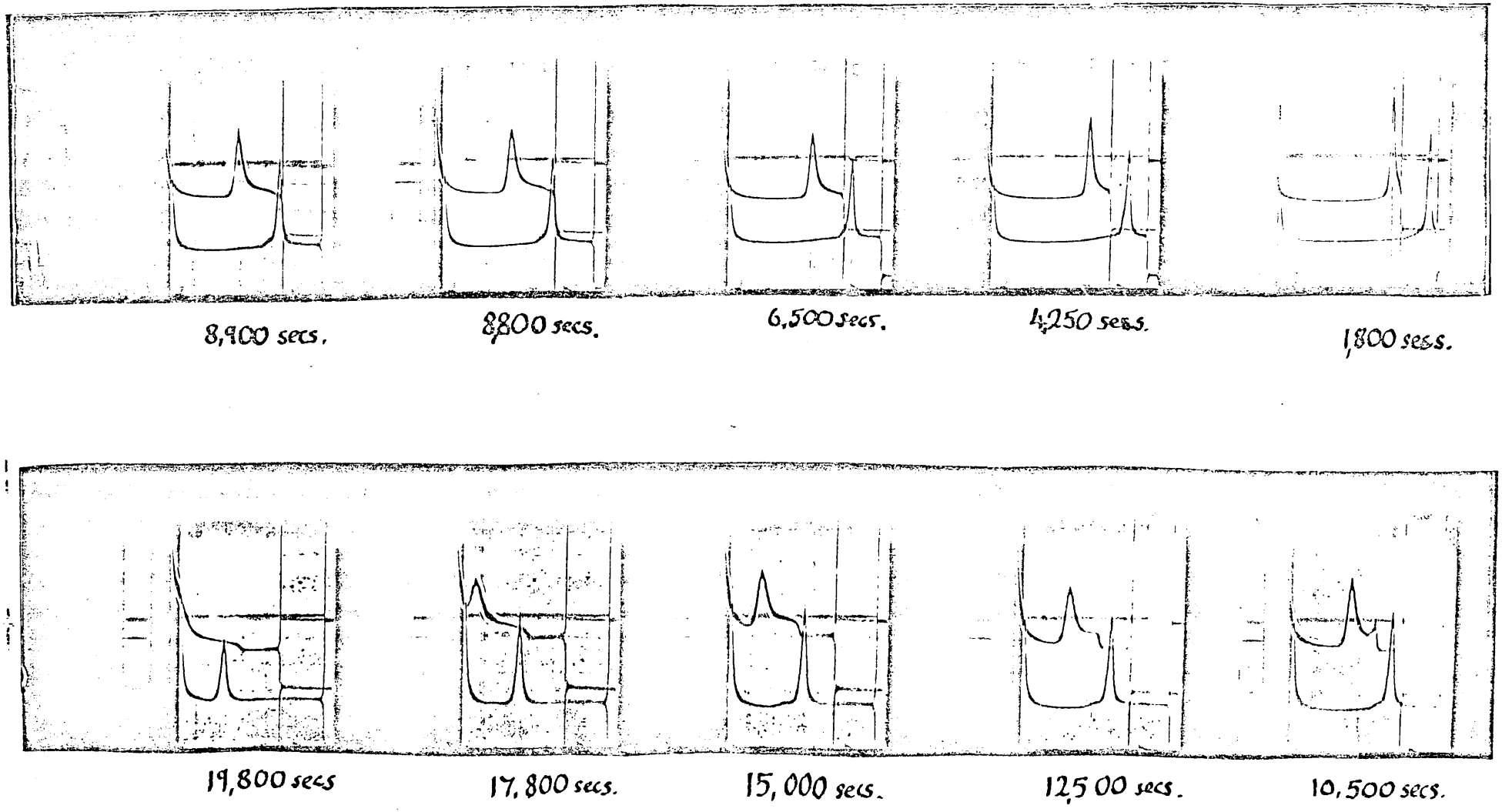
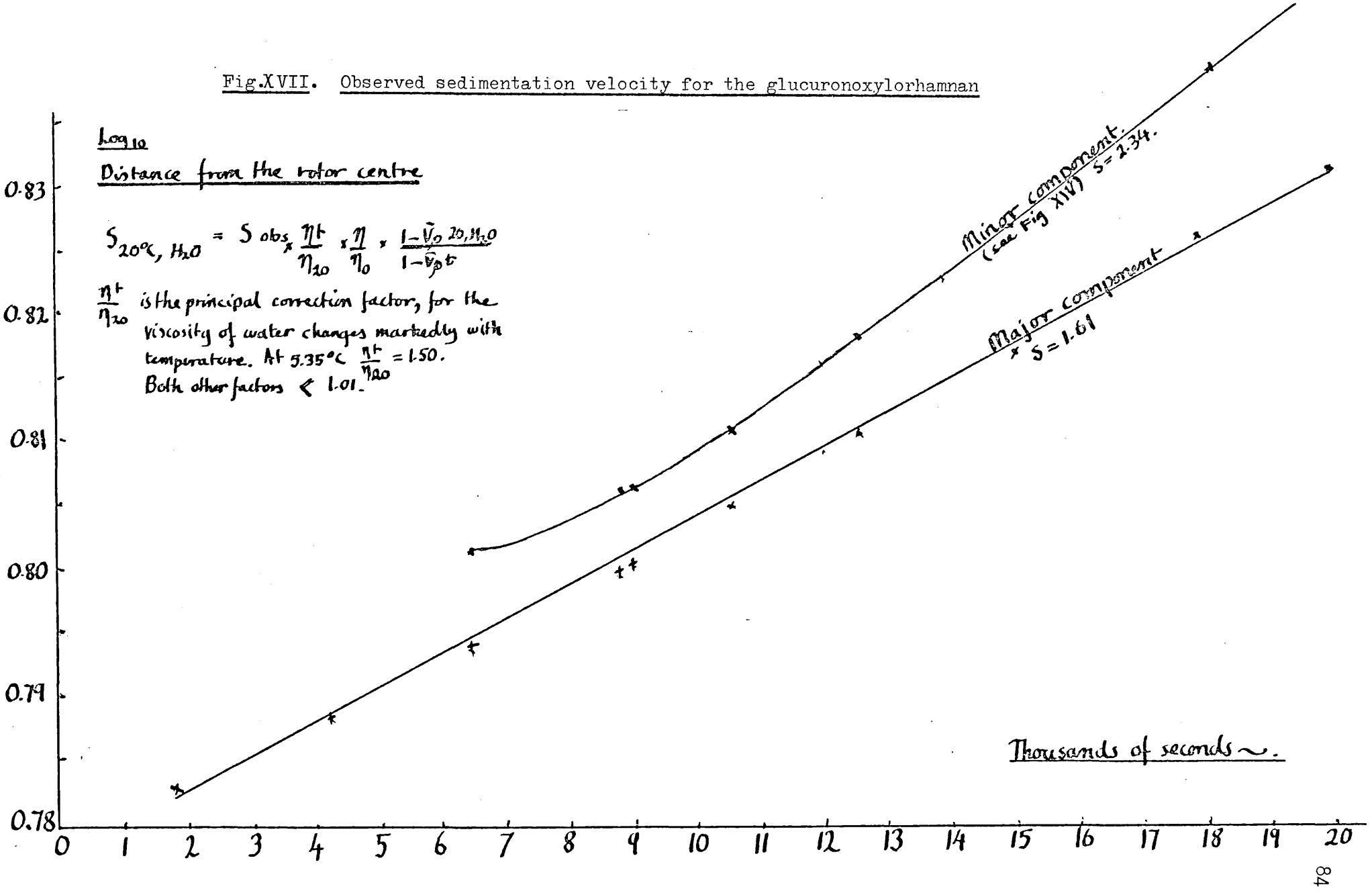


Fig.XVII. Observed sedimentation velocity for the glucuronoxylorhamnan



$$S_{20^\circ C,\, H_2O} = S_{obs} \times \frac{\eta_t}{\eta_{20}} \times \frac{\eta}{\eta_0} \times \frac{1-\bar{V}_{0\,20,H_2O}}{1-\bar{V}_p \rho_t}$$

$\frac{\eta_t}{\eta_{20}}$ is the principal correction factor, for the viscosity of water changes markedly with temperature. At 5.35°C $\frac{\eta_t}{\eta_{20}} = 1.50$. Both other factors < 1.01.

The sedimentation coefficients were $S[5.3^{\circ}, 0.2M\ KCl]=1.61$ (major component) and 2.34 (minor component).

The partial specific volume ($\bar{V}$) of the major peak was estimated from a xylose:rhamnose:glucurone ratio of 3:4:1 + 25% by weight of $KHSO_4$ (= 17-18% SO_4''), $=\rho 1.608=\bar{V}\,0.623$ in aqueous solution (see Fig. XVII).

Experiment XIX. Degree of Polymerisation (DP)

The (DP) was determined from the difference in absorption (A), given by the phenol-sulphuric acid reagent, by equal amounts of the 0.8M KCl fraction both before (O) and after reduction (R). The formula A_O/A_O-A_R gave a minimum value of at least 45 for the (DP)-since this is the limit of the sensitivity of the method, the experiment was repeated five times and the results averaged.

Experiment XX. Autohydrolysis and analysis of the products

The unfractionated aqueous extract (2 + 3 p.96) (4.0 g., carbohydrate content ca. 1.8 g) was dissolved in water (250 ml) and treated with IR $120H^+$ Amberlite resin. When the pH had dropped from 7.7 to 1.7, the resin was filtered off, and the filtrate transferred to a glycerine-free dialysis sac and heated in distilled water at 70° with stirring for 10 hr. The dialysate was removed, neutralised with barium carbonate, filtered and concentrated. A precipitate (1.1 g. carbohydrate content 0.19 g) had formed inside the dialysis sac. This was removed, hydrolyzed, analyzed by paper chromatography and discarded. The solution from the dialysis sac was placed inside a fresh glycerine-free dialysis tube and heated for 6-8 hr at 70°. The dialysate was removed and neutralised as before. The heating in fresh dialysis water was repeated 5 more times and then the

residual solution inside the dialysis sac was neutralised and concentrated to dryness (0.675 g, carbohydrate content 0.65 g.)

The dialysates were examined by paper chromatography (see p.112) and then combined. The combined material was dissolved in water (10 ml) and methanol (100 ml) added. The precipitate (0.85 g ca. 0.55 g carbohydrate) was removed by filtration and washed with methanol. The precipitate was then separated into nine fractions on 3MM paper (3 sheets, GM III a-5 (i)).

The filtrate was concentrated and examined by paper chromatography. It had small amounts of monosaccharides, together with some 7 oligosaccharides, which were not examined further.

Experiment XXI. Desulphation of the 0.8 molar fraction of the glucuronoxylorhamnan

(i) By N-NaOH[156]

The polysaccharide [100 mg, ca. 66 mg carbohydrate + 11.5 mg sulphate] was dissolved in water [10 ml], and ca. 90-100 mg of sodium borohydride added. The solution was set aside for 20 hr after which time sodium hydroxide solution (5 ml, 3N) and more sodium borohydride (ca. 200 mg) were added. The solution was then heated at 80°C for 7 hr, after which time it was cooled, dialyzed and freeze-dried to a white solid [88 mg, 63 mg carbohydrate + 11.1 mg sulphate]. An aliquot of the original and of the alkali-treated material were hydrolyzed separately (GM IIa) and analyzed by paper chromatography.

(ii) By pyridine and dimethyl sulphoxide[157]

The polysaccharide [500 mg, ca. 330 mg carbohydrate + 58 mg sulphate] was added to dimethyl sulphoxide [500 ml] in which it slowly dissolved to form a clear viscous solution. When it had completely dissolved pyridine [10 ml] was added. The solution was then heated at 100°C for 8 hr,

cooled, dialyzed and freeze-dried to a white solid [388 mg, 256 mg carbohydrate + 34 mg sulphate]. An aliquot of the original polysaccharide and of the desulphated polysaccharide were each analyzed for uronic acid and 6-deoxy hexose, while further aliquots were separately hydrolyzed and examined by paper chromatography.

(iii) Desulphation of the pyridinium salt by method (ii)

The polysaccharide [500 mg] was converted into its pyridinium salt by dissolution in water (200 ml) and treatment with Amberlite IR 120 Pyr^+ resin (formed by neutralising IR 120 H^+ with pyridine.) The resin was removed by filtration. The filtrate was then concentrated and freeze-dried to a white solid. This white solid was added to dimethyl sulphoxide (500 ml), in which it rapidly dissolved, and the resulting solution was treated as described for (ii). The solution, which was brown by the end of 8 hr, on dialysis and freeze-drying gave 25 mg of a fawn solid which on hydrolysis and paper chromatography gave xylose, rhamnose and glucuronic acid. It was not examined further.

(iv) Desulphation with methanolic hydrogen chloride[158]

The polysaccharide [500 mg, 330 mg carbohydrate 58 mg sulphate] was dispersed with vigorous stirring into dry 0.04M-methanolic hydrogen chloride [200 ml]. The stirring was continued for 48 hr in a sealed container at 25°C. The polysaccharide [406 mg, 326 mg carbohydrate + 43 mg sulphate] was then filtered off and washed extensively with dry methanol. After dissolution in water and dialysis, the product was freeze-dried to a crisp white solid [192 mg, 170 mg carbohydrate + ca. 8-9 mg sulphate]. This solid (which, with others derived from repetitions of this experiment, is referred to in future experiments as the 'partly desulphated polysaccharide') was analyzed by infrared (Insert 1, p.81). An aliquot was assayed for uronic acid while another

aliquot was hydrolyzed and examined by paper chromatography and the proportion of sugars found (GM IVf).

Experiment XXII. Methylation of the 0.8M KCl fraction and of the partly desulphated polysaccharides.

(1) Preliminary experiment

The polysaccharide (34 mg, 0.8M KCl fraction) was methylated (GM VIIb(1)) dialyzed and freeze-dried. The product was checked for full methylation (GM VIIb(2), thin layer). It was then hydrolyzed and the hydrolysate analyzed by paper chromatography in solvents A, B & D with locating reagent (ii).

(2) Later experiment

Both the 0.8M KCl polysaccharide (97 mg) and the partially desulphated polysaccharide (98 mg) were methylated as described above. The fully sulphated polysaccharide took 18 hr to dissolve in the dimethyl sulphoxide and then formed a viscous solution, whereas the partially desulphated material dissolved very readily. After methylation the products were dialyzed and freeze-dried to yellowish solids (ca. 133 mg each). The freeze-dried materials were each suspended in tetrahydrofuran [100 ml]. Lithium aluminium hydride [100 mg][159] was then added to each solution, which was then set aside for 0.5 hr before being refluxed for 2 hr under nitrogen. More lithium aluminium hydride [60 mg] was then added and the refluxing continued for a further 0.5 hr.

