STRUCTURAL INVESTIGATIONS

ON

ALGAL POLYSACCHARIDES

THE CARBOHYDRATES SYNTHESISED BY THE DIATOMS

PART I. PHAEODACTYLUM TRICORNUTUM

AND

PART II. MONODUS SUBTERRANEUS

by

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TO MY MOTHER AND ANNE

CONTENTS

GENERAL INTRODUCTION

ALGAE

Algae are among the most interesting plants produced by nature. The variety of colour, shape and size among the thousands of species is enormous. The smallest algae measure scarcely a micron in diameter, whereas the giant kelps often exceed a hundred feet in length. Algae are found in every conceivable habitat; in the oceans, rivers, ponds, the soil, and even in the arctic and antarctic regions, where it is considered that life ultimately depends upon their presence. In the matter of pigmentation, perhaps no other group of plants exhibits so many different colours. The common names applied to the various classes of algae - greens, blue-greens, yellow-greens, browns, golden-browns, reds - merely represent what is commonly the manifest colour of the plants. This gives little clue to the possible colour changes during normal growth. The greens, for example, may be vivid green (when actively growing) and nearly yellow or brown during spore formation. The blue-greens, perhaps, exhibit the widest range of colour, hues from red through the spectrum to violet may be observed. The variation range in the algae is among the greatest in the plant kingdom. They may be unicellular or multicellular. The colonial forms may have definite shapes, or they may be merely irregular aggregates of cells. The cells may be spherical, cylindrical, club-shaped, spiral, sigmoid, wedge-shaped, or

even amoeboid. The plants are filamentous or plate-like, branched or unbranched, free floating or attached, microscopic or macroscopic, highly ornamented and sculptured or smooth, annuals or perennials.

Some of the pigments found in algae, and their chemical compositions, are: chlorophyll a, $C_{55}H_{72}O_5N_4Mg$, chlorophyll b, $C_{55}H_{70}O_6N_4Mg$, carotin, $C_{40}H_{56}$, xanthophyll, $C_{40}H_{56}O_2$, and fucoxanthin, $C_{40}H_{56}O_6$. Phycoerythrin, of unknown chemical composition is thought to be allied to proteins. Phycobilin is also proteinaceous in composition. Chlorophylls c, d, and e, and perhaps others, are known from their spectrum analysis. Their chemical composition is as yet unknown. The distribution of some pigments in the algae may be seen in Table 1 p.3.

Man's interest has centred mainly around the larger species of marine algae, commonly known as seaweeds. For centuries, certain species have been used for human and animal consumption, especially in the Far East and the islands of the Pacific Ocean. In the eighteenth and nineteenth centuries they were sources of iodine, potash and soda, but with the advent of cheaper methods of producing these materials, the once flourishing kelp industry of Scotland, Ireland and France soon faded and died. Industrial interest in seaweeds was revived during the second world war, and carbohydrate extracts of the Rhodophyceae and Phaeophyceae have, to-day, considerable everyday applications in the food, cosmetic, pharmaceutical, textile, brewing and paper industries.

The seaweeds have been divided into four classes, accord-

Table 1

CLASS OF ALGA	Chlorophyll a	Chlorophyll b	Chlorophyll c	Chlorophyll d	Chlorophyll e	α - Carotene	β - Carotene	Xanthophylls	Phycobilins
Chlorophyceae (Green Algae)	X	X	O	O	O	X	X	X	O
Xanthophyceae (Yellow-greens)	X	O	O	O	X	?	X	?	O
Bacillariophyceae (Diatoms)	X	O	X	O	O	O	X	X	O
Chrysophyceae (Golden browns)	X	O	?	?	?	?	X	X	?
Dinophyceae (Dinoflagellates)	X	O	X	O	O	O	X	X	O
Euglenophyceae (Euglenids)	X	X	O	O	O	?	X	?	O
Phaeophyceae (Browns)	X	O	X	O	O	O	X	X	O
Rhodophyceae (Reds)	X	O	O	X	O	X	X	X	X
Myxophyceae (Blue-greens)	X	O	O	O	O	?	X	X	X

X = present, O = absent, ? not known

ing to their photosynthetic pigments, which also happen to coincide with differences in chemical nature, exemplified by the different types of polysaccharides synthesised. These will be discussed in some detail.

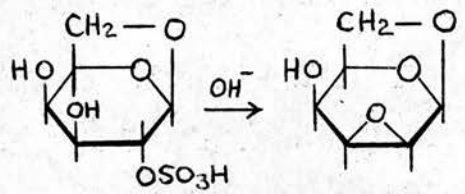
Examples of what are considered to be food reserve materials are found in the β-1,3-linked glucan, laminarin, of the Phaeophyceae, and the glucans resembling starch in the green and red seaweeds. The proportions present of these reserve materials vary considerably with the season. They are usually at a maximum in the autumn.

The environmental conditions of marine algae, contrasting so much to those of land plants, give rise to considerably different skeletal requirements. The normal constituents of the cell walls of land plants, cellulose, hemicellulose and lignin, giving the characteristic rigid structure, are either absent or present in only very small quantities. Seaweeds require flexibility and ease of movement, and in keeping with this is the mucilaginous nature of many of their polysaccharides. Common features of algal structural polysaccharides are their complexity, being heteroglycans, and their acidic nature. This latter feature arises from the presence of uronic acid residues, and/or sulphuric acid half ester groups. No polysaccharides of the land plants have as yet been found to contain sulphate ester residues, but some of the animal polysaccharides are sulphated. Thus, heparin (the anticoagulant), the chondroitin sulphates (found largely in cartilage) and keratan sulphate (cornea), all have ester sulphate. The reason for the presence of sulphate

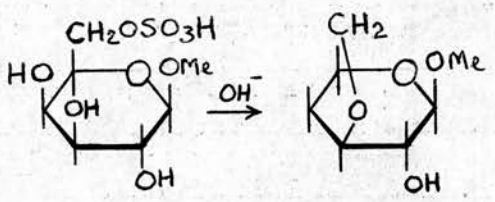
residues in algal polysaccharides has not been definitely established. One view is that their hydrophilic nature may keep the plant from dehydration at low tides, another is that they regulate the ionic equilibrium of the cells.

SULPHATED POLYSACCHARIDES

The presence of sulphuric acid half ester groups in seaweeds was first reported by Haas in 1921 (1). These esters hydrolyse under acid conditions, but with alkali they are either stable or are eliminated to form anhydro sugars. Studies on monosaccharide sulphates (2) have shown that hydrolysis in alkali occurs if an adjacent -OH group is in a '<u>trans</u>' configuration to the sulphate residue (Fig.<u>1</u>), or if removal of the sulphate group can result in the formation of a 3,6-anhydride (Fig.<u>2</u>).



<u>Fig.1</u>



<u>Fig.2</u>

Thus, 1,6-anhydro-β-D-galactose-2-sulphate with a trans -OH group on C_3 hydrolyses to 2,3—1,6-dianhydro-β-D-talopyranose (Fig.<u>1</u>), whereas methyl-β-D-galactopyranoside-6-sulphate, with a free 'cis' -OH group on C_3, gives rise to

methyl-3,6-anhydro-β-D-galacto-pyranoside (Fig.2).

In general, the sulphate esters of seaweeds are stable to alkali, a fact which hinders elucidation of the polysaccharide structure, and the site of the sulphate. Methylation of such materials is difficult to accomplish, is seldom complete, and the results ambiguous. Recently, yields of oligosaccharides from partial enzymic hydrolysis of some polysaccharides were found to be improved if the oligosaccharides were removed continuously from the reaction solution by dialysis (3). A similar method has been applied to the autohydrolysis of the sulphated polysaccharide from the red alga Furcellaria fastigiata (4). The polymer contains D-galactose (43.1%), 3,6-anhydro-D-galactose (30.3%) and half ester sulphate (as-SO_3Na, 20.1%) (5). An aqueous solution of the polymer, previously stirred with a cation exchange resin, was dialysed in presence of barium carbonate. It was found that a yield corresponding to 60% of the original polysaccharide could be recovered, with a loss of only 20% of the sulphate ester content.

Rees, (6), in studies on galactose 4- and 6-sulphates and glucose-3-sulphate has shown that the rate of acid hydrolysis of the sulphate residues varies according to where the sulphate is situated. Stability increases in the order as follows : equatorial < axial < primary.

FUCOIDIN

Fucoidin is a heavily sulphated fucose containing polymer (7) of the Phaeophyceae. The high sulphate content (ca 33%)

corresponds to an average of one sulphate residue on every fucose unit. Most of this sulphate is alkali stable (8). Methylation studies on the sulphated polymer have led to the isolation of methyl 3-O-methyl-L-fucoside (58%), methyl L-fucoside (ca 20%) and methyl 2,3-di-O-methyl-L-fucoside. These results indicated that positions C_2 and C_4 were involved in linkages with adjacent L-fucose units or with sulphate ester groups. Acetolysis and reduction of fucoidin (9) resulted in the characterisation of 2-O-α-L-fucopyranosyl-L-fucitol, (Fig.3), consistent with methylation results, and indicated 1,2' linked fucose units with sulphate residues probably on C_4 positions.

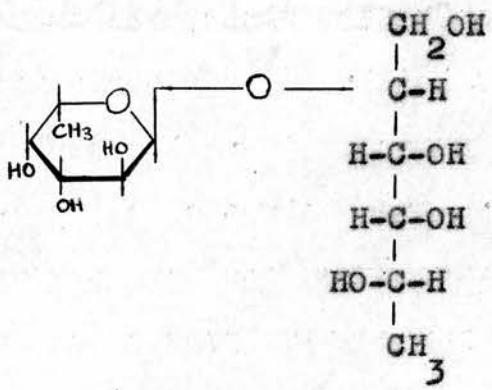


Fig.3

The stability of the sulphate to alkali also indicates this position, for C_2 sulphate in a 1,4'-linked fucan would be alkali labile. However, further studies have led to the isolation and characterisation of 4-O-α-L-fucopyranosyl-L-fucose (10). Thus, there is some degree of 1-4'L-fucose linkages and a possibility that some of the sugar units are disulphated.

CARRAGEENAN

Carrageenan, a sulphated galactan, can be extracted with hot water from the red seaweed Irish moss, (Chondrus crispus and Gigartina stellata). Forming a reversible gel with water, carrageenan finds many uses, and is extracted commercially as a stabilizer and homogeniser (protective colloid) for chocolate milk, ice cream, toothpaste and pharmaceutical products.

Carrageenan appears to be a mixture of polymers containing D-galactose (30-40%) and 3,6-anhydro-D-galactose residues (ca 12%). There is also an ester sulphate content of ca 30% (11) (12) (13). The two principal polymers have been named κ-carrageenan and λ-carrageenan. They have been fractionated (14) (15) using potassium chloride, the former polymer being precipitated from aqueous solution, the latter remaining dissolved. The proportions of κ : λ are ca 2:3. The isolation of the disaccharide 4-O-β-D-galactopyranosyl-3,6-anhydro-D-galactose, (carrabiose) (Fig.4) using partial mercaptolysis (16) led to the tentative suggestion for the repeating unit of the κ-polymer (Fig.5).

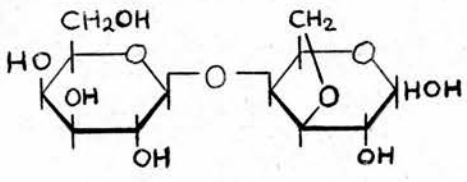


Fig.4

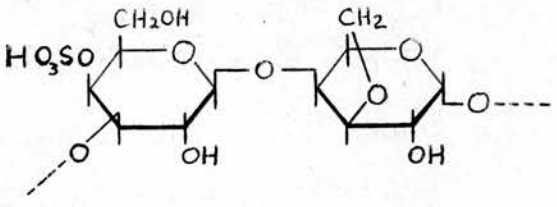


Fig.5

λ - Carrageenan has been shown to consist almost entirely of sulphated-D-galactose residues. Evidence for 1,3'- linkages has been supplied by Morgan and O'Neill (17) who isolated and characterised 3-O-α-D-galactopyranosyl-D-galactose (Fig.6).

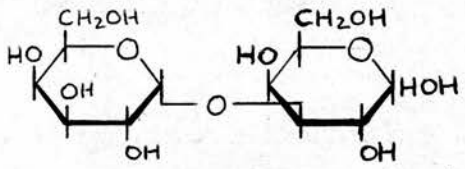


Fig.6

The same workers also isolated a crystalline trisaccharide tentatively identified as O-α-D-galactopyranosyl-(1-3)-O-α-D-galactopyranosyl-(1-3)-D-galactose.

More recently Rees (18)(19) has shown that a sample of λ - carrageenan contained galactose-6-sulphate, which by alkali treatment was converted into 3,6-anhydro galactose. Partial mercaptolysis resulted in the isolation and characterisation of a derivative of 4-O-β-D-galactopyranosyl-3,6-anhydro-D-galactose, showing the presence of 1,4'- linkages. Rees also supplied evidence for presence of sulphate on C_2 and C_6 of the galactose residues linked through the C_4 positions. The characterisation of galactose-6-sulphate from a partial acid hydrolysate established some sulphate on C_6. Sulphate on C_2 was deduced from the rate of acid hydrolysis of the sulphate (6), periodate oxidation on the partially hydrolysed and reduced polysaccharide, and a

tentative identification of 3,6-anhydro-galactose-2-sulphate.

AGAR

Agar is the gel-forming, major polysaccharide from Gelidium amansii and other red algae. It is used as a food ingredient in the Orient, and elsewhere it is used extensively as a laboratory culture medium for micro-organisms.

Agar contains residues of both D- and L-galactose, the latter as the 3,6- anhydro derivative (20)(21). Pyruvic acid and small amounts of L-galactose (22) as well as ester sulphate (0.3-2.2%) are also present (23).

Acetylated agar has been separated into a chloroform soluble fraction agarose (70%) and a chloroform insoluble fraction agaropectin (30%) (24). Araki has suggested that agarose is composed of 1,3' linked β -D-galactopyranose and 1,4'- linked 3,6-anhydro-α-L-galactose, the residues being alternately repeated to form a chain (25). This structure has been based on the chemical composition of agar, the identification of the scission products of methylated agarose (26)(27), the isolation of agarobiose (4-O-β-D-galactopyranosyl-3,6-anhydro-L-galactose) (Fig.7) and its derivatives from the products of partial acid hydrolysis (28), mercaptolysis (29), and methanolysis (30) of agar, and the isolation of neoagarobiose (3-O-3,6-anhydro-α-L-galactopyranosyl-D-galactose) (Fig.7) from the enzymatic hydrolysis of agar (31). An agar digesting bacterium Pseudomonas kyotensis was used as an enzyme solution, and as well as neoagarobiose, a tetrasaccharide was isolated,

crystallised, and characterised (32). The name given to the tetrasaccharide was neoagarotetraose and the structure assigned as in Fig.7.

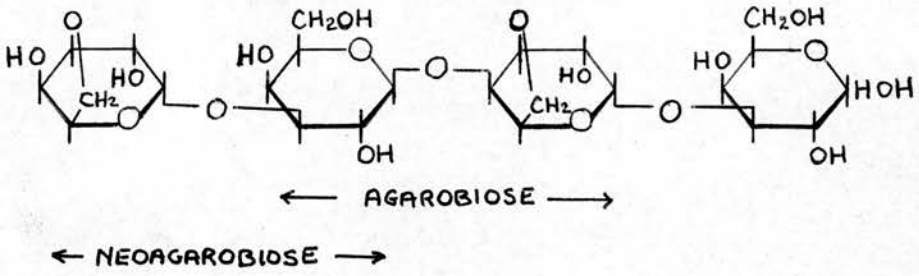


Fig.7

Agaropectin contains β -D-galactose, 3,6-anhydro-L-galactose as well as sulphate ester, pyruvic acid and D-glucuronic acid. Partial methanolysis of agar (33) has led to the isolation and characterisation of a disaccharide as 4,6-O-1' carboxyethylidene-β -D-galactopyranosyl-(1-4)-3,6-anhydro-L-galactose (Fig.8). This evidence partially establishes the position of the pyruvic acid.

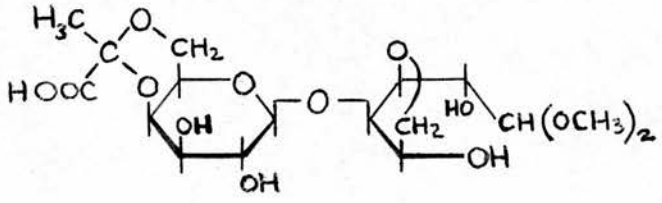


Fig.8

The location of the sulphate residues and the position of the glucuronic acid has not yet been determined.

FUNORIN

The Gloiopeltis family of red algae, under cultivation in Japan for food, synthesises a sulphated polysaccharide, similar to agarose (Fig.7) in consisting of alternating residues of 1,3'-linked β-D-galactopyranose and 1,4'-linked 3,6-anhydro-α-L-galactopyranose residues. This polysaccharide, termed funorin, although forming highly viscous solutions does not possess the property of forming gels, characteristic of agar and carrageenan. From this polymer Hirase and Araki have isolated the dimethyl-acetal of 3,6-anhydro-L-galactose and methyl D-galactoside after complete methanolysis, and the dimethyl acetal of agarobiose (Fig.7) after partial methanolysis.

GALACTAN from Dilsea edulis

This polymer also forms highly viscous solutions. There is a sulphate ester content of ca 11%, and the sugar residues present are D-galactose (ca 70%), D-glucuronic acid (ca 10%) D-xylose (7%), together with small amounts of 3,6-anhydro-D-galactose (34)(35). Methylation of the sulphate free, degraded polysaccharide acetate has indicated that it is mainly a 1,3'-linked D-galactose polymer, with some 1,4'-linkages also present (36).

Application of the Barry degradation to the oxidised polysaccharide (37) has confirmed a backbone of 1,3'-linked D-galactose residues, to which are attached highly sulphated

1,3'- and 1,4'- linked D-galactose chains, xylose, and glucuronic acid.

GALACTAN from Porphyra umbilicalis

Another sulphated galactan, it resembles agar and carrageenan in its constituents. Most of the sulphate esters are sited on C_6 of the L-galactose residues (38). One feature distinguishing this polymer from both agar and carrageenan is the presence of 6-O-methyl-D-galactose residues (39). Rees (40) has shown the simultaneous enzymic conversion of the L-galactose-6-sulphate to 3,6-anhydro-L-galactose. It is thus probable that this occurs as part of the normal metabolism of the plant.

SULPHATED HETEROPOLYSACCHARIDES

The major polysaccharides synthesised by the green seaweeds appear to be water-soluble, complex, sulphated polymers. They can be divided into two main groups: those which contain uronic acid residues, and those which do not. The former category will be discussed later. Polysaccharides of the second type are synthesised by the Cladophoras and the Chaetomorphas. These polymers have been found to consist mainly of arabinose and galactose, together with smaller proportions of xylose, glucose and trace quantities of rhamnose (41). Evidence has been obtained indicating 1,3'- and 1,6'- linked galactose residues to be present, while galactose-6-sulphate and arabinose-3-sulphate have been isolated and characterised (42). The Codiums and Caulerpas (43) synthesise similar water-soluble polysaccharides containing galactose with less arabinose

together with mannose and xylose. Studies on Codium fragile (44) have given proof of the presence of 1,3'-linked galactose and arabinose, and have led to the isolation and characterisation of galactose 4- and 6-sulphates.

POLYURONIDES

ALGINIC ACID

Discovered by Stanford (45) in 1883, alginic acid, to-day, still provides a source for structural investigations. In low concentrations (ca 0.1-2%) it forms stable gels, the reason for its great commercial importance in the food industry, and production of pharmaceuticals and cosmetics wherever an emulsifying or thickening agent is required.

Alginic acid is the major polysaccharide of the Phaeophyceae. Because of the stabilising influence of the uronic acid residues on the interglycosidic linkages, hydrolysis of the polymer requires very rigorous conditions resulting in considerable degradation. Although the acidity also hinders methylation, Hirst et al (46) succeeded in establishing the presence of 1,4'-linked mannuronic acid units when they isolated 2,3-di-O-methyl mannuronic acid from methylated alginic acid. A chain length of over 100 was indicated for methylated degraded alginic acid from studies by Chanda, Hirst, Percival and Ross (47) on the reduced methyl esters. Further studies on alginic acid hydrolysate by Fisher and Dörfel (48) provided evidence for the presence of L-guluronic acid residues (I), in addition to D-mannuronic acid (II). This has been confirmed by Drummond and Percival (49), who also established 1,4'

glycosidic linkages using the scheme below (Fig.9).