The excess hydride was destroyed by the cautious addition of a little water to each solution, during which a voluminous precipitate of aluminium hydroxide formed. More water (100 ml) was added, and the solutions were acidified with dilute sulphuric acid until the precipitates had dissolved (pH 3.5 - 4.0). They were then extracted thrice with

chloroform, the chloroform extracts being combined. It was found that the majority of the carbohydrate was extracted from the partly desulphated material by this means, but very little of the fully sulphated material. Accordingly the aqueous solution containing this was treated with IR 120H$^+$ and dialyzed until it gave a negative test for aluminium [Alizarin sulphonic acid sodium salt + Aluminium ions give a purple colour]. It was then concentrated and freeze-dried.

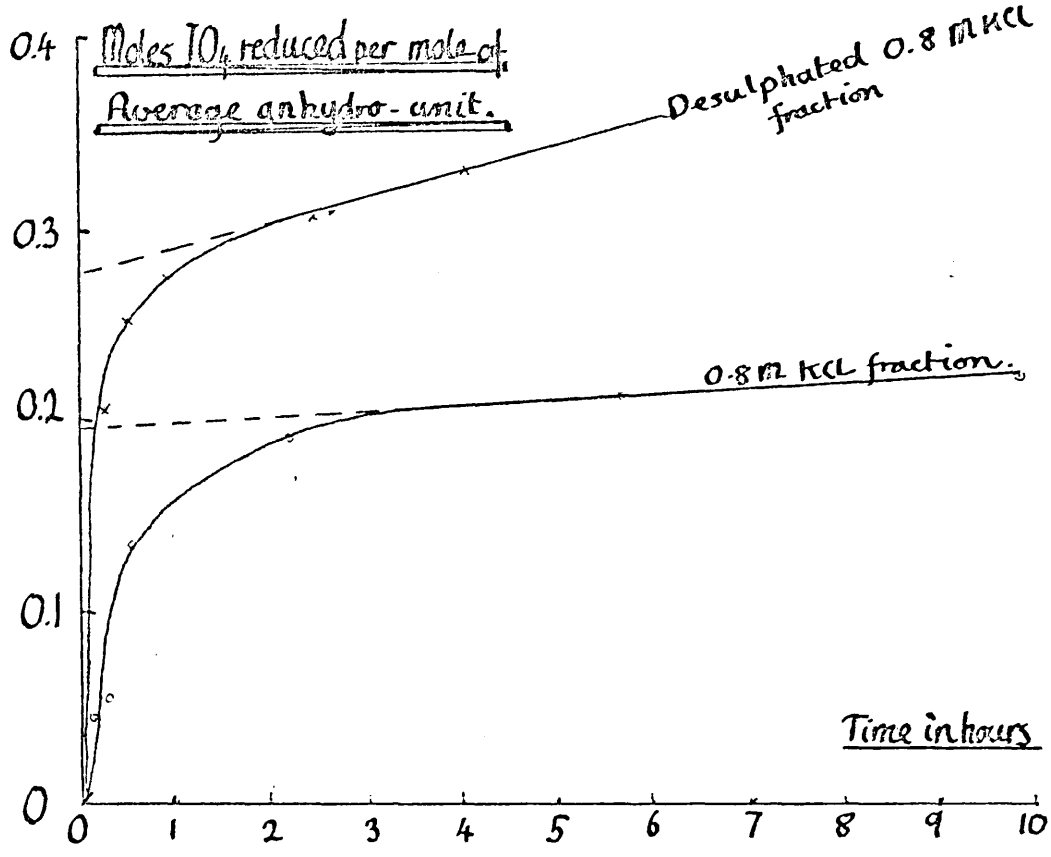
Both extracts were then separately re-methylated (twice) and checked for full methylation (GM VII2b - infrared). They were then hydrolyzed and examined by paper chromatography [Solvent A, B and D, locating reagents (ii) and (iii)]. An aliquot of each was then glycosided (GM VIIc) and the derived glycosides examined by GLC on columns (1) and (2). The rest were reduced (GM VIId) and converted into their alditol acetates (GM VIIe) and analyzed by GLC and GLC/MS on column 13 (see p. 110).

Experiment XXIII. Periodate oxidation of the glucuronoxylorhamnan

(i) Oxidation of the 0.8M-KCl fraction

The polysaccharide [1.18g, 790 mg carbohydrate + ca. 134 mg sulphate] was dissolved in water (250 mL). A solution of 0.03M sodium periodate (250 ml) was then added, and the reaction monitored for 24 hr (GM VIIh) during which 0.20 moles of periodate per anhydro unit were reduced (see Fig. XVII).

Fig. XVIII Oxidation of the 0.8M KCl fraction and the desulphated 0.8M KCl fraction with 0.15M sodium periodate



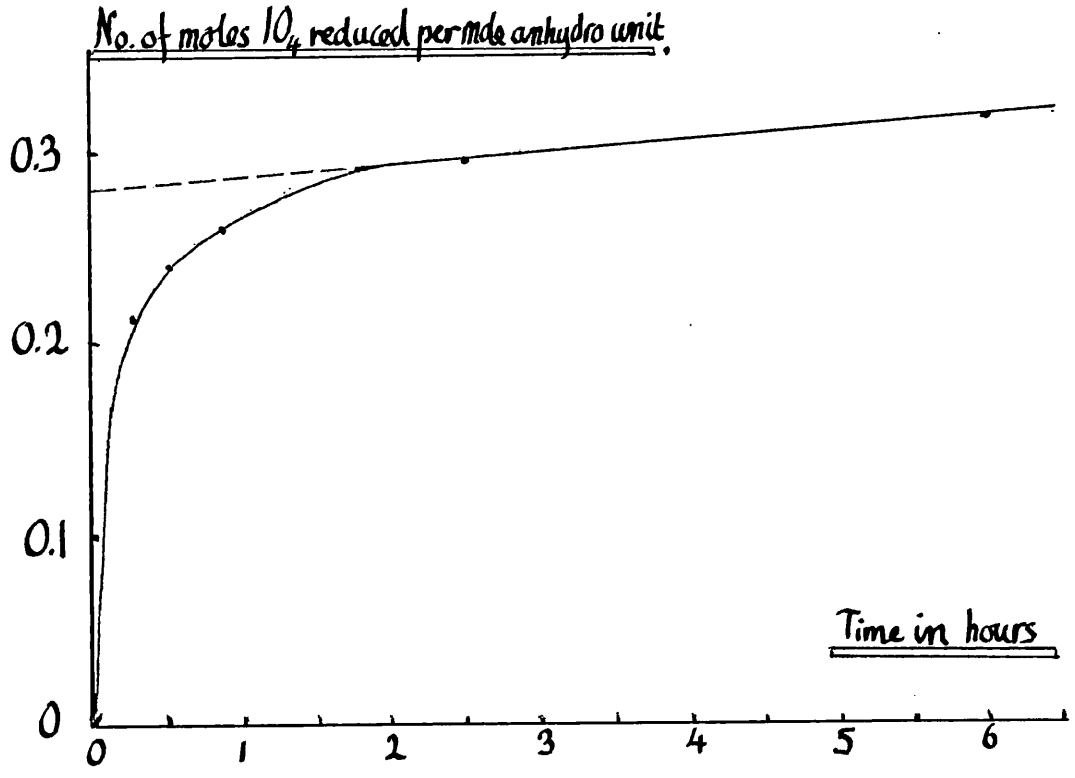
The periodate was destroyed by the addition of ethylene glycol (1 ml). The solution was reduced, dialysed and freeze-dried to a crisp, readily soluble white solid [Polyalcohol I - 973 mg, 526 mg carbohydrate]. Oxidation of an aliquot of this polyalcohol I resulted in a negligible reduction of periodate. An aliquot was then analyzed by hydrolysis and paper chromatography and the proportion of the constituent uncleaved sugars found (GM IVf - see p.107). The uronic acid in the polyalcohol was assayed by the carbazole method (GM IVd).

The polyalcohol I (390 mg) was then hydrolyzed with dilute sulphuric acid [pH 1.3, 48 hr, 23°C, the conditions being found by preliminary experiments[160]] after which the solution was neutralized with a 5% solution

of N,N dioctylmethylamine in chloroform. The excess base and its sulphates were removed from the solution by shaking with chloroform and discarding the lower chloroform layer. The upper aqueous solution was dialyzed and freeze-dried to a white solid (degraded polymer I, 247 mg, ca. 180 mg carbohydrate). An aliquot (20 mg) was hydrolysed and analyzed by paper chromatography and GLC. The dialysate [about 135 mg carbohydrate] was concentrated and separated into three major fractions by chromatography on 3MM paper in solvent B [GM IIIa) - 5(i)]. Aliquots of the three fractions were separately analyzed by paper chromatography, while another aliquot of each was hydrolyzed (GM IIa) and examined by paper chromatography.

Meanwhile the remainder of the degraded polymer I [225 mg 162 mg carbohydrate] was oxidized with periodate solution as described above (0.28 moles periodate were reduced per mole anhydro unit) (see Fig. XIX)

Fig. XIX. Oxidation of degraded polymer I with 0.15M sodium periodate solution.



after which the solution was reduced, dialyzed and recovered as a crisp white readily soluble solid [185 mg, 92 mg carbohydrate]. This was analyzed after complete and mild hydrolysis by paper chromatography.

(ii) Oxidation of the desulphated polysaccharide

A partly desulphated polysaccharide [667 mg, 595 mg carbohydrate + ca. 30 mg sulphate] was dissolved in water (200ml.) and oxidized by 0.03M periodate (200ml) as described above (p.89). After 48 hr, 0.28 moles of periodate per mole anhydro unit had been reduced (Fig. XVIII). The polyaldehyde was reduced, dialyzed and freeze-dried to a white solid [polyalcohol I]. This was reoxidized with periodate, during which a further 0.06 moles of periodate per mole average anhydro unit were reduced. This was reduced, dialyzed and freeze-dried to a crisp white solid (polyalcohol II) (540 mg, 370 mg carbohydrate.)
This was assayed for uronic acid and a hydrolysate examined by P.C. and the molar proportion of uncleaved sugars determined (GM IVf).

IV. Examination of subsequent Urospora extracts

Experiment XXIV. Preliminary examination of the extracts

An aliquot of each extract was hydrolyzed and examined by paper chromatography in solvents A, B and C with locating reagents (i), (ii), (vi) and (vii). The results of this investigation are shown on p.116 .

Experiment XXV. The Acidic Extract (p.116)

A typical extract had $[\alpha]_D^{23}$ + 58° (c = 0.5) and gave a purple colour with I_2 /KI reagent. The hydrolysate showed a large proportion of mannose with less glucose. It was possible to separate a crystalline phenylhydrazone with m.p. and mixed m.p. with authentic D-mannose phenylhydrazone, 186°. However attempts to precipitate a mannan from the

original extract with Fehling's solution was unsuccessful.

A similar acidic extract from a later batch of alga was found to have a much higher proportion of glucose. An aliquot of this was methylated (GM VIIb) and the derived methylated alditol acetates were analyzed on GLC/MS (GM VIIe and VIb). The results are given on p.116.

Experiment XXVI. The Chlorite Extract (p.116)

This extract was very crude and contained only 4% of carbohydrate and was not examined further.

Experiment XXVII. The Alkali Extract

An aliquot of material precipitated on neutralisation of the alkali extract (see p.118) was methylated (as for the acidic extract), checked for full methylation, hydrolyzed and analyzed by P.C. The derived methylated alditol acetates were analyzed by GLC/MS (see p.118).

Periodate oxidation

Another aliquot [167 mg, 39 mg carbohydrate] was suspended in water (50 ml) and oxidized with 0.03M-sodium periodate solution (50 ml) and the product reduced. The derived polyalcohol was hydrolyzed (GM IIb) and examined by paper chromatography (see p.118).

Infrared examination

The polysaccharide was examined by infrared spectroscopy (see p.117).

Experiment XXVIII. Examination of the insoluble residue

The residue gave a purplish colour with chlorzinc iodide (Schultz's solution) indicating the presence of cellulose.

An aliquot was methylated and examined as described in Expt. XXII(i) (see p.118).

Results and Discussion.

The major difficulties in this investigation of Urospora penicilliformis arose from the highly seasonal nature of the alga, and the difficulty in collecting and purifying a sufficient quantity on which to work. Thus, for example, it took well over a week to hand pick even one or two grams (dry weight) of the pure alga from the large quantities of sand and debris with which it was associated (p. 56). However, the discovery of the flotation method (p. 59) then made it possible to purify several grams of alga from its associated impurities in a day.

I. Examination of the low molecular weight materials

The low molecular weight materials were separated from the ethanolic and the initial aqueous extracts. After separation from inorganic salts and other impurities they were fractionated on No.17 paper (p.65). The first fraction, a non-reducing syrup, was identified by paper chromatography and ionophoresis as glyceric acid. The second fraction was a mixture of glucose and fructose and the third fraction contained only glucose. Sucrose was characterised by the production of equal proportions of glucose and fructose on hydrolysis, by its mobility and colour reactions on paper chromatography, and by its retention time on GLC. The fifth fraction comprised a mixture of maltose and myo-inositol. The former was hydrolyzed to glucose which was removed with glucose oxidase and the myo-inositol identified by its mobility on paper chromatograms and its retention times on different columns on GLC.

A syrupy mixture of maltooligosaccharides was also separated. That they belonged to an homologous series was shown by the straight line obtained on plotting the D.P.-1 against $\log \left(\frac{1}{R_f} - 1\right) - \log \left(\frac{1}{R_{fGlc}} - 1\right)$. Proof of the 1,4-linkage was obtained from the presence of 2,3,6-tri-O-methyl

glucose in the hydrolysate derived from a methylated aliquot of the syrup. The high positive specific rotation of 141° indicated α-linkage [cf. maltose $[\alpha]_D^{20} = 139°$]. This was further confirmed by the action of salivary α-amylase (p. 67) which produced both glucose and a higher proportion of maltose, with a corresponding decrease in the proportion of malto-triose, -tetraose and -pentaose.

The identity of this mixture of oligosaccharides was further confirmed by the action of sweet potato β-amylase and the use of two-way paper chromatography (p. 68-69). The oligosaccharide mixture was separated on a paper chromatogram after which the paper was sprayed with the β-amylase and incubated for 8 hr. The enzyme was then destroyed and the paper redeveloped at right angles to the original development. Glucose was liberated from each of the saccharides, together with maltose and malto-triose from the higher oligosaccharides.

In several other genera of the Chlorophyceae, glucose, fructose and sucrose have also all been reported.[161a] However, not all genera so far investigated appear to metabolize these sugars, the unicellular organisms Dunaliella and Tetraselmis both being devoid of fructose and sucrose, and synthesizing only small quantities of glucose. The major low molecular weight carbohydrates were found to be glycerol in Dunaliella and mannitol in Tetraselmis.[161b]

The presence of myo-inositol has been reported in Acetabularia crenulata, which alga also synthesizes a homologous series of 1,2-linked fructans.[162] While the other carbohydrates appear to be typical of the Chlorophyceae, the homologous series of maltosaccharides appears to be unique to this alga. Since they are present in the initial ethanolic extract, it is unlikely that they have arisen by degradation of the amylose

during extraction. It is possible that they arose by enzymic degradation, but this too is unlikely since the alga was plunged into ethanol immediately after harvesting. The presence of the homologous series of 2,1-linked fructose oligosaccharides in A. crenulata shows that oligosaccharide series as such are found in at least one other green alga, however.