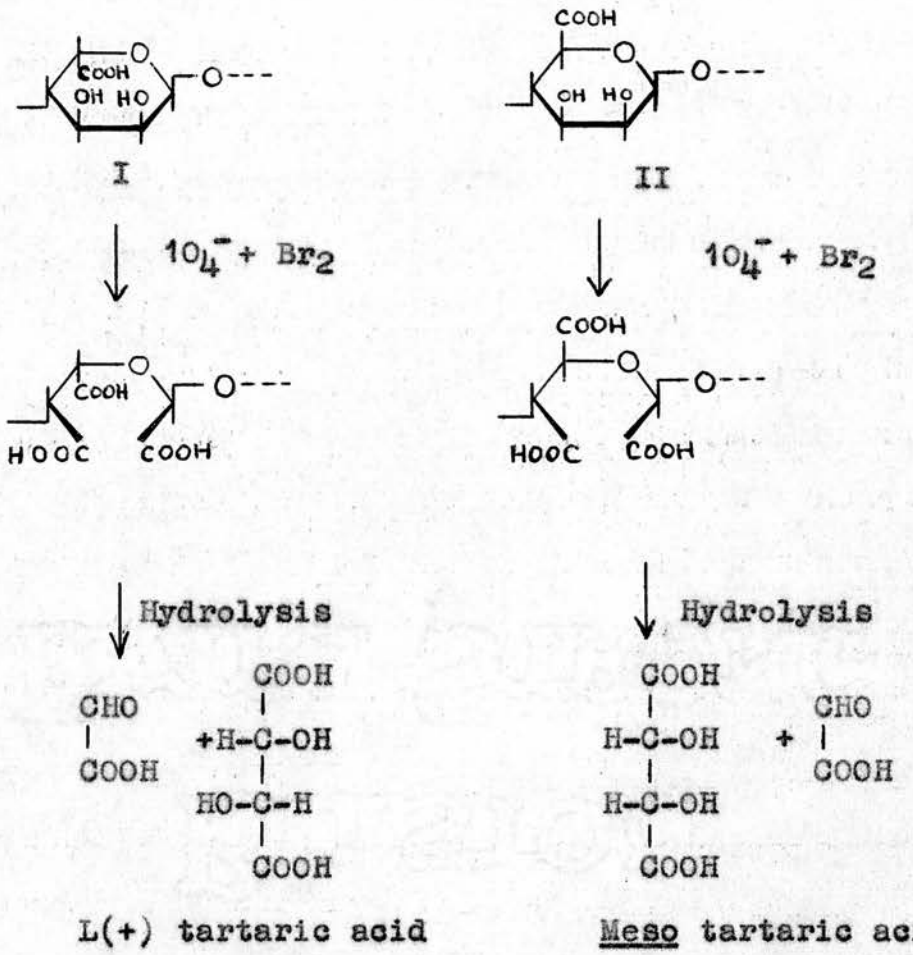


Fig.9

Attempts to fractionate alginic acid into a guluronic acid polymer and mannuronic acid polymer have not succeeded, although materials containing varying proportions of the two acids have been isolated and partial fractionation has been achieved. Evidence that both acid residues are in the same molecule has been provided by Percival and Wold (51) who isolated a crystalline gulosyl-mannose after partial acid hydrolysis of the reduced material. This was tentatively characterised as

1-4 linked.

COMPLEX POLYURONIDES METABOLYSED BY THE GREEN SEAWEEDS

Green algae in the genera, Ulva, Enteromorpha and Acrosiphonia (Spongomorpha)(52), all synthesise mucilages in which the main sugar, rhamnose, is accompanied by xylose, glucose, glucuronic acid (ca 20%) and ester sulphate (7-20%). Studies on these polysaccharides suggest a high proportion of 1,3'-linked residues or branching in the molecules or a combination of both. Direct linkage between glucuronic acid and rhamnose is also indicated (53). The majority of the sulphate appears to be linked to the rhamnose at C_2, and proof that some of the xylose residues are also sulphated at C_2 has been obtained for Ulva and Enteromorpha (54).

FOOD RESERVE POLYSACCHARIDES

The energy reserves of many algae take the form of polymeric glucose. The red and green seaweeds synthesise starch like polymers, whereas the brown seaweeds synthesise an essentially β 1,3-linked glucan called laminarin.

FLORIDEAN STARCH

First isolated by Colin (55), Floridean starch, the food reserve polysaccharide of Dilsea and Furcellaria spp., is considered to have an essentially amylopectin type of molecular structure. Early studies on the glucan from Dilsea edulis (56), using periodate, led to the opinion, from a low consumption (0.6 moles.) of periodate per glucose unit, that linkages through the C_1 and C_3 must be present. However, later studies

(57) revealed a periodate uptake of ca one mole.periodate per hexose unit, a value confirmed by nitrogen and sulphur analysis of the thio-semicarbazide and isoniazid derivatives of the oxidised polysaccharide (58). Absence of 1,3'-linkages was also indicated by enzymic studies. The percentage conversion to maltose by α-amylase (α-amylolysis limit) of 65 and a β-limit of 45 (57) are comparable to the values for amylopectin. However, the short average chain length of 9-13 glucose units corresponds more to the value for glycogen. (c.f. C.L. of amylopectin ca 21). While the evidence points to α-1,4' linked glucopyranose residues with branching on C_6 of the glucose unit as being the structure of Floridean starch, a small amount of nigerose (3-O-α-D-glucopyranosyl-D-glucose) (ca. 0.5%) has been isolated from a partial hydrolysate (59).

LAMINARIN

In some of the species of brown seaweeds, especially the Laminaria spp., the food reserve polysaccharide is the glucan laminarin. Two forms of this polysaccharide, chemically almost indistinguishable, have been isolated, differing only in their solubility. Laminarin from L.digitata remains in aqueous solution on cooling, whereas that from L.cloustoni precipitates on standing.

Methylation studies (60), supported by periodate oxidation (61), have shown that both forms of laminarin are essentially β-1,3'-linked glucans. These results were further confirmed by hydrolysis, using an enzyme from Helix pomatia (62), which led to the isolation and characterisation of 3-O-β-D-gluco-

pyranosyl-D-glucose (Fig. 10).

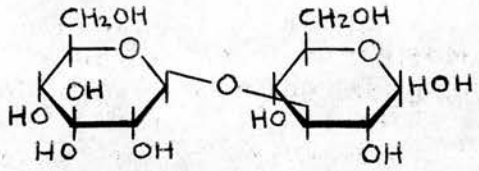


Fig. 10

More recent investigations have shown that the structure of laminarin is more complicated than previously supposed. Partial hydrolysis (63) of laminarin has produced, in addition to the laminaribiose series of oligosaccharides, D-mannitol, 6-O-β-D-glucopyranosyl-D-glucose (gentiobiose)(Fig. 11), 1-O-β-D-glucosyl-mannitol (Fig. 12) and 1-O-laminaribiosyl-mannitol.

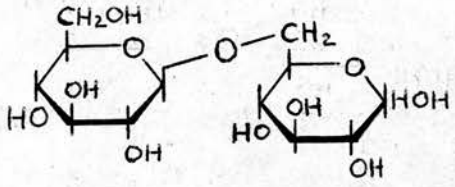


Fig. 11

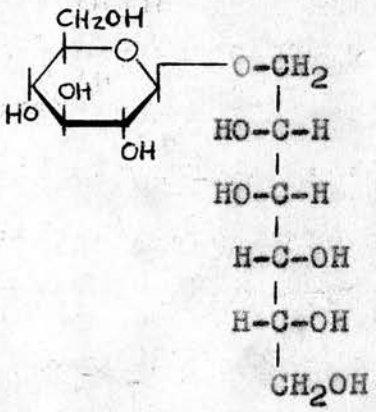


Fig. 12

The isolation of a small quantity of gentiobiose along with two isomeric trisaccharides indicated the presence of β-1,6'-linkages in the polymer.

From the above evidence it was considered that there were two types of chains in the laminarin molecules: 'M-chains' in which the reducing end was terminated by mannitol, linked through C_1 or C_6, and 'G-chains' in which the terminal units were reducing glucose residues. By measuring the formaldehyde released when laminarin is oxidised with periodate at 2°C (64) Anderson et al. obtained a value of 46% for the proportion of laminarin molecules having mannitol terminated chains.

Oxidation of the reducing molecules of laminarin to laminaric acid (65) and fractionation on anion exchange resin, separated a non-reducing, mannitol terminated entity,(M-chains) laminaritol. Mannitol was only detected in the neutral fraction. Periodate oxidation and reduction of laminaritol, followed by hydrolysis, failed to yield ethylene glycol, the expected product from a terminal mannitol residue linked only through C_1 or C_6. This evidence led Smith and Unrau to postulate that the mannitol acts as an aglycone residue for two glucose chains joined to C_1 and C_2. More recent studies have, however, provided evidence for 1-substituted terminal mannitol residues (66)(Fig.13). The isolation and characterisation of ethylene glycol from the hydrolysate of laminarin polyalcohol provided strong evidence for mannitol residues linked through C_1 or C_6. This evidence was further confirmed by periodate oxidation studies on laminarin, when it was found that three molar proportions of formic acid per mannitol residue were released.

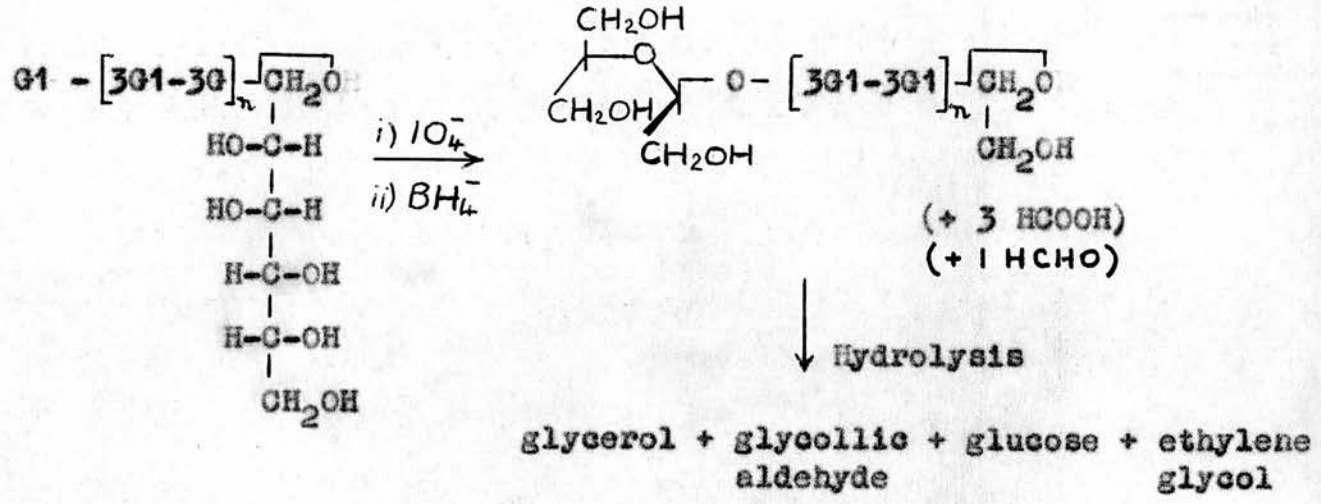


Fig.13

The existence of 1-6'-linkages in laminarin was substantiated by a series of reactions involving periodate oxidation, reduction, and finally mild hydrolysis (67). This treatment was applied to both the reducing and non-reducing components of laminarin. After two treatments, the products were fully hydrolysed. Both components of laminarin gave ethylene glycol (Fig.14) in amounts corresponding to two β 1,6'-linkages for laminaritol, and one such link for the reducing laminarose, per molecule.

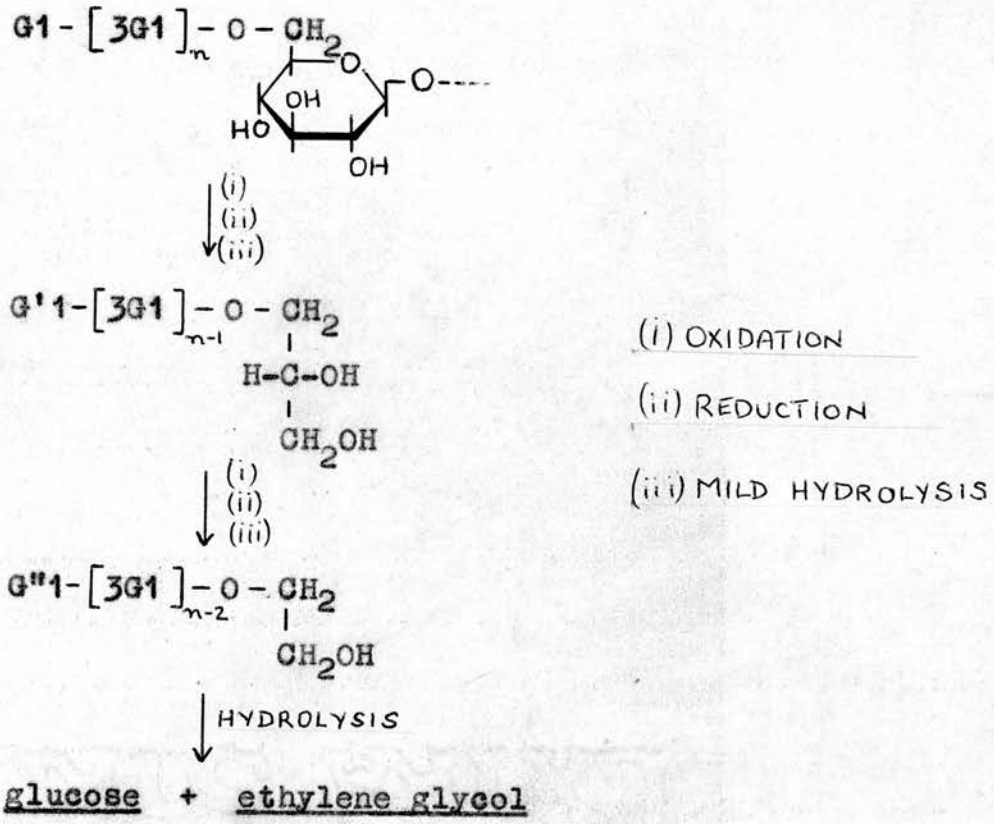


Fig. 14

This method does not detect branched points involving 1,6'-linkages. Branching in laminarin has been postulated (64) but so far has not been confirmed.

Mannose has been reported to be present to an extent of 14% of the glucose content in one commercial sample (68), but so far other workers have not been able to detect this heterogeneity in laminarin.

PART 1(a)

THE GLUCAN

FROM

PHAEODACTYLUM TRICORNUTUM

PART 1(a)

INTRODUCTION

BACILLARIOPHYCEAE (Diatoms)

Diatoms are microscopic, unicellular organisms belonging to the Chrysophyta. There are motile and non-motile species. The term 'diatom' is derived from the Greek 'diatomos', meaning 'cut through', and arises out of the organism consisting of two "flinty" shells which fit together like a box and lid.

Diatoms represent a very interesting group of organisms not only phylogenetically but also because of their great importance in the synthesis of organic substance on the earth. Historically, diatoms, along with the dinoflagellates, are the youngest of the algae, first appearing in the Triassic some 190,000,000 years ago. (c.f. blue green seaweeds 1,500,000,000-2,000,000,000 years old). Large fossil deposits of diatoms are found all over the world, and have been found to contain many species now extinct. Throughout the years, these fossils, (popularly called diatomaceous earth), have found various uses. It is recorded that Emperor Justinian, (532 A.D.), because of their lightness, instructed bricks to be made of this material in the repair of the Church of Saint Sophia in Constantinople. Present day uses include insulation for pipes and linings for walls of blast furnaces. Formerly a polishing agent, it has largely been replaced by carborundum.

Diatoms are found in seawater, freshwater and in the soil. Marine diatoms, recognised as the primary food supply of the sea, are one of the many planktonic forms of marine algae.

Seawater diatoms differ from their freshwater counterparts only in species and genera, in variety of ornamentation, and in relative abundance of different forms. So important are they considered to be, marine diatoms are often referred to as the grass of the sea. Like the larger forms of algae they contain chlorophyll and photosynthesise complex materials. It has been shown that 'bottom' diatoms in shallow waters (< 100 ft.) are preeminently valuable to fisheries. Fish breed in shallow water and the young thrive on an abundance of diatoms.

Although this type of organism has been known for many years, harvesting single species in the quantities needed for chemical investigations was quite impossible. It is only recently that methods have been devised for growing them under artificial culture. Furthermore, it is only during the past few years that experimental techniques in sub-micro analysis and structural studies have made it possible to characterise milligram quantities of material. Consequently, up till 1956 (69) the composition of the carbohydrates of diatoms had not been studied chemically. Only their cytological reactions had been taken into account. For example, Mangin (70) deduced the presence of pectic substances in diatoms on the basis of characteristic staining reactions of the cell wall. The same conclusion was reached by Liebisch (71) after staining the membranes with a weak solution of methylene blue. The presence of fucosan in the cells of the marine diatom Licmophora Lyngbyi Kut. was established by Chadefaud (72) who stained the cells with vanillin hydrochloride. The presence of cellulose in the

cells of diatoms has been postulated by Whistler and Smart (73). Barashkov (69) has investigated alcohol and water soluble carbohydrates of a mixture of diatom species. Complete removal of carbohydrate after ten extractions with water at 40°C was reported. No structural investigations of any polysaccharide material was attempted. Only reducing sugars were detected by paper chromatography. Two oligosaccharides were isolated, and from comparison of spot intensities of hydrolysed and unhydrolysed material, they were reported to consist of two and three glucose units respectively. Chromatographic investigation of the aqueous hydrolysate showed spots with mobilities of galactose, glucose, arabinose, xylose, ribose, and rhamnose. The absence of uronic acid, sucrose, insoluble hemicellulose and cellulose was observed.

LEUCOSIN

The Chrysophyceae (Table 1) comprise a large number of species of planktonic algae of fresh and seawater, characterised by their golden-brown pigmentation. Along with the bacillariophyceae, and Xanthophyceae (Table 1 and Introduction to Part II p.120) they belong to the phylum Chrysophyta. The reserve-food substance synthesised by these golden browns has been termed leucosin by the phycologists. Since the time of Klebs (74) this substance has been regarded as a polysaccharide. However, Pringsheim (75) considers the term 'leucosin' has been applied to very different substances. It has been described as a polyfructose resembling inulin, and a poly-

glucose of the laminarin type. Volutin is a substance found in the cytoplasm of various cells and believed to contribute to the formation of chromatin, a readily stained substance of the cell nucleus. Volutin has been stated to occur in various species of Chlorophyceae, as well as in Diatoms, Euglenophyceae and Myxophyceae (Table 1). Smith (76) has identified the volutin of diatoms with the leucosin of the Chrysophyceae, while Garaudin (77) states that chromatin and leucosin are identical.

An insoluble and a soluble form of leucosin has been isolated from marine diatoms (78), (c.f. laminarin). Von Stosch (79) found that the refractive index of the cell was proportional to its leucosin content, and calculated the content of Schroederella cells of the Meeres plankton to be 20% of the fresh weight.

The difficulty of isolating sufficient leucosin from a pure species of Chrysophyceae has held up systematic structural investigations on this material. Only a few isolated examples of chemical studies on leucosin are available.

Quillet (80) found that leucosin from Hydrurus foetidus had $[\alpha]_D$ -6°, gave no stain with iodine, and only glucose on acid hydrolysis. Partial hydrolysis showed six evenly spaced spots on a paper chromatogram, from which Quillet deduced the polysaccharide contained at least eight anhydroglucose units. In view of the ambiguity of the term leucosin and of its chemical similarity to laminarin, he suggested the name chrysolaminarin for this material.

It is of interest to note that a leucosin type of polysaccharide has been extracted from the flagellated protozoon Ochromonas malhamensis. Structural investigations on this polysaccharide (81) have indicated a resemblance to laminarin in the presence of β -1,3' linked glucose units. A small proportion of other linkages are also present.

A comparative study of leucosin, (hereafter called chrysolaminarin), isolated from several mixed species of the Chrysophyceae, and laminarin, extracted from L. cloustoni, has been made (82). The major difference is apparently the absence of mannitol residues in chrysolaminarin. There is also a slightly higher degree of branching and in the number of 1,6'- linkages in the main chain.

PHAEODACTYLUM TRICORNUTUM

Phaeodactylum tricornutum is a microscopic marine diatom with relatively unexacting growth requirements, having been found to be tolerant of large changes of pH and temperature (83)(84). Previously confused with Nitzschia closterium var. minutissima, Phaeodactylum is now botanically classed in a genus of which it is the only member. Since the isolation of the original culture (85) the rate of growth in relation to nutritional and environmental conditions has been widely studied. Phaeodactylum was found to be the only specimen of several examined which grew well under tank conditions (86). Interest in growing Phaeodactylum artifically no doubt stems from its being widely used as a food for zooplankton, and because it is apparently a major constituent in the diets of

mussels and oysters (87). Phaeodactylum is pleomorphic, producing ovoid, fusiform, triradiate and cruciform cells (88) of which only the ovoid form possesses a siliceous valve (89). In contrast to most diatoms, Phaeodactylum is reported to have a silica content of only 1%. The presence of chrysolaminarin (leucosin) has been reported from studies on the staining reactions of the cells (91), but it has not previously been investigated chemically.