II. Examination of the water soluble polysaccharides

(a) Extraction

It was found necessary to give repeated extractions with cold and then hot water to separate the majority of the water soluble polysaccharides from this alga. Since the cold and hot water extracts were almost identical the cold water step was omitted from the final procedures. The result of a typical extraction is given in Table II.

Table II. Sequential aqueous extraction of Urospora harvested in 1972

Extract	Conditions (vol.)	Weight	Protein	Proportions Carbohydrate*					
				%	Rha	Xyl	Man	Glc	GlcUA
1	70°C, 1hr, 1.56l.	6.6g	25%	62-64%	+++	+++++	+++	++	++
2&3	100°C, 4hr, 2.56l.	4.0g	-	39-42%	+++	+++	++	++	++
4	" 42hr, 1.06l.	4.0g	45%	39-40%	++++	++++	++	++++	++
5&6	" 22hr, 1.56l.	1.7g	-	18-21%	+++	++	++	++++	++
7	" 8hr, 1.86l.	0.6g	79%	10%	++++	++	+	+++++	++

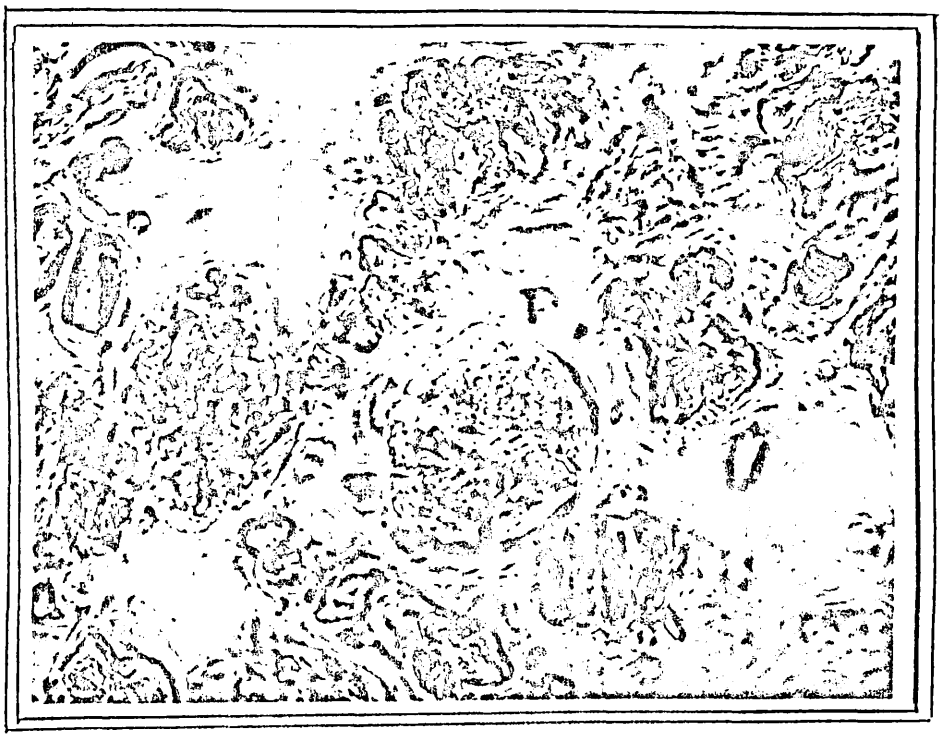
* Traces of galactose and ribose were present in all the extracts, and they all gave the blue colour with I_2/KI characteristic of amylose.
Each of the extracted carbohydrates carried half-ester sulphate.
The sugars were separated and characterized as crystalline materials or derivatives (p.73).

Determination of the relative proportions of the major sugars in the aqueous extracts from different batches of weeds showed only minor variations. A typical initial extract comprised the following percentage proportions of sugars: Rhamnose 26, Xylose 35, Mannose and Glucose 22, Glucuronic acid 17, Galactose trace.

It can be seen from Table II that as the extraction proceeds the carbohydrate of each successive extract decreases, while that of the protein increases. These extracts also show an increasing proportion of glucose and a decreasing proportion of mannose.

The effect of the prolonged aqueous extraction on the alga can be seen in Fig. XVII. (cf. Fig. Xb and frontispiece), which shows that most of the extracellular mucilage has been removed.

Fig. XVII. The alga after prolonged aqueous extraction



In view of the complexity of these extracts and the variation in the relative proportions of glucose and mannose (Table III) it was suspected that more than a single polysaccharide is present. However attempts to separate a xylan or a mannan by copper precipitation (p.71) were unsuccessful. A fairly pure glucuronoxylorhamnan was however separated with Celite and strong salt solution, as shown below:-

Table III. Fractionation achieved with Celite

Sugar	Rhamnose	Ribose	Xylose	Mannose	Glucose	Galactose
Precipitated with Celite (1,200 mg)	+++++	-	++++	+	+	-
In supernatant solution (300 mg)	+++	++	+++	+++++	+++	+

It was found that a more complete fractionation could be achieved with D.E.52 cellulose eluting with water followed either to a gradient-or by a stepwise-increase in KCl concentration.

The neutral fraction eluted with water comprised mainly mannose together with less glucose. The composition of the various peaks (see p.99) eluted with increasing concentration of KCl is given in Table IV.

Table IV. Analysis of the fractions obtained on gradient elution of an aqueous extract

(Chromatographic analysis)

Fraction	0.0 — 0.4 M KCl	0.4 — 0.8 M KCl	0.8 — 1.0 M KCl
Total weight	5.4 mg	50 mg (25% protein)	8 mg
% carbohydrate	100%*	51% +	63%+
% sulphate	trace	17%	21%
Rhamnose	++	+++++	+++++
Xylose	++	+++++	+++++
Mannose	+++++	trace	trace
Glucose	++	-	-
Galactose	-	-	-
Uronic acid	trace	++	++

* as glucose. Based on a mannose:glucose ratio of 3:1 it becomes 71% carbohydrate.

+ calculated from a synthetic mixture of glucuronic acid xylose and rhamnose.

A similar pattern was found when the hot water extract was fractionated by stepwise increases in the concentration of KCl(p.100) Table V.

Table V. Stepwise fractionation of an aqueous extract

The aqueous extract [1.08g] had 73% carbohydrate content.

	eluate			
Fraction	Water	0.3 M KCl	0.8 M KCl	1.0 & 1.3 M KCl
Weight (mg)	22	158 mg	730 mg	184 mg
Carbohydrate (mg)	15	47	585	109 + 25
Sulphate %	trace	2%	15.5%	21%
Uronic acid	trace	+	17%	17%
Rhamnose	trace	trace	+++++	+++++
Xylose	+++	+	+++++	+++++
Mannose	+++++	+++++	+	+
Glucose	+++	+	trace	trace
Galactose	+	-	-	-
(Ribose)	-	-	+	trace

It can be seen from Table V that there was 100% recovery of carbohydrate from the fractionation, but it must be remembered that the carbohydrate contents of the different fractions are read from different standard graphs, and while the results are as accurate as possible with such a mixture of polysaccharides, there may be small differences, due to this, from the absolute content. However, it is clear that an excellent recovery is obtained.

Other aqueous extracts were fractionated by this stepwise elution with essentially similar results. Occasionally a larger proportion of material was found in the aqueous eluate.

The presence of a xylose containing polysaccharide in the aqueous eluate suggests the presence of a xylan, but since it failed to precipitate with Fehling's solution it was not investigated further.

Determination of the proportions of the sugars in the hydrolysates of the neutral and 0.3M-KCl fractions from the DE-cellulose column showed them to be essentially similar with about five times as much mannose and glucose as xylose and thirty times as much hexose as rhamnose. The glucose corresponded to about 25% of the combined hexoses. These two fractions constituted about 8% of the aqueous extract. They were devoid of uronic acid and sulphate.

Characterisation of the Amylose

The presence of a reasonably high proportion of glucose in the aqueous eluate from the DE-cellulose column suggested the presence of starch in this fraction, and the presence of amylose was further indicated by the blue colour given by starch-iodine complex. This was also found to be true for the 0.3M fraction, and for the residual polymer left after autohydrolysis of an aqueous extract (2 and 3) (p.85). Digestion of these latter two materials with salivary α-amylase (specific for α-1,4-linked glucose units), followed by dialysis gave principally maltose and glucose; while the contents of the dialysis sacs were found to be devoid of glucose- containing carbohydrate. All the glucose units in the autohydrolysis residue were cleaved on periodate oxidation and the hydrolysate of the derived poly-alcohol contained erythritol, the expected product from a 1,4-linked glucan such as amylose, together with a very small amount of glycerol derived from the oxidation of the non-reducing end-group glucose.

A pure amylose was separated from a later extract (p.78) which gave the same results with α-amylase and periodate oxidation. However the residual material after removal of the amylose still contained, in addition to the mannan and glucuronoxylorhamnan, polysaccharide comprising glucose units. This is probably low molecular weight cellulose (see later) which has been removed from the cell-wall by the exhaustive extraction procedure.

Methylation of the 0.3M-KCl fraction yielded on hydrolysis methylated mannoses and about 25% of 2,3,6-tri-O-methyl glucose. This was characterised by its mobility on paper chromatography and by the retention time and characteristic peaks on GLC/mass spectrometry of the derived alditol acetate. The absence of amylopectin type glucan is surprising since starches extracted from other Chlorophyceaen genera[163] comprise both amylopectin as well as amylose fractions.

Characterisation of the Mannan

The crude mannan from the 0.3M-KCl fraction had $[\alpha]_D^{123}$ + 92° [c = 1.0]. However the essentially pure mannan obtained from this 0.3 M-KCl fraction after digestion with α-amylase (p.77) had $[\alpha]_D^{21}$ + 50° (c = 0.2) indicating α-linkage (cf. methyl α-D-mannopyranoside $[\alpha]_D$ + 79° and Chlorophycean amyloses + 157 - 197°.[164]) It gave nearly pure mannose on hydrolysis, which was characterised as the crystalline phenylhydrazone. Methylation of the crude mannan [p 76 Expt XIV(3)] containing 25% amylose and about 5.1% of other polysaccharides gave on hydrolysis as the major product 2,4,6-tri-O-methylmannose. This was by far the largest spot on a paper chromatogram and the major peak on GLC. This was confirmed by the retention time of its alditol acetate on GLC and its fragmentation pattern on mass spectrometry. In addition a small peak (ca. 2% abundance) also ocurred which gave the fragmentation pattern of a 2,4-di-O-methyl hexitol, suggesting that while most of the mannose residues are 1,3-linked, some are also 1,3,6-linked.

The evidence for the 1,3-linked mannose residues was supported by periodate oxidation, which consumed 0.23 moles per mole anhydro unit, and which on hydrolysis of the polyalcohol showed principally mannose, all the glucose having been cleaved to give erythritol together with a small amount of glycerol.

The evidence suggests that most, if not all, of the mannose in Urospora occurs as an α-1,3-linked mannan. This could well explain its inability to form an insoluble complex with copper since previous mannans which form this complex have been the β-1,4-linked polymers. This is the first example of an α-1,3-linked mannan to be found either in the algae or the higher plants.

It is more readily soluble in water than the more common β-1,4-linked mannans which usually require an alkaline pH for solubilisation.

The true sedimentation coefficient,[165] $S_{20,w}$, was 2.66 with the minor component (amylose) having $S_{20,w}$ of 1.14. Both, however, are probably somewhat degraded by the mild hydrolysis procedure (p.85). The minor component (p.105) in the glucuronoxylorhamnan has $S_{20,w}$ of 3.74. This value is probably the true sedimentation coefficient of the undegraded mannan.

Examination of Glucuronoxylorhamnan

The hydrolysates of the 0.8 M-KCl and 1.0 M-KCl fractions were very similar and all appeared to comprise a glucuronoxylorhamnan. The major sugars in the hydrolysate were rhamnose, xylose and glucuronic acid, together with traces of mannose, galactose and ribose. Each of these sugars was separated from a hydrolysate of the unfractionated material and characterised (see p.73). The mannose is derived from the mannan found in the neutral fraction and in the residue from the partial acid hydrolysate (pp 76, 85). A very small proportion of this mannan persisted as a contaminant in the KCl fractions of the glucuronoxylorhamnan. The ribose is considered to be derived from contaminating ribonucleic acids. The quantity of galactose is very small and was not found in the fractionated glucuronoxylorhamnan.

Determination of the proportions of the constituent sugars in the 0.8 M-KCl fraction isolated both by gradient and graded elution determined by GLC (p.79) and by colorimetric methods (p.80) gave similar results (Table VI).

Table VI. Percentages of sugar residues in the Glucuronoxylorhamnan fractions

Method	Fraction	Rhamnose	Xylose	Glucuronic acid
	0.4 - 0.8 M-KCl 1970	31.9	49.6	18.5
	0.4 - 0.8 M-KCl 1971	36.6	47.2	16.2
	0.4 - 0.8 M-KCl 1972	35.6	47.6	16.8
By GLC (p.)	0.8 M-KCl 1971	40.5	43	16.5
By GLC (p.)	0.8 M-KCl 1971	41	42.5	16.5
	0.8 - 1.0 M-KCl 1972	37.8	45.1	17.1

These correspond roughly to a molar ratio of rhamnose; xylose, glucuronic acid of 5:7:2. The ester sulphate varied from 17-22% in the different fractions.

Evidence for the presence of a single polysaccharide

Both gel electrophoresis (p.81) and the fractionation experiments (p.72) provided evidence that the majority of the protein was present as a contaminant and was not covalently linked to the polysaccharide. It was however gradually precipitated from solution on mild hydrolysis of the aqueous extract suggesting some kind of association of the two polymeric materials in the natural state.

Attempts to separate a xylan by Fehling's precipitation were unsuccessful indicating that the xylose is a constituent of the heteropolysaccharide. That this is a single polydisperse heteropolysaccharide is also supported by the fractionation experiments (Fig. XIII p. 72).

Ultracentrifugation of this fractionated extract gave a sharp symmetrical peak[165] $S_{20,w}$ 2.57 with a minor peak $S_{20,w}$ 3.74 (Fig XVII p.84). This is further confirmation that the glucuronoxylorhamnan is a single polysaccharide, the minor peak being due to the contaminating mannan. By analogy with the fucan sulphate[166] of M.W. 78,000, $S_{20,w}$ 4.5 and $\bar{V}$ 0.53 this polysaccharide would have an M.W. in the region of 38,000 corresponding to a degree of polymerization of 200+.

Evidence for the presence of β-linkages.

The optical rotation of the various extracts was found to range from -87° to -93°. This small variation could be explained by the difference in purity and determination of the carbohydrate content of the different extracts. The highly negative rotation suggests that both the xylose and the glucuronic acid are β-linked while the rhamnose may be α-linked (see Table VII).

Table VII. Rotations of standard sugar glycosides

Methyl β-D-xylopyranoside -66°
Methyl α-D-xylopyranoside $+153^{\circ}$
Methyl α-L-rhamnopyranoside -63°
Methyl β-L-rhamnopyranoside $+95^{\circ}$

Evidence for the β-linkage is also indicated by the infrared spectrum (Inset 1, p.81). The absorption at ca. 890 cm^{-1} has been correlated with β-linkage in various glucans, galactans and mannans, the α-linkage giving an absorption at ca. 850 cm^{-1}. [171]

Desulphation of the Polysaccharide

Various methods of desulphation were investigated. Treatment with -sodium hydroxide (p.86) resulted in virtually no reduction of the sulphate content, and the hydrolysate of the derived polymer contained no additional sugars [see p.27 and Fig. V. p.28] a result which shows the absence of 1,4-linked or end-group xylose residues sulphated at either C-2 or C-3 and of 1,2-linked or end-group rhamnose residues sulphated at C-3 or C-4 provided these units are not at branch points or disulphated. The Russian workers[167] recently published two methods for desulphation; desulphation of the pyridinium salt of the polysaccharide in dimethyl sulphoxide resulted, in the present experiments (p.87), in extensive degradation of the polysaccharide. However, the action of pyridine on a dimethyl sulphoxide solution of the 0.8 M-KCl fraction reduced the sulphate from 17→11% and gave an 80% recovery of the polysaccharide.

More extensive desulphation was, however, achieved with methanolic hydrogen chloride, the sulphate content being reduced from 17.6% to about 5% with a yield of about 53% of the polysaccharide after desulphation followed by dialysis of the residue.

Infrared Analysis of the Polysaccharide

Few conclusions, apart from the anomeric linkage and the presence of half-ester sulphate can be drawn from the infrared spectrum of this polysaccharide. Tentative identifications are indicated on the spectrum (Insert 1 p.81).

The presence of half-ester sulphate is confirmed by absorptions at 1240 cm^{-1}, 850 cm^{-1} (axial) and 830 cm^{-1} (equatorial), all of which are reduced in the partly desulphated polysaccharide shown in green on the spectrum. From the size of the respective peaks there appears to be a greater proportion of axial than equatorial sulphate suggesting that C-2

of rhamnose is the more highly sulphated. However the absorptions at 935 cm^{-1}, 890 cm^{-1} and 790 cm^{-1} are also reduced by desulphation. This might be due to a certain amount of degradation of the polysaccharide, but could also be due to the presence of sulphate groups.

Periodate Oxidation (p89)

Periodate oxidation of the 0.8M-KCl fraction resulted in the reduction of the 0.2 moles of periodate per average anhydro unit. The recovery of the derived polyalcohol and its proportions of uncleaved sugars are given below.

0.8 M-KCl fraction carbohydrate content 67% → (1) Periodate 0.015M (2) BH_4^- reduction → Polyalcohol I Recovery 80% by weight 53% by carbohydrate

Molar proportions			Molar proportions		
Rhamnose	xylose	glucuronic acid	Rhamnose	xylose	glucuronic acid
0.9	1.0	0.35	1.84	1.0	0.15

Accurate determination of the uncleaved sugars proved difficult but it can be seen that the proportion of xylose and glucuronic acid relative to that of rhamnose has decreased. The presence of 1,4-linked xylose was confirmed by the presence of glycerol in the polyalcohol hydrolysate. The cleavage of the glucuronic acid was supported by the presence of glyceric M_{GlcUA} 1.52 and erythronic M_{GlcUA} 1.34 acid spots on an electrophoretogram of the hydrolysate indicating some end-group and some 1,4-linked glucuronic acid units.

That some rhamnose was also cleaved was indicated by the presence of a non-reducing spot with R_{Glc} 2.2 (solvent A) and R_{Glc} 1.4 (solvent C) on paper chromatograms. This corresponds to the mobility expected of $CH_3(CHOH)_2\ CH_2OH$, which would be derived from 1,4-linked rhamnose.

A second oxidation of the polyalcohol failed to reduce any more periodate showing acetal linkage formation had not hindered the first oxidation.[168]

On mild hydrolysis (p.90) of the polyalcohol and fractionation of the derived hydrolysate, three major non-reducing fractions were obtained (Table VII).

Table VII. Oligosaccharides obtained on mild hydrolysis of the polyalcohol

	R_{Glc} of spots				Analysis of hydrolysate				
	(Major) Solvent		(Minor) Solvent						
	A	B	A	B	Rhamnose	Xylose	Glc UA	Aldobiuronic Acid	Glycerol
Fraction I	0.03	0.35	0.08	0.29	+++++	++++	++	++	+++
Fraction II	0.45	0.24	0.30	0.35	++++	+++++	+++	trace	++
Fraction III	0.50	0.60	0.73	0.18,0.43	+++	++++	++++	trace	++

A = solvent 18:3:1:4
B = solvent 40:11:19
} GM IIIa 2

It seems very probable since each of the fractions contain traces of mannose together with all three sugars that they are mixtures of oligosaccharides. No evidence for the presence of acid fragments was obtained and the presence of glycerol indicates that they were each liberated as the result of the cleavage by periodate of a 1,4-linked xylose unit.

The degraded polymer was recovered and subjected to a second oxidation. It reduced 0.28 moles of periodate per average anhydro unit. The derived polyalcohol II (recovered in 82% yield) on complete hydrolysis gave uncleaved rhamnose, xylose and glucuronic acid (trace) together with glycerol, indicating that the mild hydrolysis of polyalcohol I had

produced fresh 1,4-linked xylose units susceptible to periodate oxidation. Mild hydrolysis of polyalcohol II and paper chromatography of the hydrolysate gave the same series of spots as were obtained from polyalcohol I.

In the total hydrolysate of polyalcohol II an additional spot with a mobility faster than that of glycerol was detected. The quantity was insufficient for characterisation, but it could be 1-deoxyglycerol derived from end-group rhamnose.

The partly desulphated polysaccharide (SO_4^{2-} ca. 5%) reduced 0.28 moles of periodate per average anhydro unit on oxidation and the derived polyalcohol, unlike that from the sulphated polysaccharide reduced a further 0.06 mole of periodate per average anhydro unit on a second oxidation indicating the formation of a small amount of acetal linkage between the uronic acid and cleaved units which had hindered complete cleavage in the initial oxidation.

Polyalcohol II was recovered in an overall yield of 57% carbohydrate and analysis of a hydrolysate showed a considerable proportion of uncleaved rhamnose, some xylose and a very small proportion of uncleaved uronic acid. Uncleaved mannose persisted throughout the oxidations supporting the deduction that this was derived from the 1,3-linked mannan found in the neutral and 0.3 M-KCl fractions.

Methylation

The R_G and retention times of the various methylated sugars are given on pp 143-5.

An initial methylation of the 0.8 M-KCl fraction (p.88) and analysis of the hydrolysates by paper chromatography and of the derived glycosides and alditol acetates by GLC showed a small amount of 2,3,4-tri-O-methylxylose; indicating end-group xylose and a considerably larger amount of 2,3-di-O-methylxylose confirming the periodate oxidation results that a

substantial proportion of the xylose is 1,4-linked.

Both 2,4-di- and 2,3-di-O-methylrhamnoses were obtained-the former in larger proportion, showing that both 1,3 and 1,4-linked rhamnose are present. However, the major rhamnose derivative was a monomethyl-rhamnose with a different chromatographic mobility from that of 4-O-methyl-rhamnose. G.L.C. analysis confirmed that this could be 2 or 3-mono-O-methylrhamnose. A small amount of free rhamnose was detected in the alditol acetates. This could have arisen from slight undermethylation or from a disulphated or doubly linked residue. The methylated uronic acid residues were not characterized.

In a second methylation experiments, after partial methylation, the glucuronic acid residues were reduced and the derived neutral poly-saccharide remethylated. Analysis of the derived alditol acetates by mass-spectrometry confirmed the previous methylation results but showed in addition the presence of a fair amount of 3-O-methyl- and a little 2,4-di-O-methylxylose together with a trace amount of 2,3,4-tri-O-methyl-rhamnose indicating 1,3-linked xylose and xylose at branch points ~~or xylose linked at C-2 and sulphated on C-4~~ together with end-group rhamnose. It also showed that the mono-methyl-rhamnose was in fact 2-O-methylrhamnose.

In addition 2,3,4,6-tetra-O-methyl glucose, and 2,3,6-tri- and 2,4,6-tri-O-methyl glucose, were also found on P.C. and GLC analysis. They could not be confirmed by M.S. of the alditol acetates owing to overlap with 2,4 di-O-methylrhamnose and 2,3,di-O-methyl xylose. In some cases it is possible by mass-spectrometry to show the presence of two sugars in a single peak while in others when one constituent is in great excess the small peaks generated by the other sugar could equally well be generated by the background of the former, since they fall below 10% of the base peak m/e 43.

On analysis of the hydrolysate from the methylated desulphated polysaccharide (p.88) similar results were obtained, with the exception that the 2,4-di-O-methylrhamnose peak was considerably enhanced while both the mono-methyl- and the free rhamnose were both reduced to trace amounts. This shows that the 1,3-linked rhamnose was mono-sulphated at C-4 and also disulphated at C-2 and C-4, desulphation removing a large proportion of the sulphate from these residues. There appeared to be no change in the other sugars confirming that both the glucuronic acid and xylose are essentially devoid of sulphate. The increased reduction of periodate by the desulphated polysaccharide therefore does not appear to result in the formation of additional hydroxyl groups by removal of sulphate. It can only be explained by considerable degradation and the formation of additional end-groups. This is supported by the comparatively low recovery of the original polysaccharide (p.89) after desulphation (recovery 98 %) and dialysis (recovery 55%) which indicates considerably degradation since 45 % passes through the dialysis sac.

Oligosaccharides isolated after Partial Hydrolysis.

Autohydrolysis of the free-acid form of the unfractionated aqueous extract in a dialysis sac (p.85) gave a number of oligosaccharides. As the hydrolysis proceeded the pattern of oligosaccharides released remained constant and only small amounts of free sugars were released. This suggests a repeating-unit type of structure for the macromolecule and absence of furanoside linkages, since these are easily hydrolysed.

The neutral and acidic oligosaccharides were separated by precipitation of the latter as their barium salts. The major neutral and acidic oligosaccharides were then separated. These, together with their constituent sugars and some of their properties are detailed in the following Table.

Table of fractions

Fraction	Chromatographic mobility ($R_{sucrose}$) solvents A	B	C	D.P.	$[\alpha]_D^{23^\circ}$ (g/100ml)	Constituents Rha	Xyl	GlcUA	SO_4	others
1 +++	0.12	0.035	0.16	-	-	✓	✓	✓	✓	glucose
2 ++	0.17	0.085	0.08	4	-36°(0.16)	[1]	[1]	[1]	✓	
3 ++++	0.22	0.07–0.11	0.13	3	-53°(0.24)	1	1	1	2	
4 +	0.38	0.4	0.74	4	-52°(0.055)	[1]	[1]	-	2	
5 +	0.53	0.5	0.95	2	-9° (0.009)	[1]	[1]	-	2	Glc [1+]
6 ++++	0.53	0.68	0.95	2	-56°(0.40)	1	1	-	1	
7 +	0.67	-	1.43	2	-6.1°(0.013)	-	-	-	-	Rib. only
8 +	0.76	-	-	2	-6.2°(0.013)	[1]	[1]	-	-	Glc[1] Gal[1]
9 +	0.83	-	-	2	-48° (0.078)	-	-	-	-	Glc[1] Gal[1]

✓ = present

Bracketed [ ] figures are visual estimations from paper chromatography. Rotations are based on the carbohydrate content and do not include the weight of sulphate. The small amounts of material available did not permit the usual sulphate determination. The sulphate contents were therefore determined by the method given in Appendix II. The presence of sulphated oligosaccharides (1–6) was also inferred from chromatography in solvent B and ethylmethylketone:water 10:1, both solvents being modified by the addition of 3% w/v cetylpyridinium chloride. The sulphated sugars complex with the latter and have a considerably increased ($\rangle$ 2.5x) mobility.

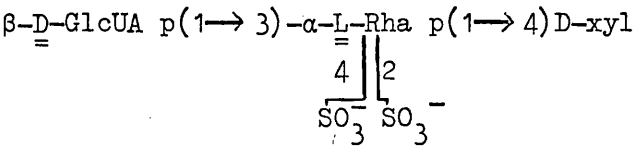
Hydrolysis of fraction 1 with oxalic acid (GM II d) gave the same series of oligosaccharides which could be seen when the hydrolysate was examined on a paper chromatogram. The major spot had the mobility of oligosaccharide 5 and this was followed in colour intensity by oligosaccharides 3 and 2. This shows that the smaller oligosaccharides are derived from the higher oligomer and that the latter does not represent a different polymeric structure in the macromolecule but again denotes a repeating pattern. The glucose present in this and the hydrolysates of (5), (8) and (9) probably indicates contamination with maltosaccharides derived from the partial hydrolysis of the amylose present in the unfractionated extract.

Only oligosaccharides (3) and (6) were separated in sufficient quantity to allow further structural investigation. Examination of the hydrolysate obtained after reduction showed that in both of these xylose constituted the reducing end-group.

Methylation of the esterified and reduced trisaccharide (3) followed by GLC analysis of the derived glycosides and alditol acetates showed 2,3,4,6-tetra-O-methylglucose, 2,3-di-O-methylxylose and free rhamnose as constituents of the hydrolysate from the methylated material.

Assuming from the earlier methylation studies on partly desulphated material that the rhamnose is 1,3-linked and the earlier deductions concerning the anomeric linkages, the formula (I) is in agreement with the determined properties (Table 12) and constitution of trisaccharide (3).

Formula I. Probable structure of oligosaccharide 3



In view however of the 1,4-linkages also found for rhamnose it is possible for the linkage and position of sulphate to be different in this trisaccharide.

Disaccharide (6) was subjected to a similar methylation and 2,3-di-O-methylxylose and 2,3- and/or 3,4-di-O-methylrhamnose were characterized as constituents of the methylated hydrolysate. Unequivocal characterization of the dimethylrhamnoses from the mass spectra fragmentation pattern was not possible due to the presence of overlapping peaks of contaminating non-carbohydrate material. The $[\alpha]_D^{23^\circ}$ -56° (cf. methyl α-L-rhamnopyranoside $[\alpha]_D^{20^\circ}$ -63° and the equilibrium value of D-xylose $+19^\circ$) is evidence for α-linkage of this disaccharide. Disaccharide (6) therefore has the structure II and/or III.

Formulae II and III. Structures of Disaccharide 6

II α-L-Rha p(1→4)D-Xyl.
4
SO_3^-

III α-L-Rha p(1→4)D-Xyl
2
SO_3^-

The structures determined for these oligosaccharides are in agreement with those found from the periodate oxidation and methylation studies.

The results of periodate oxidation, methylation and partial hydrolysis are all in agreement. Repeated periodate oxidation after a Smith Degradation cleaves the greater part of the xylose and glucuronic acid units whereas the proportion of rhamnose steadily increases. This is in agreement with end-group and 1,4-linked xylose residues and end-group and 1,4-linked glucuronic acid units which were found to be present from methylation studies.

The presence of 1,3-linked xylose units and xylose at branch points found from methylation studies, does not at first glance, fit in with the removal of the greater proportion of the xylose on repeated oxidation. However, the removal of cleaved units on the mild hydrolysis (Smith

Degradation) could well produce end-group xylose from units which were previously linked through C-3 and from branch units previously linked through C-2 and C-4 which would then be vulnerable to a second oxidation.