The sample of Phaeodactylum tricornutum studied was grown under bacteria-free conditions by Mr. M.Droop at the Marine Biological Station in Millport, Isle of Cumbrae, Scotland. The process was slow, (the rate of growth of this sample was 0.24 divisions per day) and the yield of culture low (630 litres of culture yielded 85g. freeze-dried material including ca 11g. inorganic salts). Hence it was necessary to work on a rather smaller scale than is usually necessary for marine algae.

EXPERIMENTAL SECTION

General Procedures

G.P.1

a) Principal Chromatographic Solvents

A) Ethylacetate - acetic acid - formic acid - water (18:3:1:4)

B) Ethyl Acetate - pyridine - water (10:4:3)

C) Butanol - ethanol - water (40:11:19)

D) Ethyl methyl ketone - water (+ 1% NH_4OH)(10:1)

E) Butanol - Acetic acid - water (2:1:1)

F) Butanol - Acetone - water (5:3:2)

G) Butanol - pyridine - water (6:4:3)

H) Ethyl methyl ketone - acetic acid - water (sat.with boric acid) (9:1:1)

b) The following chromatographic sprays were used, where appropriate, to develop chromatograms, which were run, unless otherwise stated, on Whatman No.1 paper, with control sugars, and air dried.

1) A saturated aqueous solution of aniline oxalate followed by heating at 120°C for ca five minutes.

2) A 5% solution of p-anisidine hydrochloride in butanol, followed by heating at 120°C for ca five minutes.

3) A 10% solution of silver nitrate (1 ml) diluted with acetone (100 ml) used as a dip (92). The silver nitrate impregnated chromato-

grams were dried in air and dipped in an alcoholic solution of sodium hydroxide (0.5M). This latter reagent was prepared by first dissolving the sodium hydroxide in the minimum water, and making up to the required volume with either ethanol or methanol. The background colour of the chromatograms was removed by immersion in 0.1M sodium thiosulphate.

4) A 2% aqueous solution of sodium metaperiodate + 1% potassium permanganate in 2% sodium carbonate (4:1)(93). The chromatograms were allowed to dry at room temperature, the spots appearing, usually after ca 15 minutes, as yellow blobs on a purple background. The papers were preserved by washing with water.
5) Bromocresol green (0.1% solution in 95% ethanol made just alkaline with sodium hydroxide).
6) Bromocresol purple (0.1% solution in 95% ethanol made just alkaline with sodium hydroxide).

The following abbreviations have been used to describe the distances travelled by sugars on paper chromatography and ionophoresis :

$$Rg = \frac{\text{distance travelled by the sugar.}}{\text{distance travelled by glucose.}}$$

$$R_G = \frac{\text{distance travelled by the sugar.}}{\text{distance travelled by tetra-O-methyl glucose.}}$$

$$R_F = \frac{\text{distance travelled by the sugar.}}{\text{distance travelled by the solvent front.}}$$

$$M_G = \frac{\text{distance between sugar and tetra-O-methyl glucose.}}{\text{distance between glucose and tetra-O-methyl glucose.}}$$

$$M_{GA} = \frac{\text{distance between sugar and glucose.}}{\text{distance between glucuronic acid and glucose.}}$$

G.P.2

Ionophoresis

Principal buffers used were:

a) Borate at pH 10

b) Phosphate at pH 7.2

c) Molybdate, 1.5%, pH 5

Separation was effected on either Whatman No.1 or No.3 paper. The ionophoretograms were developed with either aniline oxalate in 5% acetic acid, or the silver nitrate reagent.

G.P.3

Acid Hydrolysis. Complete acid hydrolysis was carried out by heating the polysaccharide with N sulphuric acid at 100°C for four hours.

G.P.4

Dialysis of polysaccharide solutions was carried out in cellophane tubes against running water (ca 3 days), with the addition of a few drops of toluene to prevent bacterial contamination.

G.P.5

Optical rotations were measured in water in a 1 dm. tube at room temperature.

G.P.6

Moisture content of polysaccharides was obtained by drying in a vacuum ~~desiccator~~ over phosphorus pentoxide at 60°C.

G.P.7

Sulphate ester

a) Qualitative detection of sulphate ester was achieved using

azur II and toluidine blue reagents (94). The former reagent was prepared by dissolving azur II (0.1 g.) in water (25 ml.) and mixing with acetone (200 ml.) and methanol (900 ml.). The latter reagent consisted of a near saturated solution of toluidine blue in ethanol. Polysaccharide containing sulphate was spotted as a 2% solution on filter paper which was then immersed in the reagent for 5 minutes followed by washing with aqueous ethanol (40%). Presence of sulphate is indicated by a dark-blue colour.

b) Quantitative estimations of sulphate were carried out by digesting the carbohydrate overnight with Analar nitric acid, in a sealed tube, on a boiling water bath. The tube was opened, sodium chloride (1 mg.) added and the solution cautiously evaporated on a bunsen flame. The residue was further heated in an oven at 130°C for one hour. After cooling, the residue was dissolved in a known volume of water, and the sulphate content determined by the micro-colorimetric method of Jones and Letham (95).

G.P. 8

Concentration of solutions was effected, unless otherwise stated, under reduced pressure, at 40°C.

G.P. 9

Ash content was determined by igniting polysaccharide material (ca 50 mg.) in a platinum crucible until constant weight was obtained.

G.P. 10

Nitrogen content was determined by a semi-micro modification of the Kjeldahl method (96).

G.P.11

Reducing Sugar Content:

a) Cuprimetrically, using the method of Somogyi (97)

Aliquots of hydrolysed polysaccharide solution were made up to 5 ml. Somogyi reagent (5 ml.) was added, and the solution heated on a boiling water bath for ca 20 minutes. The amount of copper sulphate reduced to cuprous oxide was determined by titration with $\frac{N}{100}$ sodium thiosulphate, using starch as indicator. This titre was, with the use of previously prepared standard graphs for the appropriate monosaccharide, directly convertible into an equivalent weight of carbohydrate.

b) The Phenol-Sulphuric Acid Method (98)

Aqueous sugar solution containing 10-100 µg. was pipetted into test tubes (6 x $\frac{5}{8}$") and diluted to 1 ml. with water. Phenol solution (4%, 1 ml.) was added to each tube, followed by Analar sulphuric acid (5 ml.) from a fast delivery pipette, the stream of acid being directed on to the solution surface to obtain a maximum rise in temperature. When cool, the optical density of the solution was measured on a Unicam Spectrophotometer SP 500 against a blank, prepared by using water in place of the sugar solution, at 487 mµ.

A standard graph was obtained by plotting µg sugar/ml. against the optical density (E).

G.P.12

Gas-liquid partition chromatography

This was carried out on a "Pye Argon Chromatograph"

using argon as the mobile gas phase. The methyl glycosides from the total methanolysis of the glucan (Part 1(a)) were kindly analysed by Dr. Aspinall. The stationary liquid phase was supported on celite and consisted of :

(a) Butanediolsuccinate ester, or

(b) Polyphenol ether

(The operating temperature for (a) was 175°C and for (b)200°C). The retention times "T" of the methyl ether methyl glycosides were quoted relative to that of methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside.

G.P. 13

Methanolysis was carried out by refluxing the sugars with dry methanolic hydrogen chloride (3%) for ca 6 hrs. (i.e. till the rotation of the solution attained a constant value). The solution was neutralised with silver carbonate, filtered, the silver salts washed thoroughly with dry methanol, and the combined filtrate and washings were concentrated to a syrup.

G.P. 14

Demethylation (99)(100)

The sugar sample in 2 ml. of dry dichloromethane was cooled to -80°C. Boron trichloride (1-2g) cooled to -80°C was added and the mixture kept at -80°C under anhydrous conditions for thirty minutes. The solution was then allowed to warm to room temperature and allowed to stand under anhydrous conditions for sixteen hours. The remaining solvent was removed under vacuum and methanol (3 x 3 ml.) was added and evaporated. The syrup was examined chromatographically.

G.P.15

Uronic acid

(a) Qualitative detection (101). Uronic acid was detected by boiling the substance (2 mg.) and naphthoresorcinol (5-10 mg.) with concentrated hydrochloric acid for a few minutes. After cooling, the solution is extracted with benzene or ether, a blue-purple colour in the upper organic layer indicating the presence of uronic acid.

(b) Quantitatively, uronic acid was determined by the decarboxylation method of Axel, et al.(102).

PART I(a)

Experimental

Preliminary Experiments

Experiment 1

Total Hydrolysis of the Organism

P.tricornutum organisms (200 mg.) were completely hydrolysed. The residue,filtered off, and washed, was rehydrolysed but the second hydrolysate failed to give any positive test for reducing sugars (97). The initial hydrolysate was neutralised with Analar barium carbonate, filtered, treated with Amberlite IR 120(H+) resin, and made up to 10 ml. with water. This solution was then divided into two parts, (a) and (b) of 5 ml. each.

(i)Solution (a)

After concentration to a syrup, the products were examined chromatographically (Solvents A and B, spray 1)). Spots with mobilities of xylose, mannose, glucose, were observed, in addition to incompletely hydrolysed material near the base line in both solvents. Traces with the speeds of ribose and rhamnose were also observed.

(ii)Solution (b)

This solution was quantitatively analysed for reducing sugar content by the method of Somogyi (97). A value of ca 20% of the dry weight of organism was obtained.

Experiment 2

Extraction with cold water

The organism (100 mg.) was directly extracted with water

(*ca* 5 ml.) at room temperature for five days. The residual organism was filtered, washed, and the combined filtrate and washings made up to 10 ml. The solution was analysed for reducing sugars (97), and gave a value of 2.3% with respect to the original weight of organism. Chromatographic investigation (solvent A, spray 1)) revealed a spot with the mobility of glucose. Two slower spots were also observed in addition to material on the base line of the chromatogram.

Hydrolysis of the cold water extraction

The above solution (2 ml.) was hydrolysed completely, neutralised with sodium hydroxide, and analysed for reducing sugars (97). Based on the original weight of dry organism a value of 9.5% for the quantity of sugars present was obtained. Chromatography (solvents A and H, sprays 1 , and 4), revealed spots with mobilities of glucose (strong) and mannose, xylose and ribose (traces). There was also traces of material running just behind glucose. No hexitols could be detected.