The small proportion of uncleaved glucuronic acid after Smith Degradation and a second oxidation favours the presence of 1,3-linked units tentatively characterized by methylation studies.

The linkages and degree of sulphation of the rhamnose units found from methylation are in agreement with the small proportion of rhamnose which is vulnerable to periodate and are in agreement with the oligosaccharides isolated on partial hydrolysis.

These results indicate an essentially linear molecule with a small degree of branching at C-2 of some of the xylose units, and both the periodate oxidation and the partial hydrolysis studies indicate a chain of repeating units comprising all three sugars, with the rhamnose variably sulphated, as forming the most probable structure for the macromolecule.

The strong negative change in optical rotation from -93° to -138° (see p.80) with the reverse change to -102° on cooling indicates a change in the conformation of the macromolecule. The rotation at the higher temperature is probably the rotation of the "random" structure, while that at 23° is the rotation of a definite conformation of the polysaccharide. This suggests that the repeating unit of the polysaccharide allows the macromolecule to take up the definite conformation required for its biological function.

III. Examination of the acid and alkali extracts

The results of paper chromatographic analysis of hydrolysates of these extracts are given in Table VIII.

Table VIII. Results of paper chromatographic analysis of each extract derived from extraction by:—

Extract	Carbohydrate %	Glucose	Mannose	Xylose	Rhamnose	Glucuronic acid
Acid (pH 2.70°C)	28%	+++	++++	+	+	+
Sodium chlorite (0.5%)*	4%	++	+	++	+	
Sodium hydroxide (13%) (soluble at pH 5)	24%	+++	+	++	++	+
Sodium hydroxide (13%) (precipitated at pH 5)	27%	+++++		+	trace	
Residue	73%	++++				

* Was not examined further.

It was found that the proportion of carbohydrate and the ratio of glucose to mannose in the acidic extract varied considerably in the different batches of weed.

The mannose was characterized from a mannose rich extract by the formation of the crystalline phenylhydrazone and the glucose with glucose oxidase reagent.

2,3,6-Tri-O-methylglucose was the major product from the methylation of a glucose-rich extract together with a smaller proportion of a 2,4,6-tri-O-methylhexose (MS of the hexitol) and 2,3,4,6-tetra-O-methylhexose (MS of hexitol).

From this it was concluded that this extract was comprised mainly of a degraded amylose, and this conclusion was supported by the purple colour given by I_2/KI reagent, together with the ~~addition of the~~ water soluble 1,3-linked mannan found in the earlier extracts.

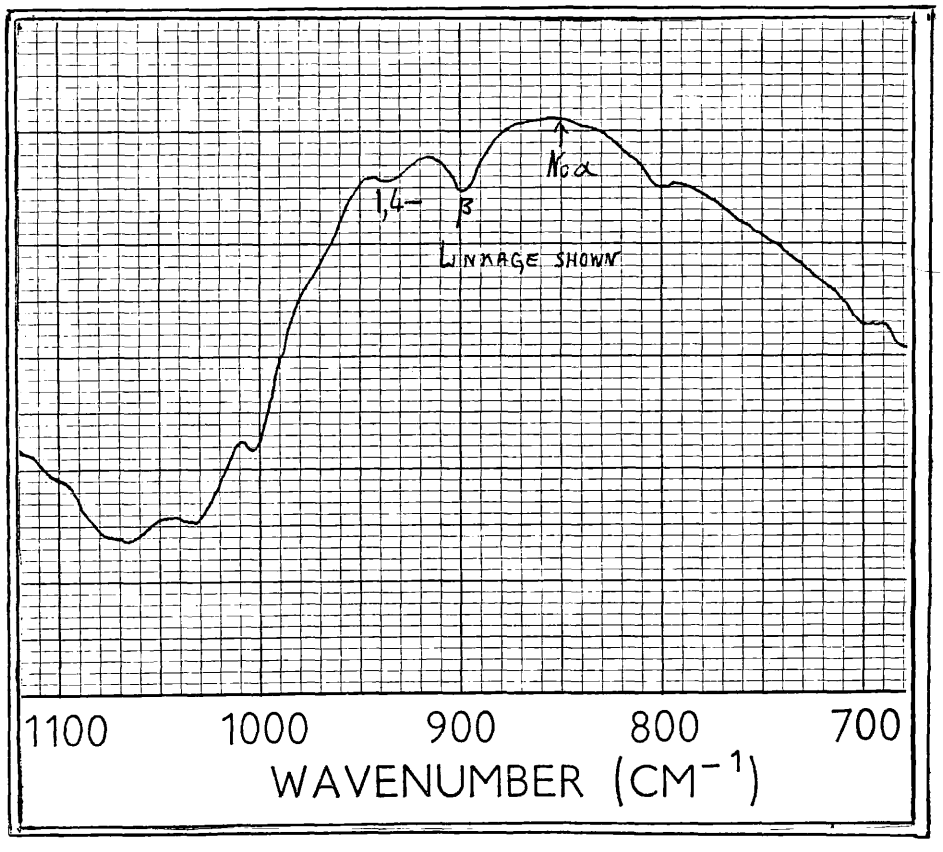
Since the soluble part of the alkali extract (see Table VIII) appeared to be a mixture of the glucuronoxylorhamnan already characterized together with a glucan, probably degraded cellulose, which also appeared in the insoluble part in an almost pure form, it was not examined further.

The insoluble part was shown to contain D-glucose. On infrared analysis it gave a strong absorption at 3400 cm^{-1} (OH, H-bonded) and in the region of 1200-1000 cm^{-1} (also OH^-).

The strong absorptions at 1640 cm^{-1} and at 1560 cm^{-1} were derived from the secondary amide absorptions of the contaminating protein. In the region 750 - 950 cm^{-1} (see spectra insert p. 117) it showed weak absorptions at 940 cm^{-1} (suggesting 1,4-links)[171] and at 890 cm^{-1} (β linkage)[171]. The weak absorption at 790 cm^{-1} is probably due to the sulphate absorptions derived from the small amount of contaminating glucuronoxylorhamnan (see Table VIII).

Insert II

Infra-red spectrum of cellulose: 1120-680 cm^{-1}



Further support for the 1,4-links of this polysaccharide came from GLC/MS analysis of the alditol acetates derived from the reduced hydrolysate of an aliquot of the methylated polysaccharide, which showed 2,3,6-tri-O-methyl hexitol (major) and 2,3,4,6-tetra-O-methyl hexitol together with small peaks derived from the contaminating glucuronoxylorhamnan.

This was confirmed by periodate oxidation followed by hydrolysis of the derived polyalcohol. Paper chromatography of the hydrolysate showed the complete absence of glucose and large amounts of erythritol, derived from the cleaved 1,4-linked units, together with a small amount of glycerol.

These results provide evidence for the presence of cellulose in this extract although it did not give the characteristic purplish colour given by cellulose with chlor-zinc iodide,-this is thought to be due to the extensive degradation of the macromolecule during the extraction with alkali.

Examination of the Final Residue

The residue left after alkali extraction gave the characteristic purplish colour with the chlor-zinc iodide reagent, showing cellulose. A hydrolysate showed only D-glucose (glucose oxidase reagent). The 1,4-linkages were confirmed by GLC/MS of the derived alditol acetates of the methylated material, which showed 2,3,6-tri-O-methyl glucose, derived from 1,4-linked and non-reducing end-group residues respectively.

V. General Conclusions and comparison with other members of the Chlorophyceae

It has been shown that the low molecular weight carbohydrates metabolized by Urospora are comprised mainly of a maltooligosaccharide series, with lesser amounts of glucose, fructose, sucrose and myoinositol. While the last four carbohydrates have been found before in green algae, this is the first time that the presence of the maltosaccharides has been shown in a member of the Chlorophyceae.

The aqueous extracts were shown to consist principally of a mixture of amylose, an α-1,3-linked mannan and a complex sulphated glucuronoxylorhamnan.

While starches comprising amylose and amylopectin have been reported from other members of the Chlorophyceae,[172] this is the first example of a member of this class which appears to synthesize only amylose.

The occurrence of β-1,4-linked mannans in algae has long been known, a β-1,4-linked mannan having been isolated from Porphyra umbilicalis[173] and in several genera of the Chlorophyceae.[174a 24 (2nd. ref.)] However the presence of an α-1,3-linked mannan has not been demonstrated before. In this context it is worth recording that mannose has been isolated and characterized from the aqueous extract of Acrosiphonia arcta,[175] and it also occurs in the aqueous extract from Ulva lactuca[176] but no mannan as such has been separated.

The structure of the glucuronoxylorhamnose metabolized by Urospora is similar to that metabolized by Ulva, although there are some differences. In both polysaccharides the uronic acid appears to be principally 1,4-linked with smaller proportions of 1,3-linked and end-group residues. Although evidence was obtained for both glucuronosyl-rhamnose and glucuronosyl-xylose linkages in Ulva, no evidence for the latter linkage was found for Urospora.

Again, both polysaccharides show xylose which is principally 1,4-linked though small amounts of 1,3-linked and end-group xylose also occur. However, it has been shown[177] that part of the 1,4-linked xylose (15%) of Ulva was monosulphated at C-2 but no evidence of sulphate on the xylose residues of Urospora could be obtained.

The rhamnose in both algae appeared to be 1,3-linked with some 1,4-linked and end-group rhamnose. In Ulva[176] evidence was also obtained for small quantities of 1,2,3-, 1,3,4-, and 1,2,3,4-linked or sulphated rhamnose; the bulk of the sulphate appeared to be attached to C-2 of the 1,4- and 1,3-linked residues.[177] The principal differences for Urospora are shown by the larger proportion of 1,3-linked compared with the 1,4-linked residues. Consequently the sulphate is principally linked to C-2 of the 1,3-linked rhamnosyl residues. A substantial proportion of these were also sulphated at C-4.

It is worth noting that the presence of this type of polysaccharide is rather more widely spread than just in the Acrosiphoniaceae and the Ulvales. A preliminary examination of a Ulothrix sp.[178] has revealed the presence of a sulphated rhamnose:xylose:glucuronic acid-containing polysaccharide in this genus.

The presence of both the α-1,3-linked mannan and a degraded amylose in the acidic extract is at first glance somewhat surprising. However the photograph at Fig.XVII (p.97) shows that not all the cells were broken, indicating the possibility that these polysaccharides were "locked up" inside the cell until a sufficiently drastic extraction method could release them.

The presence of rhamnose and xylose in the soluble part of the alkali extract (p.116) suggests that while the bulk of the glucuronoxylorhamnan is easily removed as the mucilage with hot water, some of it is deeply embedded in the cellulosic/protein matrix of the cell wall, and thus is difficult to extract.

Cellulose I has been shown in a number of the Chlorophyceae.[179] Within the "Cladophorales" in all the species examined it is absent only

from the Acrosiphoniaceae. This lack of Cellulose I is also shared by a number of other genera, including Ulothrix (Ulotrichales) and Ulva, Enteromorpha and Monostroma (Ulvales).

The taxonomic implications of this work show that whatever the other taxonomic characteristics of Urospora may be, its carbohydrate metabolism certainly jusifies placing it with Acrosiphonia in a separate family or even order, and that its affinities lie with the Ulvales rather than the Cladophorales.

Part II MOUGEOTIA

Introduction

This alga is widely spread as a constituent of the flora of neutral or slightly acidic ponds. Although present all the year round, it attains its maximum development towards the end of April, when the whole pond surface may be covered with bright green mats of the alga buoyed up by the oxygen it releases on photosynthesis.

In its relationships it is classed with algae such as Spirogyra, Zygnema and Zygogonium in the order Zygnematales (Conjugales),[180] since its highly characteristic habit of reproduction by conjugation is also shown by these algae. As a class they occupy similar habitats, though some are terrestrial, Zygogonium ericetorum in particular being very widespread as a constituent of damp heathland floras. Many of these algae often have a purplish tinge,[181a] which in Spirogyra sp. has been shown to be a galloyl glucose tannin complex with ferric ions, which ions would occur dissolved in the rather acid waters of their habitats.

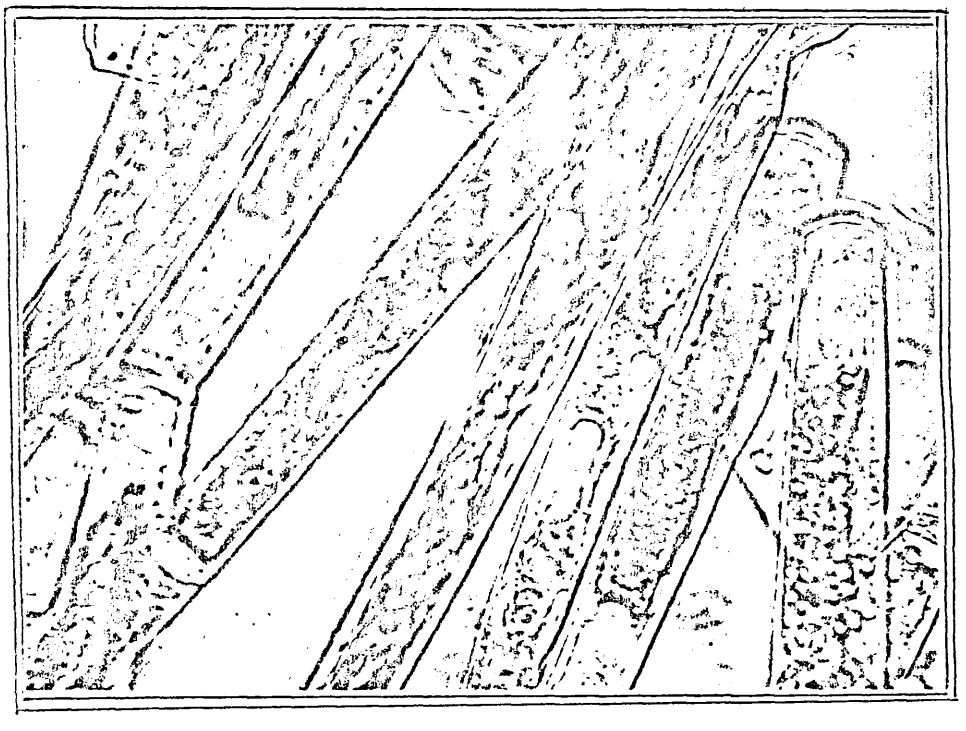
Mougeotia itself has the interesting property of being able to adjust its plate-like chloroplast to the light intensity,[181b] a fact which has excited the majority of research into this alga. This chloroplast moves from face to profile view in high light intensities[182] and in the reverse

direction when the light intensity decreases.[183] This reaction has been shown to be dependent on the age of the cell and the colour of the light employed among other factors.

X-ray studies[185,181c] on the cell wall of this alga have shown the presence of patterns characteristic of cellulose [not cellulose I] but apart from a reference to the existence of polyphosphates in the cell[181d] the literature appears to be largely devoid of all reference to the alga except as a constituent of the flora of a particular habitat or as a new species.

The Mougeotia sp. investigated was harvested from a tree-shaded pond in the College grounds. The period of harvesting varied from January to April. It started to grow in January as fine rather slippery green filaments which towards March became detached from the substratum and formed floating green "mats" usually contaminated with a small proportion ($\prec 5\%$). of a filamentous diatom together with some sand and organic debris.

Fig. Filaments of Mougeotia from young "mats" X400



Experimental

I. Extraction Methods

Experiment I. Complete extraction of Mougeotia sp.

Floating mats of Mougeotia were collected from a pond in the College grounds at about 11 am on a bright day in early March, 1970. On microscopic examination these proved to consist almost entirely of Mougeotia (98%), the main contaminants being fialmentous diatoms and leaf debris. These "mats" were then freeze-dried to fibrous bright green masses (6.4 g), which were then ground to a powder under liquid nitrogen. This powder was freeze-dried (5.9 g).

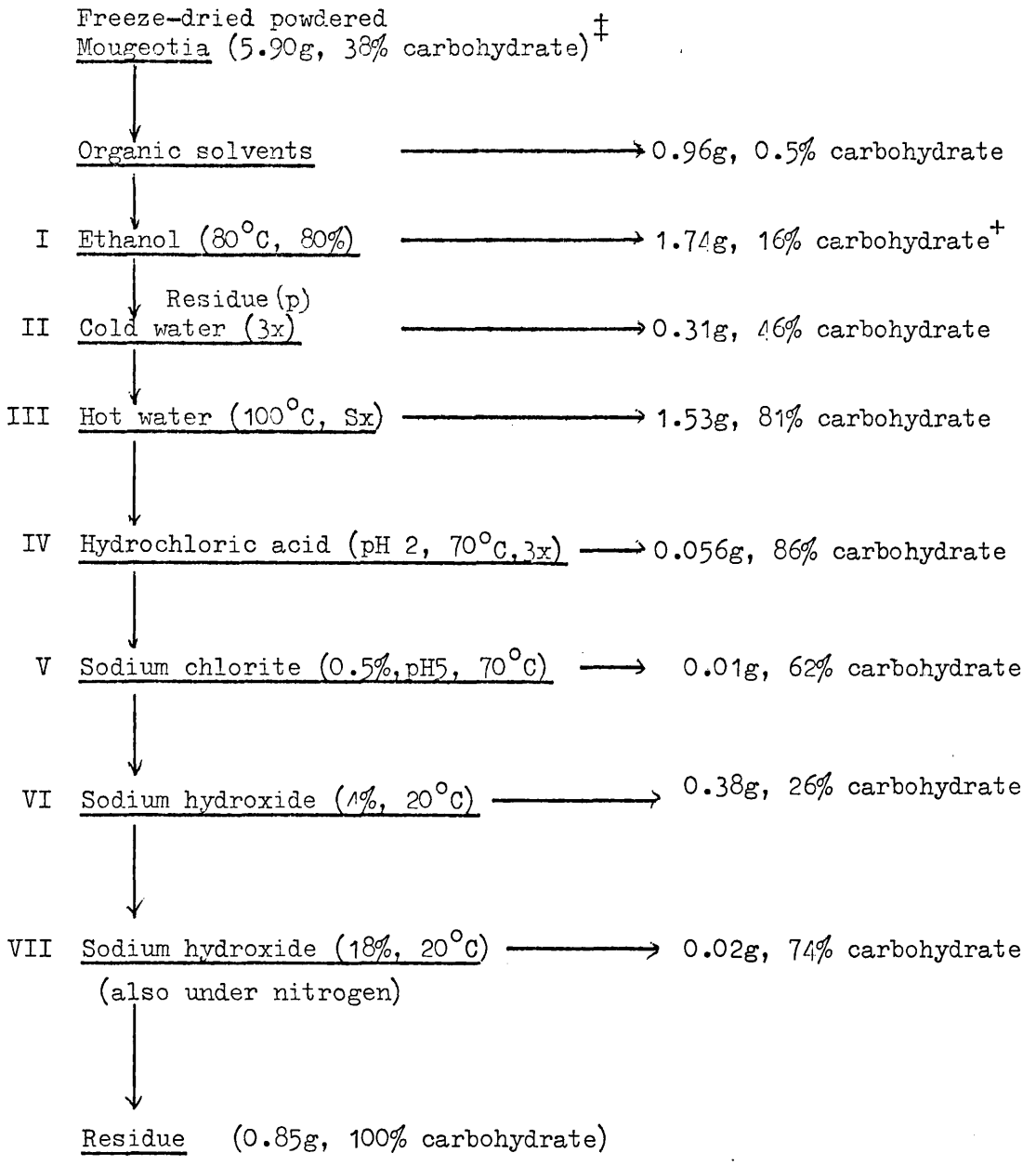
The freeze-dried powder was put into a Soxhlet thimble and extracted continuously with petroleum ether ($60-80^{\circ}$ b.p. fraction) followed by chloroform until the respective extracts were colourless. The residual pale-green alga was dried and exhaustively extracted with 80% ethanol ($80^{\circ}C$). The derived ethanolic solutions were combined and partitioned between toluene and water (cf. Urospora, p. 60) as shown in Flow Chart II.

The residue (P) , a pale purple powder, was then successively extracted as shown in Flow Chart II (cf. Urospora p63).

Each extraction step was continued until no further carbohydrate was obtained. The final residue left was hydrolyzed (GM IIc). Some of the initial extracts were coloured purple from fine cell debris.

Each of the extracts subsequent to the aqueous extraction in the Flow diagram was hydrolyzed and the sugars present in each are detailed in Table 1 with the approximate proportions deduced from visual examination.

Flow Chart II. Sequential extraction of Mougeotia



‡ All carbohydrate contents for Mougeotia unless otherwise stated are based on a standard glucose graph

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+ The ethanolic extracts were partitioned between water and toluene:

Water layer	1.21g	(carbohydrate 3.5%)
Toluene layer	0.47g	(carbohydrate 1.0%)
Insoluble residue	0.06g	(carbohydrate 22%)

Table I

Extract	Acids	Gal	Glc	Man.	Ara.	Xyl.	Fuc.	Rha.
HCl 70°	4 spots	4	4	1	3	5	3	3
$HClO_2$ 70°	1 spot	4	4	2	2	3	2	3
4% alkali	2 spots	4	3	1	trace	5	1	1
18% alkali	3 spots	4	3	2	1	5	1	1
Residue		-	5	1	-	1	-	-

These extracts were not examined further.

Experiment II. Aqueous extraction of Mougeotia

The Mougeotia was collected not only from the surface as floating mats but also from the bottom of the pond. The alga [blotted weight ca. 400g] was freeze-dried to a fibrous green mass [50.2 g] which was then ground to a powder under liquid nitrogen. The powder was extracted with cold (4 x 14 hr) and hot (1 x 1 hr at 85°, then 3 x 16 hr at 100°C) water. The residual material was freeze-dried to a mauve powder [12.0 g] and was not examined further.

The combined cold aqueous extracts were freed from sand and other debris by decantation. The polysaccharides were precipitated from solution by acetone (4 volumes). The precipitate resuspended in water and the precipitation repeated and the mauve product (1.54 g), after dissolution in water, was further purified by centrifugation followed by filtration through microbiological and glass fibre filters, after which it was recovered as a buff coloured solid(Q) (1.4 g) by freeze-drying.

The combined hot aqueous extracts were treated similarly, the purified material (S) (0.76g carbohydrate) again being recovered by freeze-drying. Both extracts failed to give a colour with the I_2/KI reagent.

Examination of the extracts

Samples of hydrolysates for paper chromatographic analysis were run on No.1 Whatman paper in solvents A, B and C. The separated spots were

then located with reagents (i), (ii) and (vii).

Experiment III. Ethanolic Extracts

The water soluble fraction of the 80% ethanol extract (1.2g) (p.123) was freed from ionic materials by passage through IR120H$^+$ followed by I.R. 45B OH$^-$ resin columns (p.47). The eluant was concentrated and chromatographically examined using all the locating reagents except (iii) - and the results are given on p.131 .

Experiment IV. The aqueous extracts

Both the cold and hot water extracts given on Flow Chart II and in experiment II were extensively dialyzed, concentrated and freeze-dried. Chromatographic examination by paper and GLC of hydrolysates of each of the cold and hot water extracts showed their essential similarity and all structural studies were carried out on the hot-water extracts. The above dialysates were also concentrated, deionized and examined by paper chromatography, and shown to contain malto-oligosaccharides.

Experiment V. Attempted fractionation of the Aqueous Extract

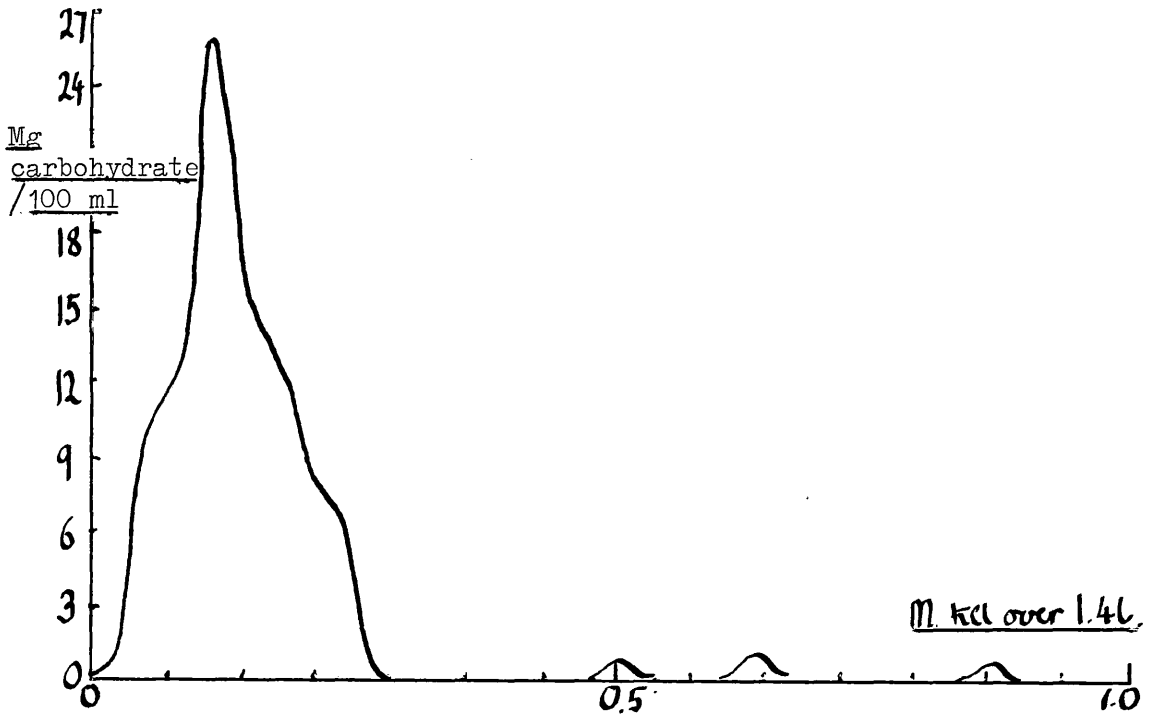
(i) By formation of a copper complex

The addition of Fehling's solution to an alkaline solution of the extracts gave no precipitates.

(ii) By D.E.52 cellulose

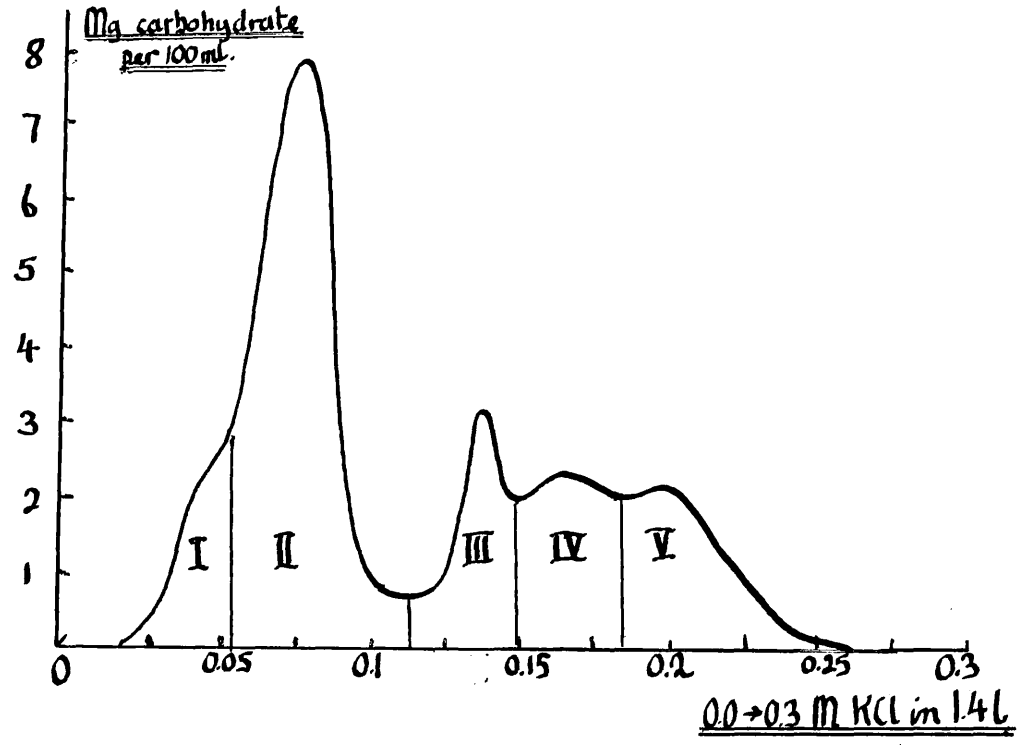
The column was prepared (GM V) and the dialyzed hot water extract [100 mg, 81 mg carbohydrate] was layered onto the surface, and eluted with a 0 — 1.0 M-KCl gradient over 1.4l, with a 75% recovery of carbohydrate (Fig. I).

Fig. I. 0.0 → 1.0 M-KCl fractionation of Mougeotia



Accordingly the major peak was refractionated using a 0.0 — 0.3 M-KCl gradient, approximately 55 mg of material (as glucose) being separated. (Figure II).

Fig. II



Each fraction was assayed for carbohydrate and uronic acid before being hydrolyzed and examined by paper chromatography. An electrophoretogram of each hydrolysate was also run. The results are shown in Table III p.132. The total recovery of carbohydrate was 45.3 mg as glucose.