Experiment 3

Hydrolysis of the residual organism

The residual organism from the cold water extractions was completely hydrolysed. Analysis for reducing sugars revealed a further quantity corresponding to 8.1% by weight of the starting material.

Chromatographic investigations (solvent A and H, sprays 1, and 4), revealed spots with mobilities of glucose, mannose (strong), and xylose (trace) with traces of material just slower than glucose. No hexitols could be detected.

Experiment 4

Decolourisation Experiments

As a preliminary treatment, the cells (1 g.) were frozen in liquid nitrogen, followed by grinding to a fine powder.

(i) Repeated extractions with ether failed to remove any significant quantity of colouring matter.

(ii) Acetone, as a decolourising agent was slightly better than ether, but still too inefficient to be considered for large scale extractions.

(iii) When the cells were stirred overnight in a mixture of acetone and dimethylsulphoxide (1:3) almost complete decolourisation was achieved. The coloured solution gave a small reducing power with Fehling's solution, but addition of more acetone failed to precipitate any significant amount of material.

The residual cells, centrifuged off, were further extracted with cold water, then with hot water.

(a) Cold water extraction

The decolourised organism was stirred overnight with water (200 ml.) at room temperature. After centrifuging and washing the residue, the solution was allowed to stand for several hours at 2°C. A white precipitate (ca 30 mg.) was formed, which after hydrolysis had a reducing power of 15%. Treatment with ethanol (5 volumes) gave a further precipitate of similar quantity and carbohydrate content.

(b) Hot water extraction

The residual cells, suspended in water (200 ml.),

were heated on a boiling water-bath for six hours in an atmosphere of nitrogen. After centrifuging, the solution again gave an insoluble white precipitate on standing at 2°C. The carbohydrate content was negligible. A slight precipitate was produced on further treatment of the solution with ethanol (5 volumes) which also had a negligible carbohydrate content. Hence it would appear that the decolourising solvents had removed all the water soluble carbohydrates.

(c) Hydrolysis of residual material

A portion of the residual organism (ca 20 mg.) was hydrolysed completely. After neutralisation and concentration to a syrup, the hydrolysate was examined chromatographically (solvent A, spray 1)). A heavy spot with mobility of mannose was detected, along with spots of lesser intensities at the levels of xylose and glucose. Material just behind glucose, and apparently incompletely hydrolysed material near the starting line of the chromatogram were also present.

(d) Mild Chlorite Extraction (103)

The residual material from the hot water extraction in aqueous acetic acid (0.2 ml. glacial acetic acid in 50 ml. water) was treated with solid sodium chlorite (2 x 0.25 g.) added at hourly intervals, at 70°C. This treatment bleached the cell material. The mixture was centrifuged, and the solid matter washed thoroughly with water. The centrifugate and washings were treated with ethanol (5 volumes). A white precipitate (ca 15 mg.) was formed, which on hydrolysis was found to have a very low reducing power.

(e) Dilute Alkali Extraction

The bleached residue was stirred with 4% sodium hydroxide solution (25 ml.) under an atmosphere of nitrogen for fifteen minutes. Undissolved material was centrifuged down, and reextracted overnight under the same conditions.

The solutions from both alkaline extractions were brought to pH 6 with acetic acid. White precipitates in both cases were deposited which on hydrolysis had negligible reducing powers. Treatment of the supernatants with ethanol (5 volumes) gave more alcohol insoluble material, again with negligible reducing powers after hydrolysis.

Hydrolysis of the slight residual material (ca 20 mg.) and chromatographic investigation showed a strong spot with the mobility of mannose together with traces at the levels of xylose and glucose and some slower substances.

(iv) n-Butanol saturated with water

Since it was very difficult to recover material from dimethylsulphoxide, another decolourising system, butanol saturated with water, was investigated. P. tricornutum organisms (10 g.) were stirred with the decolourising agent (200 ml.) for two hours. The resulting dark green solution was decanted and replaced by fresh solvent. After stirring overnight, the green liquid was again decanted. A third treatment removed most of the residual colour from the cells. The cells were centrifuged to remove as much as possible of the aqueous butanol, before washing with a little fresh solvent.

Examination of the Aqueous Butanol

The dark green solution was vigorously stirred with an equal volume of water. Two layers were allowed to separate overnight. The upper (butanol) phase, after concentration, was found to contain negligible carbohydrate material. The lower phase (aqueous) was very green and contained carbohydrate. Chromatography (solvent A, spray 1)) revealed a spot just ahead of glucose (faint), one at glucose level and some higher molecular weight material near the base line. Hydrolysis and chromatography in the same solvent gave a similar chromatogram but with the intensity of the spot at the glucose level increased, and a faint pink spot with the mobility of xylose.

(a) Cold water extraction of residual organisms

The decolourised cells were exhaustively extracted with water (3 x 150 ml.) for a total of twenty four hours. The cells were centrifuged down, and the supernatant concentrated and dialysed against a closed system. The solution inside the dialysis bag was treated with ethanol (ten volumes) producing an off-white, flocculent precipitate which was centrifuged down, dried, and freeze-dried. (Yield 560 mg.)

Examination of the alcohol precipitated material

Hydrolysis of an aliquot of the freeze-dried material and estimation of reducing sugars gave a carbohydrate content of 60%. Chromatographic analysis (solvent A, spray 1)) revealed spots with mobilities glucose (very strong) and xylose, mannose and rhamnose (traces).

Examination of the dialysate

The dialysate after freeze-drying weighed 1.62 g., and on hydrolysis had a reducing power of 20%. Chromatographic investigation (solvent A, spray 1)) of the dialysate revealed a strong spot with the mobility of glucose along with much slower moving material. After hydrolysis the intensity of the spot at the level of glucose increased, but there was still some material near the base line of the chromatogram, though apparently reduced in quantity.

(b) Hot water extraction

The organism from the cold water extraction was stirred with water (250 ml.) in an atmosphere of nitrogen, on a boiling water bath for five hours. After centrifuging, the solution was concentrated and dialysed against a closed system. The solution inside the dialysis tube was treated with ethanol (10 vols.) giving a white precipitate, which was centrifuged down, dried, and freeze-dried (Yield 410 mg.). The freeze-dried precipitate was found to contain 31% by weight of reducing sugars. Chromatographic examination of the hydrolysate gave a similar pattern to the cold water extracted material.

Examination of the dialysate

Freeze-dried material (620 mg.) was obtained from the dialysate, containing 13% by weight of reducing sugars. Chromatographic investigation of the material hydrolysed and unhydrolysed, revealed the same pattern as that obtained in the cold water extraction.

(c) Sodium Chlorite extraction

The hot water-extracted residual material in aqueous acetic acid (1 ml. glacial acetic acid in 250 ml. water) was heated at 70° and solid sodium chlorite (2 x 0.5g.) was added at hourly intervals. The bleached residue was centrifuged down, and the supernatant dialysed, concentrated, and treated with ethanol (10 volumes). A white precipitate was isolated (140 mg.), of which 18% was found to be carbohydrate material. Chromatographic analysis revealed much the same pattern as found from the hydrolysate of the cold water extracted polysaccharide, except for an increase in the intensity of the spot with the mobility of mannose.

(d) Dilute Alkali extraction

The residual material from the chlorite extraction was stirred in 4% sodium hydroxide (100 ml.) in an atmosphere of nitrogen for fifteen minutes. Undissolved residue was centrifuged down and treated with a fresh quantity of sodium hydroxide, still under nitrogen, for twenty four hours. The residual material was again centrifuged, and both supernatant solutions were dialysed against running water. Addition of glacial acetic acid to each of the dialysed solutions to pH 5 gave white precipitates, (0.49g. and 0.27g. respectively) each with a negligible carbohydrate content. Further precipitation was brought about with ethanol (10 volumes). The derived white materials (0.69g. and 0.10g.) also contained very little carbohydrate.

(e) Residual material after alkali extraction

After washing, the material was suspended in water and freeze-dried to a white amorphous solid (1.79g.), with an estimated content of 18% carbohydrate, after hydrolysis (97). Chromatographic investigation of the hydrolysate revealed a very strong spot with the mobility of mannose with a faint spot at the level of glucose in addition to incompletely hydrolysed material.

Attempted extraction of residual carbohydrate with 20% Sodium Hydroxide at room temperature

The dilute alkali extracted residue was stirred with 20% sodium hydroxide (50 ml.) for twenty four hours under an atmosphere of nitrogen. The undissolved residue, apparently little reduced in quantity, was centrifuged down, and the supernatant dialysed against running water. The dialysed solution was brought to pH 5 with glacial acetic acid giving a white deposit (100 mg.) of negligible carbohydrate content. Treatment of the solution with ethanol (10 volumes) failed to produce any further precipitation.

(f) Extraction with 20% Sodium Hydroxide on boiling water

Heating the residual material with 20% sodium hydroxide in an atmosphere of nitrogen on a boiling water bath caused almost complete dissolution of the solid. The slight residue was removed, and the solution dialysed against running water. Addition of glacial acetic acid failed to produce a precipitate. Treatment with alcohol (10 volumes) caused a precipitate to form which on freeze-drying weighed 170 mg. and had a reducing

sugar content of 20% (97). Chromatographic investigation revealed exactly the same pattern as that given by the hydrolysed material after dilute alkali extraction ((e)p.43)

(v) Decolourisation with pure n-butanol

In an attempt to reduce loss of carbohydrate material without loss of decolourising efficiency, butanol saturated with water was replaced by n-butanol itself.

P. tricornutum organisms (10g.) were stirred with n-butanol (250 ml.). After one hour the very dark green solution was decanted off and replaced with fresh solvent (250 ml.). More colour was removed by this treatment, and after four hours a third extraction overnight gave a butanol solution of a lighter green than the first two treatments. Three fresh lots of butanol were added at twenty four hourly intervals, the last of which was barely coloured after being in contact with the organisms for twenty four hours. The Phaeodactylum cells were not completely decolourised, but the butanol extracts, on examination, revealed very little carbohydrate.

(a) Cold water extraction

Exhaustive treatment of the decolourised organisms with water (6 x 200 ml.) over a period of three days at room temperature, followed by concentration of the extract and treatment with ethanol (10 volumes), produced a flocculent precipitate which, on freeze-drying, weighed 1.43g., and had N_2 1.95%, SO_4 ester nil., uronic acid negative (101), ash 4.2%.

Analysis for reducing sugars gave a carbohydrate content of 55%. Chromatographic analysis revealed spots with

mobilities of glucose (very strong), xylose, mannose and rhamnose (faint).

Examination of the supernatant

The alcoholic supernatant was concentrated to dryness, taken up in water, then freeze-dried to a green, rather gummy solid, weighing 2.01g. The carbohydrate content and chromatographic pattern was the same as the dialysate of the previous extraction (p.41).