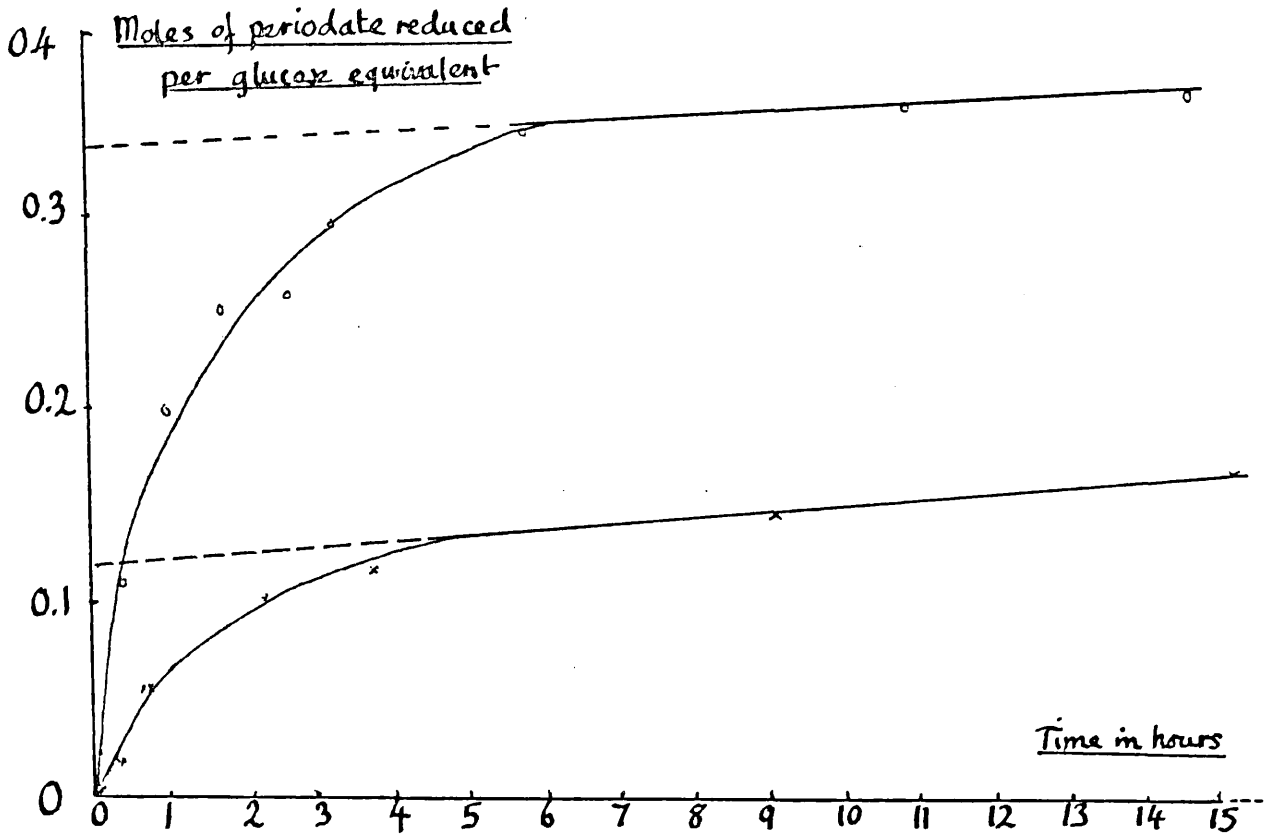
A second fractionation of a fresh sample gave a similar fractionation pattern. The recovery of carbohydrate in this case was 74%.

Experiment VI. Periodate Oxidation

Although it was found that the various fractions differed in little more than their uronic acid content the fractionation procedure removed a considerable amount of non-carbohydrate material and the recovered material was pure carbohydrate (estimated as glucose). This material (291 mg containing 275 mg carbohydrate) was dissolved in water (100 ml) and oxidised with 0.03M-sodium periodate solution (100 ml) [GM VIIh]. The reduction of 0.12 mole periodate per/anhydro unit and a cleavage of 13% of the carbohydrate occurred.

A second oxidation on the crude extract (containing fine particles of cell-wall debris) was oxidized in the same way. The periodate reduction corresponded to 0.345 mole per anhydro unit and a third of the apparent carbohydrate was oxidized by the periodate. The derived polyalcohols were hydrolyzed (GM IIb) and examined by P.C.

Fig. III. Periodate Oxidation of Aqueous Extracts



Experiment VII. Isolation of a disaccharide.

The alga (12g) was hydrolyzed (GMIIb) and the hydrolysate was filtered from the voluminous solid material still present. The solution was then neutralized [$BaCO_3$] and filtered, treated with IR $120H^+$ and filtered and again neutralized with barium carbonate. It was then concentrated to ca. 5 ml and poured into methanol (100 ml). The white precipitate [ca. 100 mg] was collected.

Experiment VIII. Characterization of the Precipitated Material

This precipitate was analyzed by PC and ionophoresis and shown to consist of a major spot with the mobility of an aldobiouronic, — together with a number of faint oligouronic, acid spots. The degree of polymerisation was found to be 2.05 and it had $[\alpha]_D^{23}$ 56.4° (c, 0.77). The uronic acid was assayed (GM IVd.).

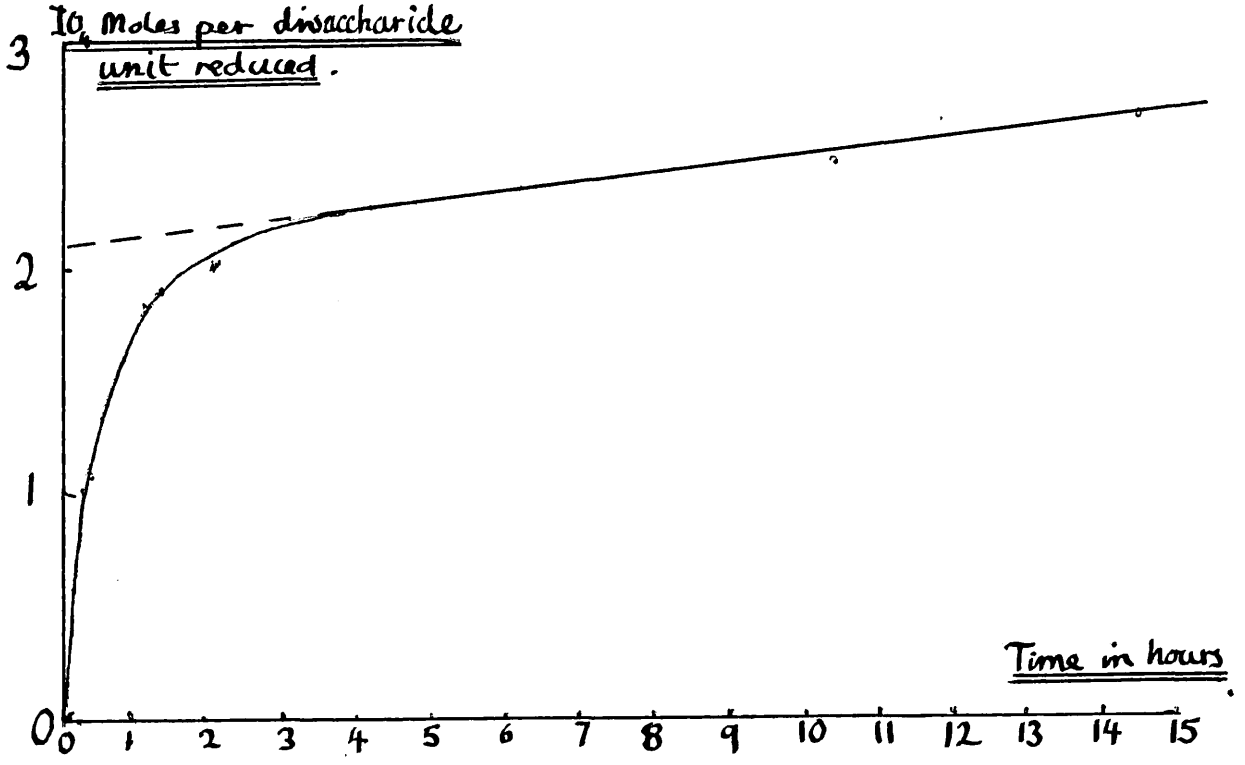
One aliquot was hydrolyzed while a second was reduced and hydrolyzed. The derived hydrolysates were examined by paper chromatography and also by paper electrophoresis (GM.IIb).

Another aliquot was treated with IR 120H$^+$ and the resulting acid neutralized with sodium bicarbonate. This sodium salt was incubated at 37°C for 24 hr with β-Glucuronidase (pH 6.8 in 0.004M $Na_2H\ PO_4$ buffer). The solution was then heated to destroy the enzyme, filtered, and the solution evaporated to small volume and examined by paper chromatography (see p.134).

Experiment IX. Periodate oxidation of the above aldobiouronic acid

An aliquot of the precipitate (ca. 6.0 mg) was converted into its methyl ester glycoside (5.28 mg) and dissolved in water [1 ml]. This solution was then treated with an equal volume of 0.03 M-periodate in 10% n-propanol solution. There was a reduction of 2.08 moles of periodate per mole. The course of the reaction is shown in Fig. IV.

Fig. IV. Periodate oxidation of the disaccharide



Experiment X. Methylation of the aldobiouronic acid

An aliquot (6.0 mg) was converted into its methyl ester glycoside, which was then reduced. The derived disaccharide glycoside was then methylated (GMLC) and hydrolyzed.

A portion of the hydrolysate was glycosided while the residue was reduced and acetylated. The derived alditol acetates were then examined by GLC/MS.

Results and Discussion

Ethanolic Extracts.- The purified water-soluble ethanolic extract and the deionized aqueous dialysates from the aqueous extracts were shown by paper chromatographic analysis to contain galactose, glucose, sucrose, maltose and maltosaccharides. Myo-inositol was tentatively identified.

Aqueous, followed by acidic extraction, chlorite treatment and alkali extraction all gave rise to complex materials which on hydrolysis were found to comprise galactose, glucose, mannose, arabinose, xylose, fucose, rhamnose and uronic acids. (see Table 1 p.125). As extraction proceeded the proportion of xylose increased indicating a separate xylan. The final residue consisted almost entirely of glucose suggesting a cellulose cell-wall. Only the aqueous extracts were examined further.

Aqueous Extracts.- The cold and hot water soluble-extracts after dialysis gave on hydrolysis very similar paper chromatograms (see Table 2).

Table 2. The Proportions of Sugars in the cold and hot water extracts (visual examination)

Extract:	Gal.	Glc.	Man.	Ara.	Xy.	Fuc.	Rha.	Acids*
Cold water	5	3	2	3	2	3	3	3
Hot water	5	4	1	3	4	3	3	4

* Includes oligouronic acids.

Although glucose was present in the hydrolysate neither extract gave a positive test for starch. All attempts to separate a mannan or xylan by copper precipitation were unsuccessful.

Fractionation on DE-cellulose with increasing concentration of potassium chloride gave a single large peak between 0.1 and 0.3 M-KCl (see Fig. I). The material in this peak was separated and refractionated with 0 — 0.3 M-KCl gradient elution (Fig. II). Each of the fractions indicated in the figure was isolated hydrolyzed and analyzed (Table 3).

Table 3. Constituents of the Fractions separated from the Cellulose Column

Peak	I	II	III	IV	V
Carbohydrate mg as glc	3.9	25.6	4.8	4.1	6.0
% Glc UA of carbohydrate content	11.5	12.8	16	17	20
Rhamnose	trace	trace	++	++	+
Fucose	"	++++	+++	+++	++
Arabinose	++	++++	++	+++	++
Galactose	++++	++++	++++	++++	++++
Acids	trace	++	+	+	+

Although there is no fractionation into separate polysaccharides the variations in the proportions of the individual sugars is strongly indicative of the presence of more than one polysaccharide. The absence of glucose from all the fractions is very surprising and it can only be assumed that the glucose-containing polysaccharide was bound on the column and not eluted under the conditions used. This was also true of impurities, the recovered material having a higher carbohydrate content.

A second fractionation of fresh extract on DE-cellulose gave the same fractionation pattern with a 74% recovery of carbohydrate.

In view of their similarity and increased purity fractions from the cellulose column were combined and subjected to periodate oxidation. The extent of oxidation was small (13%). The recovered polyalcohol still contained the same complex mixture of sugars although the proportion of arabinose and uronic acid appeared to have decreased and erythritol, glycerol, erythronic acid and glyceric acid could all be detected in the hydrolysate of the polyalcohol, confirming that the uronic acid, the arabinose and also the galactose had been cleaved.

When the initial water-soluble extract was oxidized with periodate, the extent of oxidation was considerably higher and the proportion of erythritol in the polyalcohol hydrolysate was greatly increased and glucose was absent, indicating a 1,2 or 1,4-linked glucan. Microscopic examination indicated that this extract contains a high proportion of tiny cell-wall fragments. These were shown by hydrolysis of the residue to comprise glucose. This could explain the high glucose content of the extract hydrolysate and its absence in the fractions recovered from the cellulose columns.

Linkage analysis of such a complex mixture of sugars by methylation is extremely difficult and it was decided that the probable resulting mixture of methylated glycosides and alditol acetates would defy unequivocal characterisation and it was not therefore attempted.

Hydrolysis of the whole alga gave a single major oligosaccharide in approximately 1% yield. It had a DP of 2 and on hydrolysis gave glucuronic acid and D-galactose (confirmed with D-galactose oxidase). Reduction, after glycosidation and esterification, followed by hydrolysis gave equal quantities of galactose and glucose. The latter was confirmed as the

D-sugar with D-glucose oxidase. This glycoside reduced two moles of periodate per mole of aldobiouronic acid and after reduction of the derived aldehydic compound to alcohol and hydrolysis uncleaved galactose was obtained.

These results indicate that the aldobiouronic acid is a D-glucuronosyl(1 — 3)D-galactose. This was confirmed by reduction to the neutral disaccharide followed by methylation and hydrolysis. GLC analysis of the derived glycosides and of the alditol acetates showed the presence of 2,3,4,6-tetra-O-methyl glucose and 2,4,6-tri-O-methylgalactose in the methylated hydrolysate. β-Glucuronidase had no action on this aldobiouronic acid suggesting that the linkage is α-.

This aldobiouronic acid must be a structural feature of the water-soluble polysaccharide, but with the results obtained no other conclusions concerning the macromolecular structure of this complex material can be drawn.

The low molecular weight carbohydrates are very similar to those of Urospora and this is the second alga in which maltosaccharides have been found, although there does not appear to be any starch.

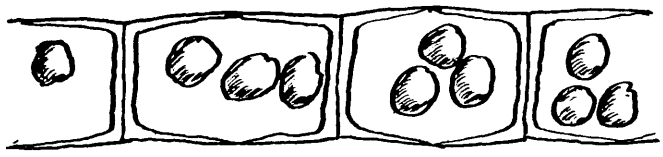
The cell wall definitely comprises a glucan which is vulnerable to periodate. and is tentatively assumed to be of the cellulose-type.

APPENDIX I

Preliminary examination of the fresh water alga Microspora

a) Introduction

The genus in the earlier years of this century suffered many classificatory changes, before it was finally put in a family of its own within the Ulotrichales by Fritsch [186] where it has since remained.[187] It resembles the alga Tribonema in that the cells are often clearly bipartite - that is, made up of cell wall sections H shaped in optical section; especially when about to sporulate:-



and for many years it was confused with that alga. Like both Mougeotia and Urospora, its walls lack cellulose I.

It normally grows best in still ponds in the coldest parts of the year. The alga investigated was collected from a cold freshwater lagoon behind a sand or shingle bar at Farr Bay in 1969. Apart from references to new species or known species in new habitats, very little work has been carried out on this alga.