(b) Hot water extraction

As before, see page 41. Yield of alcohol precipitated material was ca 460 mg., with the same carbohydrate content and chromatographic pattern.

(c) Treatment with sodium chlorite

As before, see page 42. Carbohydrate content of the alcoholic precipitate (130 mg.) was, this time, negligible. Further investigations of this material revealed a nitrogen content of ca 5% (96), and amino acids were qualitatively detected in the hydrolysate (solvent C, spray ninhydrin).

(d) Dilute alkali extraction at room temperature

The bleached residual material was stirred for twenty four hours, in an atmosphere of nitrogen, with 4% sodium hydroxide solution (200 ml.). As before, no carbohydrate material was detected in this extract.

(e) Hot dilute alkali extraction

When the residue from the previous treatment was heated on a boiling water bath with 4% sodium hydroxide, in an atmosphere of nitrogen, almost complete dissolution of the

material occurred. Neutralisation with glacial acetic acid did not produce any precipitate. The solution was dialysed, concentrated, and treated with ethanol (10 volumes). This led to the isolation of polysaccharide material (ca 450 mg. freeze-dried).

The Glucan

Experiment 5

Attempted Purification Experiments

a) Reprecipitation

Polysaccharide (126 mg., 55% carbohydrate, (p. 44)) was dissolved in water (ca 10 ml.), centrifuged and then treated with ethanol (10 volumes). The alcohol insoluble material was centrifuged down, dried and freeze-dried to give polysaccharide material (91 mg.) with a carbohydrate content of 62% (97).

b) Acidification

Treatment of a solution of the polysaccharide (1%, 10 ml.) with glacial acetic acid to pH4-5 failed to precipitate any non-carbohydrate material.

c) Extraction with toluene

Polysaccharide (85 mg.,55% carbohydrate) dissolved in 0.1N sodium chloride solution (15 ml.) was shaken with toluene (ca 10 ml.) for several hours. The lower aqueous layer was separated, dialysed and freeze-dried, yield 72 mg. containing 54% carbohydrate (97)(98).

d) Extraction with phenol

Polysaccharide (1.4g.,55% carbohydrate), dissolved in

water (35 ml.), was added to a solution of phenol (31.5g.) in water (35 ml.). The mixture was shaken for half an hour before placing in the refrigerator to allow the two layers to separate (ca 24 hrs.). The phenol layer was discarded, and the procedure repeated twice. Emulsion between the two phases was removed by warming with butyric acid. Traces of phenol contaminating the aqueous layer were extracted with ether. Polysaccharide material was finally freeze-dried and isolated as a white solid (0.65g.) with a carbohydrate content of 55% (98).

e) Fractionation on D.E.A.E.-cellulose (104)(105)

Preparation of the column

Diethylaminoethylcellulose (D.E.A.E.-cellulose)(55 g.) was made into a slurry with distilled water and poured into a glass column (diameter 1.6 inches). Water was continuously passed down the column while the modified cellulose settled under gravity. The column was then washed alternately with 0.5N NaOH (1ℓ) and 0.5N HCl (1ℓ), three times with each; the column being neutralised with water between each application of alkali and acid. Potassium chloride solution (0.5N, 500 ml.) was run through, finally, converting the column to the chloride form. After washing off excess chloride with water, the column was ready for use.

Fractionation of the glucan

The glucan (950 mg.), dissolved in a minimum of water, was applied to the top of the column and allowed to gradually sink in. The column was eluted with a gradient

system starting with distilled water, with gradual increase in the concentration of potassium chloride up to 0.5N. Eluent was collected in twenty-five ml. fractions at ca 50 ml. per hour. When no more carbohydrate material was eluted by the neutral eluent, a second gradient system of 0.5N KCl-0.5N KOH was set up. This alkaline solvent failed to elute any further carbohydrate.

Polysaccharide material was detected by the phenol-sulphuric acid method (98). The contents of the appropriate tubes were combined, concentrated, dialysed and freeze-dried. The main fraction (tubes 10-17) weighed 340 mg., had a carbohydrate content of 90% (98), and gave only glucose on hydrolysis. Analyses for ester sulphate (94)(95) and nitrogen (96) were negative and the polymer had $[\alpha]_D$ + 42° (c,0.58). The polysaccharide gave no colour with iodine.

A minor fraction (25 mg.)(tubes 18-26) was also isolated in a similar manner. Chromatographic investigation of the hydrolysed material revealed the presence of glucose with appreciable proportions of xylose and mannose, and the material had a reducing sugar content of 65% (based on glucose).

Experiment 6

Characterisation of the Glucose from the Purified Glucan

The hydrolysed polysaccharide (ca 5 mg.) was incubated in water (ca 0.5 ml.) with the enzyme glucose oxidase at 35°C for 24 hours. Chromatographic examination, (solvents A and B, sprays 3 and 6) and ionophoresis (phosphate buffer, spray 3)), revealed that the glucose had been completely converted

to gluconic acid. An authentic sample of gluconic acid was run as control.

Experiment 7

Oxidation with Sodium metaperiodate (106)

The pure glucan was oxidised with various concentrations of sodium metaperiodate at both room temperature and at 2°C. Simultaneous experiments were made with laminarin under the same conditions. All experiments were carried out in acetate buffer of pH 3.7, and in the dark. The amount of periodate reduced was measured by removing an aliquot of the reaction mixture and diluting to an appropriate volume with water to give a reasonable reading for the optical density (E). Readings were measured at 223 mμ. on a Unicam SP500 spectrophotometer against a water blank. The fraction of periodate reduced at any particular time was obtained knowing the difference in absorbance between the original sodium metaperiodate solution and a sodium iodate solution of the same molarity, under the same conditions.

(a) Oxidation with 0.1M Sodium metaperiodate

Phaeodactylum glucan (38.3 mg.) was oxidised with 0.1M sodium metaperiodate (10 ml.) at room temperature. Aliquots (0.1 ml.) were removed, and diluted to 100 ml. with water before measuring their optical densities. Reduction of periodate occurred as shown on page 50 (Table 2).

Table 2

Time (hrs.)	Moles IO_4 reduced C_6 anhydro unit
6	0.02
24	0.19
56	0.38
104	0.46
127	0.54
143	0.54

(b) Oxidation with 0.05M Sodium metaperiodate

Phaeodactylum glucan (19.9 mg.) was oxidised with 0.05M sodium metaperiodate (10 ml.) at i)2°C and ii) room temperature, in the dark. Aliquots (0.1ml.) were removed, diluted to 100 ml. with water, and their optical densities measured.

Table 3

i) Oxidation at 2°C

Time (hrs.)	Moles IO_4 reduced per C_6 anhydro unit
6	0.00
24	0.12
48	0.20
96	0.23
300	0.28

ii) Oxidation at room temperature

Time (hrs.)	Moles IO_4 reduced C_6 anhydro unit
6	0.00
24	0.20
48	0.28
96	0.47
300	0.58

(c) Oxidation with 0.015M Sodium metaperiodate

Phaeodactylum glucan (18.2 mg.) and laminarin (22.7 mg.) were oxidised with 0.015M sodium metaperiodate (20 ml.) at 2°C for two days. The reaction solutions were allowed to attain room temperature and the oxidation to proceed for a further four days. Aliquots (1 ml.) were removed and diluted to 250 ml. with water before measuring their optical densities.

Table 4

Phaeodactylum glucan		Laminarin	
Time (days)	Moles IO_4 reduced per C_6 anhydro unit	Time (days)	Moles IO_4 reduced per C_6 anhydro unit
2°C		2°C	
1	0.211	1	0.217
2	0.225	2	0.228
Room temp.		Room temp.	
3	0.277	3	0.358
4	0.416	4	0.389
5	0.484	5	0.389
6	0.526	6	0.389
7	0.538		

(d) Oxidation with 0.0015M sodium metaperiodate

Phaeodactylum glucan (21.3 mg.) and laminarin (20.9 mg.) were oxidised with 0.0015M sodium metaperiodate (20 ml.) at 2°C for two days, after which oxidation was allowed to continue at room temperature for four days. Aliquots (1 ml.) were removed, diluted to 25 ml. and the

optical densities measured.

Table 5

Phaeodactylum glucan		Laminarin	
Time (days)	Moles IO_4 reduced per C_6 anhydro unit	Time (days)	Moles IO_4 reduced per C_6 anhydro unit
2°C		2°C	
1	0.068	1	0.154
2	0.094	2	0.171
Room temp.		Room temp.	
3	0.147	3	0.206
4	0.172	4	0.213

Experiment 8

Partial acid hydrolysis of the glucan

Phaeodactylum glucan (ca 5 mg.) and laminarin (ca 5 mg.) were hydrolysed with 0.1N sulphuric acid at 100°C for 1 hour. After cooling, the acid solutions were neutralised with barium carbonate, filtered, treated with IR 120(H^+)resin, and evaporated to dryness.

Chromatographic examination of the residues, (solvent A, spray 1) , revealed the following spots from both polysaccharides: Rg 0.53, 0.38, 0.30 and traces of slower material Rg ca 0.22. Laminaribiose, gentiobiose and laminaritriose, run as controls, had Rg 0.53, 0.38 and 0.30 respectively.

Experiment 9

Enzymic Hydrolysis with β 1-3 Glucanase

The glucan (2 mg.) and laminarin (2 mg.) were suspended or dissolved in citrate buffer pH4.5 (ca 0.1 ml.). The enzyme

(ca 0.5 mg.) was added to the polysaccharide solutions which were then kept at 38°C. Chromatographic investigations (solvent A, spray 1) revealed the same pattern from both polymers. Spots with Rg 0.53, 0.38, 0.30 were the main components, matching exactly the mobilities of laminaribiose, gentiobiose and laminaritriose which were run as controls. Prolonged incubation (72 hrs.) with the enzyme removed all oligosaccharides except Rg 0.38, the one corresponding to gentiobiose.

Experiment 10

Methylation of the glucan

The glucan (200 mg.) was stirred overnight with sodium borohydride, (20 ml.,1%) at room temperature. Sodium hydroxide (20 ml.,30%) and dimethyl sulphate (8 ml.) were added over six hours with vigorous stirring at 2°C., in an atmosphere of nitrogen. When all the reactants had been added, stirring was continued overnight at room temperature. The procedure of adding sodium hydroxide and dimethyl sulphate, followed by stirring overnight was repeated another four times. After dialysing, the methylated material was freeze-dried to a white solid (250 mg.). Acid hydrolysis and chromatographic investigation, (solvent D, spray 1), revealed spots with the mobilities of 2,3,4,6 tetra-O-methylglucose, 2,4,6 tri-O-methylglucose, di-O-methylglucose and monomethylglucose.