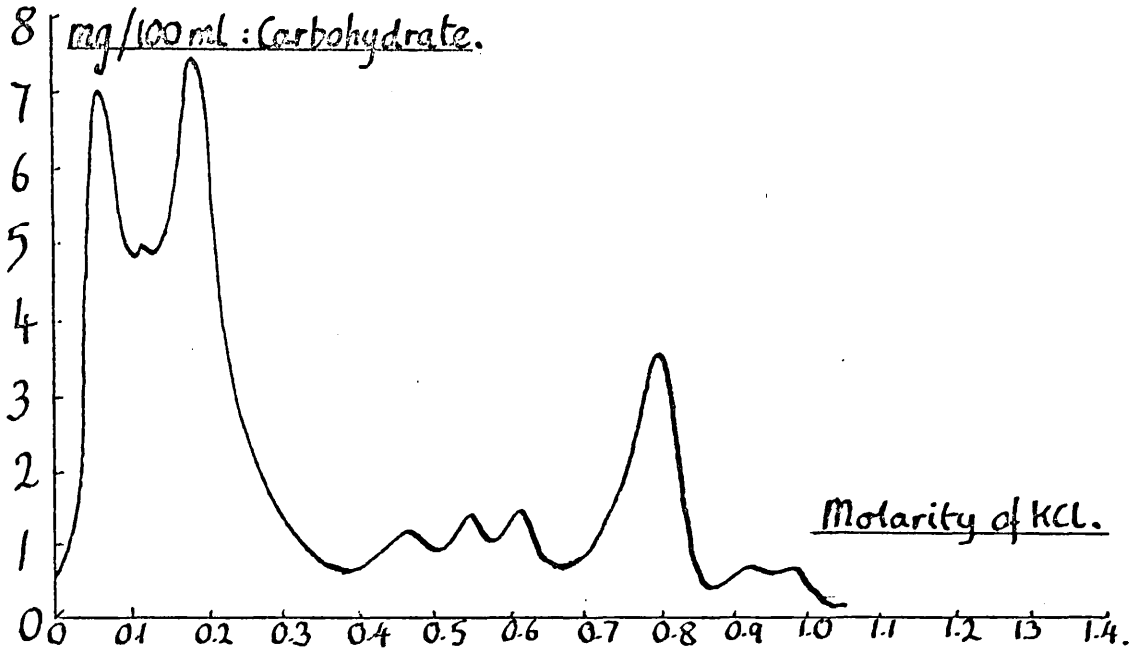
b) Experimental

Approximately 0.75 g of the alga, containing 40.5% of carbohydrate, was extracted identically to Mougeotia (see flow chart II p.124).

The aqueous extract was examined further: it gave a blue colour with the I_2/KI reagent, indicating the presence of amylose. An aliquot of this extract was digested with salivary α-amylase (see p. 136)

Fractionation of the original material on DE12 cellulose gave two major fractions I and II with similar proportions of sugars (Table I and Figure I)

Figure I. Fractionation of Microspora aqueous extract



Assay of the uronic acid content (GM IV d , $Cp^{+}Cl^{-}$ method) showed up to 53% uronic acid.

It therefore appears that the water soluble polysaccharide consists of a uronic acid containing polymer with ~~galactose, and~~ rhamnose and ~~possibly~~ glucose as the other major constituents.

The subsequent acid and alkali extracts gave hydrolysates which were similar to each other and very similar to the aqueous extracts.

TABLE II. Subsequent acid, chlorite and alkali extraction.

	Gal.	Glu.	Man.	Ara.	Xyl..	Rha.	Uronic acid.
Acid extract	++	++++	+	trace	++	+	+++
Chlorite extract	+++	+++	++	++	+++	+	+++
Alkali (4%)extract	++	+++++	+	trace	+++	+	+++
Alkali (18%) extract	++	+++++	++		++++	+	+++
Residue		+++++	+		trace		

Turn forward 2p

and dialysed. The material remaining in the dialysis sac was analysed, after hydrolysis, and conversed into the alditols. The respective proportions of the latter were determined on GLC as their Me_3Si derivatives.

c) Results and Discussion.

The chromatographic analysis of the ethanolic extract showed spots with identical mobilities and colour reactions with the standards detailed below. Sucrose was the most abundant carbohydrate present, together with lesser quantities of glycerol, myo-inositol, and galactose. There was tentative evidence for a homologous series of ketose (fructose) containing oligosaccharides.

The hydrolysed extracts, on paper chromatographic analysis, showed glucose as the major constituent sugar. Amylose was indicated by the I_2/KI reagent, and was partly proved by the considerable reduction in the intensity of the glucose spot relative to the other carbohydrates (Table I), after digestion with α-amylase and dialysis.

TABLE I. Proportions of sugars in variously treated aqueous extracts.

Extracts.	Gal.	Glu.	Man.	Xyl.	Ara.	Rib.	Rha.	Fuc.
(1) Original Extract	+	++++	+	+	trace	trace	++	trace
(2) After amylase treatment	++	+++	++	+	trace	trace	++++	+
Fractionation on DE12.								
Neutral	++	+	++	++	-	-	+	++
I 0.1 MKCl peak	+++	+	++	++	-	-	+	+
II 0.2 MKCl peak	+++	++	++	+	-	-	+	+
III 0.5 MKCl plateau	+++	++	+	++	-	-	+	+
IV 0.7 MKCl peak	++	++++	+	++	-	-	+	+

Molar proportions of sugars in the amylase treated material

Glucose	Galactose + mannose	Rhamnose	Fucose	Xylose	Arabinose	Ribose
30	3 + 3	30	6	10	1	trace

Turn back for page 137

The proportion of xylose and glucose goes up while the other sugars stay roughly constant or even drop somewhat. In the end a pure glucan is left. This maybe considered as tentative evidence for cellulose together with a structural xylan forming the skeleton of the cell wall.

When these tentative results for Microspora are compared with Tribonema aequale, (Xanthophyta) the alga which it most closely resembles morphologically, particularly in the possession of H shaped cell wall sections, some differences and similarities become readily apparent.

The low molecular weight materials synthesized by Tribonema are glycerol, erythritol, glucose, xylitol and mannitol, of which only one is common to Microspora, whose major low molecular weight carbohydrates are glucose, sucrose and fructose, together with some myoinositol.

However the polysaccharides in the latter extracts do show certain similarities. Thus, for example, both alga gave extracts which on hydrolysis yielded glucose, galactose, mannose, xylose, fucose and rhamnose, and, as the extraction proceeded from the aqueous to the alkali extracts, so the proportion of glucose and of xylose increased.

The cell wall framework in both algae is cellulose; however Tribonema lays down cellulose type I, whereas Microspora does not.

It can be seen that while there are similarities between the two algae, these are not strong enough to show a definite relationship between the two.

Appendix II

Modified sulphate estimation.

The method for sulphate assay involves the complete destruction of the carbohydrate material, with the conversion of all sulphur present into sulphate. This means a loss of material where it is precious, and the introduction of a considerable error if a lot of protein is present, since proteins contain up to 4% by weight of S. This is equivalent to an average of 2-6% estimated as sulphate. In the later aqueous extracts of Urospora it was found that the sulphate based on carbohydrate rose to 24% for a sample with an estimated 55-60% of protein and 29% carbohydrate. It can be deduced that these high values are the result of protein contamination.

The following modification enables assay of sulphate in as little as 0.5 mg of material.

The material (1-2 mg - assayed by phenol sulphuric acid method) is hydrolyzed with formic acid. The residue left after evaporation is dissolved in water (1 ml). An aliquot (0.1 ml) of this solution is then removed and added to the 4 amino 4' chlorodiphenylamine reagent (0.1 ml) in a small, pointed centrifuge tube, which is then covered and left 1-2 hr, after which the suspension is centrifuged. An aliquot (0.1 ml) of this solution is then taken and added to water (10 ml). The absorption of the derived solution is then read at 254 nm against distilled water.

APENDIX III

Table 1 Mobility of sugars on paper chromatograms

Sugars	Standard:- glucose A	B Solvents	C	colour Aniline oxalate
Rhamnose	2.23	2.0	1.75	Yellow
Fucose	1.88	1.62	1.36	Yellow
Ribose	1.91	1.59	1.54	Pink
Xylose	1.57	1.47	1.36	Pink
Arabinose	1.45	1.32	1.19	Pink
Mannose	1.19	1.25	1.16	Brown
Glucose	1.00	1.00	1.00	Brown
Galactose	0.88	0.87	0.84	Brown
Fructose	1.33	1.17	1.13	Yellow
Oligosaccharides				
Sucrose	0.58	0.72	0.74	Yellow
Maltose	0.37	0.52	0.69	brown
Maltotriose	0.13	0.28	0.48	brown
Acids				Acid spray
Glyceric	2.43		0.53	
Erythronic			0.18	
Erythronic lactone			2.07	
Glucuronic	0.85		0.09	
Glucurone	2.44		1.94	
Galacturonic	0.81		0.77	
Alcohols				Silver nitrate
Ethane diol	3.0	2.0		
Glycerol	2.30	2.47	1.76	
Erythritol	1.83	1.85	1.34	
Others				
Erythrose			2.15	
Myoinositol	0.40	0.40		

Table II Electrophoretic mobility of sugar acids in pyridine-acetic acid buffer

Standard:- Glucuronic acid

Glucuronic acid	1.0
Galacturonic acid	0.89
Gluconic acid	1.0
Glyceric acid	1.5-1.54; 1.13; 0.88; 0.74[+]
Erythronic acid	1.24.

[+]Glyceric acid appears to react with itself to form dimers, trimers and oligomers.

Table III Retention times of the Me_3Si derivatives of Carbohydrates on GLC

Column5, 9ft. 175°
Standard:- xylitol

Sugar				Alditol
Rhamnose	0.91s	1.33m		
Fucose	1.07w	1.30s	1.75s	1.13
Ribose	1.17s	1.25m	-	1.05
Xylose	1.06w	1.61s	2.2s	1.0
Arabinose	0.91s	1.14s	1.32w	1.06
Mannose	1.69s	2.89m	1.7	2.2
Glucose	3.0s	4.97m		2.34
Galactose	1.95w	2.89s	3.10m	2.37
Glucurone	2.4-2.9			
Fructose	1.67s	1.83m.	2.05m	

S = strong; m = medium; w ; weak

The retention times of the slower peaks are decreased up to 10% on a 5 ft. column

Table IV Paper Chromatographic mobilities of Methylated Sugars

Standard: 2,3,4,6-tetra-O-methylglucose

Methylated Sugar	Solvent A	Solvent B	Solvent D	Colour aniline oxalate
O-Methylxylose				
2,3,4-Tri	0.98	0.96	0.99	Pink
2,3,-Di	0.83	0.83	0.63	
2,4-Di	0.81	0.79	0.54	
3-Mono-	0.45	0.61	0.21	
Free sugar	0.19		0.13	
O-Methylrhamnose				Yellow
2,3,4-Tri-		0.97	1.07	
2,3- Di-	0.76	0.88	0.64	
2,4-Di-			0.82	
4-Mono-	0.61	0.74	0.40	
2-Mono-	0.39	0.71	0.56	
3-Mono-				
Free sugar	0.31	0.48	0.06	
O-Methylglucose				Orange brown
2,3,4,6-Tetra-	1.00	1.00	1.00	
2,3,4-Tri-	0.89	0.91	0.72	
2,3,6-Tri-	0.82	0.89	0.64	
2,4,6-Tri-	0.86	0.84	0.58	
3,4,6-Tri-				
2,4-Di-	0.68	0.68	0.28	
Free sugar	0.10	0.28	0.02	

Table V. Retention times of Methylated Glycosides on GLC

O-Methylsugar	Column 1	Column 2	Column 3
O-Methylxylose			
2,3,4-Tri-	0.48 0.61	0.41 0.50	0.52 0.63
2,3-Di-	1.48sh 1.67 1.79	0.59 0.76 0.96	1.55 1.7
3,4-Di-	1.28 1.51 1.71	0.71 0.76 0.95	
2,4-Di-			1.5 1.9
2-Mono-	4.03	0.97 1.33	
4-Mono-		1.10	
O-Methylrhamnose			
2,3,4-Tri-	0.47	0.44	0.48
2,3-Di-	1.44	0.72	1.42
2,4-Di-	1.12	0.66	1.2
3,4-Di-	1.0 1.71	0.57	1.0 1.7
2-Mono-	3.22	1.03	3.21
3-Mono-		1.26 1.1	
4-Mono-	4.36	1.03	
O-Methylglucose			
2,3,4,6-Tetra-	1.0	1.0	
2,3,4-Tri-	1.25 2.5 3.4	0.7 1.3 1.8	1.14 2.38 3.3
2,3,6-Tri-	3.2 4.3	1.75 2.3	3.07 3.37 3.96
2,4,6-Tri-	3.1 4.17	1.69 2.37 3.0	2.36 3.53 4.68
3,4,6-Tri-	3.12 3.5	1.83	
2,6-Di-		2.1 3.8	
3,4-Di-		2.85 3.0	
3,6-Di-		3.23 3.63	
4,6-Di-		3.1 3.5	
O-Methylmannose			
2,3,4,6-Tetra-	1.39	1.36 (1.68)	1.73 1.42
2,3,6-Tri-	4.65	2.27 2.46	4.42
2,4,6-Tri-	2.06 3.3@?	1.82 2.37 2.46	1.7 2.7
3,4,6-Tri-	2.9	1.75	1.9 2.7
2,3-Di-		4.23 4.39	
2,4-Di-		4.49 5.2	1.9 3.4 3.8
3,4-Di-	6.65	2.58	6.7
4,6-Di-		3.38	

Table VI Retention times of partially methylated alditol acetates

Standard: 2,3,4,6,-tetra-O-methylglucitol 1,5-acetate

Alditol	Column 12	Column 13	Column 5
O-Methylxylitol			
2,3,4-Tri-	0.55	0.58	0.58
2,3-Di-	1.38	1.19	0.87
2,4- Di-	1.27	1.05	0.81
3,4-Di-	1.36	-	0.87
2-Mono-	2.92	2.15	
3-Mono-		2.15	
Free Sugar		2.10	
O-Methylrhamnitol			
2,3,4- Tri-	0.54		0.54
2,3- Di-	0.98	1.1	1.1
2,4- Di-	0.90	0.9	0.85
3,4- Di-	0.70	0.7	0.6
2-Mono-	1.7	1.3	1.0
3-Mono-	1.94	1.67	1.15
4-Mono-			
Free sugar		1.75	
O-Methylmannitol			
2,3,4,6-Tetra-		0.99	
2,3,4-Tri		2.19	
2,3,6- Tri-		-	
2,4,6-Tri-		1.90	
3,4,6-Tri		1.82	
4,6-Di-		2.92	
O-Methylglucitol			
2,3,4,6- Tetra-		1.0	
2.3.6-Tri-		2.17	
2,4,6-Tri-		1.74	
2,3,4- Tri-		2.1	

O-Methylgalactitol see overleaf.

Table VI Continued

Alditol	Column 13
O-Methylgalactitol	
2,3,4,6,-Tetra-	1.19
2,3,4- Tri-	2.89
2,3,5-Tri-	2.76
2,4,6-Tri-	2.03
3,4,6-Tri-	2.10

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