A second series of five methylations using sodium hydroxide and dimethyl sulphate led to the isolation of freeze-dried solid (223 mg.) with a methoxyl content of

31.5% (107)(108). Infra-red analysis revealed the presence of a small amount of unmethylated hydroxyl groups.

The partially methylated material was dissolved as far as possible in re-distilled methyl iodide (10 ml.). Silver oxide (1.5g.) was added in eight half-hourly portions while the methyl iodide was gently refluxing (ca. 45°C). The mixture was heated under gentle reflux overnight, filtered, and the residue extracted exhaustively with hot chloroform. The filtrate and chloroform solution were taken to dryness. The solid residue was methylated three more times with methyl iodide and silver oxide as described above. The methylated polysaccharide was isolated by freeze-drying an aqueous suspension to give 51 mg. of material. Infra-red analysis indicated that there were no free hydroxyl groups present.

Experiment 11

Examination of the Methylated Glucan by Gas Chromatography

The methylated glucan (ca 30 mg.) was methanolised in the usual way (G.P. 13). Analysis of the derived methyl glycosides of the methylated sugars indicated the following sugar derivatives :-

Methyl 2,3,4,6 tetra-O-methyl glucoside.

Methyl 2,4,6 tri-O-methyl glucoside.

Experiment 12

Hydrolysis and Examination of the Methylated Sugars

The methyl glycosides were hydrolysed with NH_2SO_4 at 100°C for six hours. The cooled acid solution was neutralised with barium carbonate, filtered, treated with IR120(H^+) resin

and concentrated to small volume (ca 0.5 ml.). The mixture of methylated sugars was applied to two sheets of Whatman 3MM paper. After elution with solvent C for ca 15 hrs. the individual methylated sugars were located by developing side strips of the chromatograms (spray 1).

Fraction 1: 2,4,6 Tri-O-methyl glucose crystals (ca 3 mg.). Chromatographically identical (solvents C and D, spray 1) with authentic material run as control. Its melting point was 122-123°C, and a mixed melting point with the authentic compound gave no depression.

Fraction 2. Trace of syrup with mobility of a dimethyl-glucose. In borate buffer, pH10.2, ionophoresis identified the material as 2,4 di-O-methylglucose, an authentic sample being run as control. Demethylation (100) and chromatographic analysis revealed only glucose.

Experiment 13

Smith Degradation studies (109)

(i) Periodate oxidation

Phaeodactylum glucan (57.5 mg.) was oxidised with sodium metaperiodate (168.5 mg.) in the dark, in acetate buffer (5 ml.) at pH6 for 110 hours. Unreduced sodium periodate was destroyed by bubbling sulphur dioxide gas through the solution for several minutes. The oxopolysaccharide was isolated by precipitation with ethanol (10 vols.).

(ii) Reduction with sodium borohydride

The oxopolysaccharide was dissolved in boric acid buffer (110) (3 ml.,0.05M). Sodium borohydride (300 mg., in

ca 2 ml. water) was added to the buffered solution, and the reaction was allowed to take place at room temperature overnight. Excess sodium borohydride was decomposed by bringing the solution to pH7 with glacial acetic acid. After dialysis against running water, the reduced solution was evaporated nearly to dryness several times with methanol.

(iii) Mild Hydrolysis

The polyalcohol from (ii) was treated with N-sulphuric acid (ca 2 ml.) at room temperature for 48 hours. Addition of ethanol (10 volumes) led to the isolation, by freeze-drying, of polysaccharide material (30 mg.). The supernatant liquid was neutralised with barium carbonate, filtered, and concentrated.

Examination of the degraded polymer

Chromatographic analysis of the hydrolysate of the degraded material revealed only glucose.

Examination of the supernatant liquid

Chromatographic investigation (solvent B, spray 3) revealed only a trace of material with the mobility of glycerol.

DISCUSSION

The *Phaeodactylum tricornutum* organisms used in this investigation were grown under bacteria-free conditions by Mr. M. Droop at the Marine Biological Station in Millport, Isle of Cumbrae, Scotland. The culture medium used (S71) was similar to S68 employed in the cultivation of the dinoflagellate *Oxyrrhis marina* (111). The major differences were the nitrogen source (potassium nitrate instead of L-valine) and the carbon source, (atmospheric carbon dioxide instead of anhydrous sodium acetate). The rate of growth of the *P. tricornutum* organisms was 0.24 divisions per day, which is $^1/_5$ of the maximum rate in this medium. The sample investigated was spun down from 630 litres of culture medium and freeze-dried to a dark green coagulated material (84.96 g.), which included *ca* 10.6g. of inorganic salts.

Preliminary investigations (Expt. 1) revealed a carbohydrate content of *ca* 20% of the dry weight of organisms, and sugars chromatographically identified as *xylose*, *mannose*, *glucose* with traces of *rhamnose* and *ribose*. There was also other material with a mobility slower than that of glucose.

Examination of the material extracted by cold water in initial small scale investigations, indicated a presence of free reducing sugars to the extent of 2.3% (Expt. 2). Hydrolysis of the extracted material raised the reducing sugar content to 9.5%. Chromatographic analysis of the unhydrolysed extract showed the presence of free glucose along with two spots with slower chromatographic mobilities. Hydrolysis apparently

liberated considerably more glucose in addition to much smaller quantities of material with chromatographic mobilities of mannose, xylose and ribose. Mannitol, a constituent of many species of algae, could not be detected.

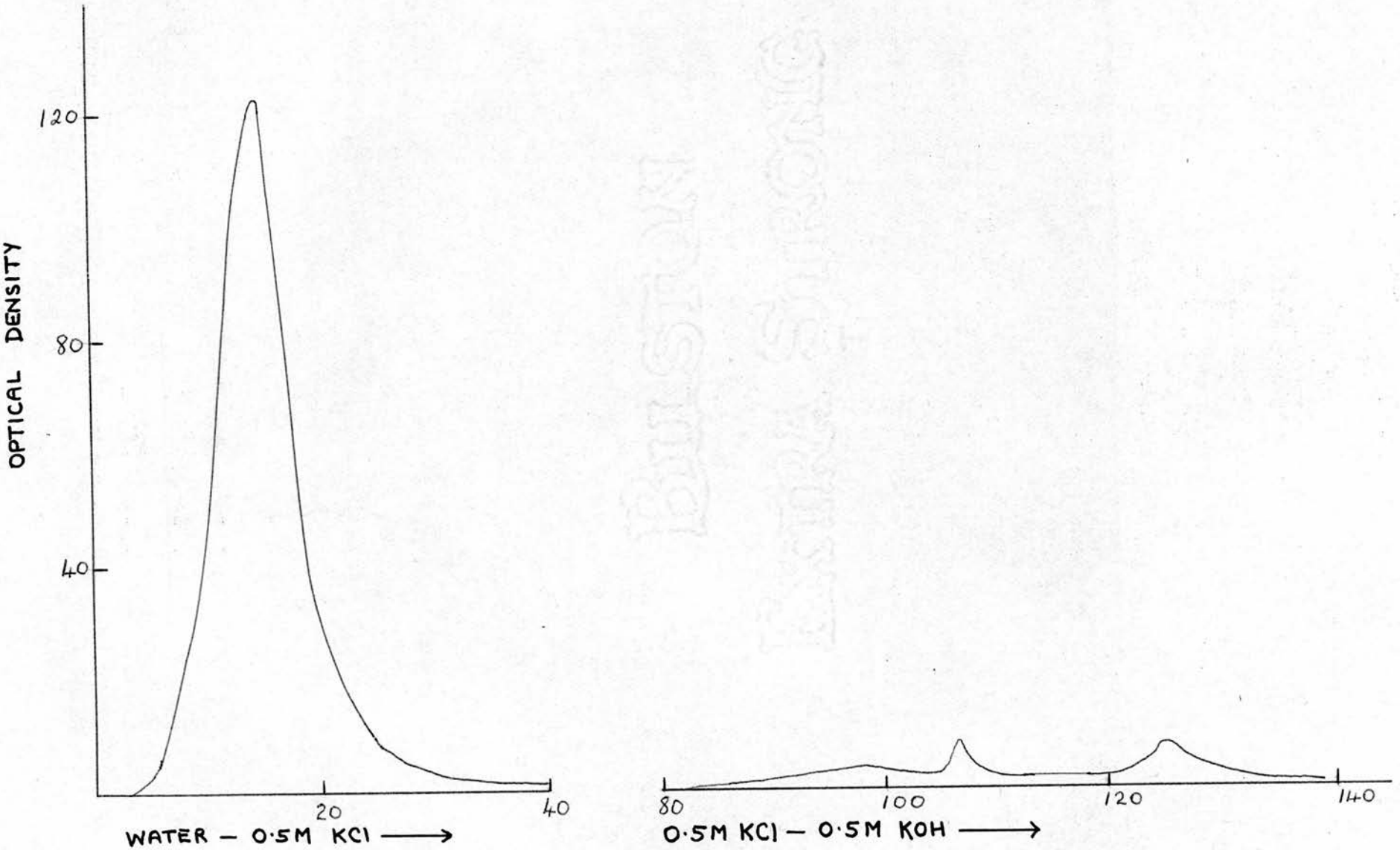
The residual organisms, after aqueous extraction, were hydrolysed and found to contain a further 8.1% of reducing sugars. Chromatographic investigations revealed a strong spot with the mobility of mannose, in addition to fainter spots with the speeds of glucose, and xylose, and some apparently unhydrolysed material near the base line (Expt.3).

The presence of a reasonable quantity of carbohydrate material in P.tricornutum organisms was thus established. Before any attempt to extract polysaccaride material was made, it was necessary to find suitable methods of preparing the cells for the most favourable release of carbohydrate. This necessitated rupturing the cell walls and preliminary removal of the colouring matter, the latter to avoid subsequent contamination of the carbohydrate. A number of techniques was investigated and it was found that the most satisfactory was by freezing the organism in liquid nitrogen, followed by thorough grinding of the frozen material. After several attempts at decolourisation (Expt.4) using ether, acetone, acetone mixed with dimethyl sulphoxide (1:3), and n-butanol saturated with water, the most suitable solvent was found to be n-butanol (Expt.4(v)p.44). While this did not completely decolourise the organism, it had the advantage that it removed only a negligible amount of carbohydrate material.

GRAPH 1

FRACTIONATION OF THE GLUCAN

ON DEAE-CELLULOSE

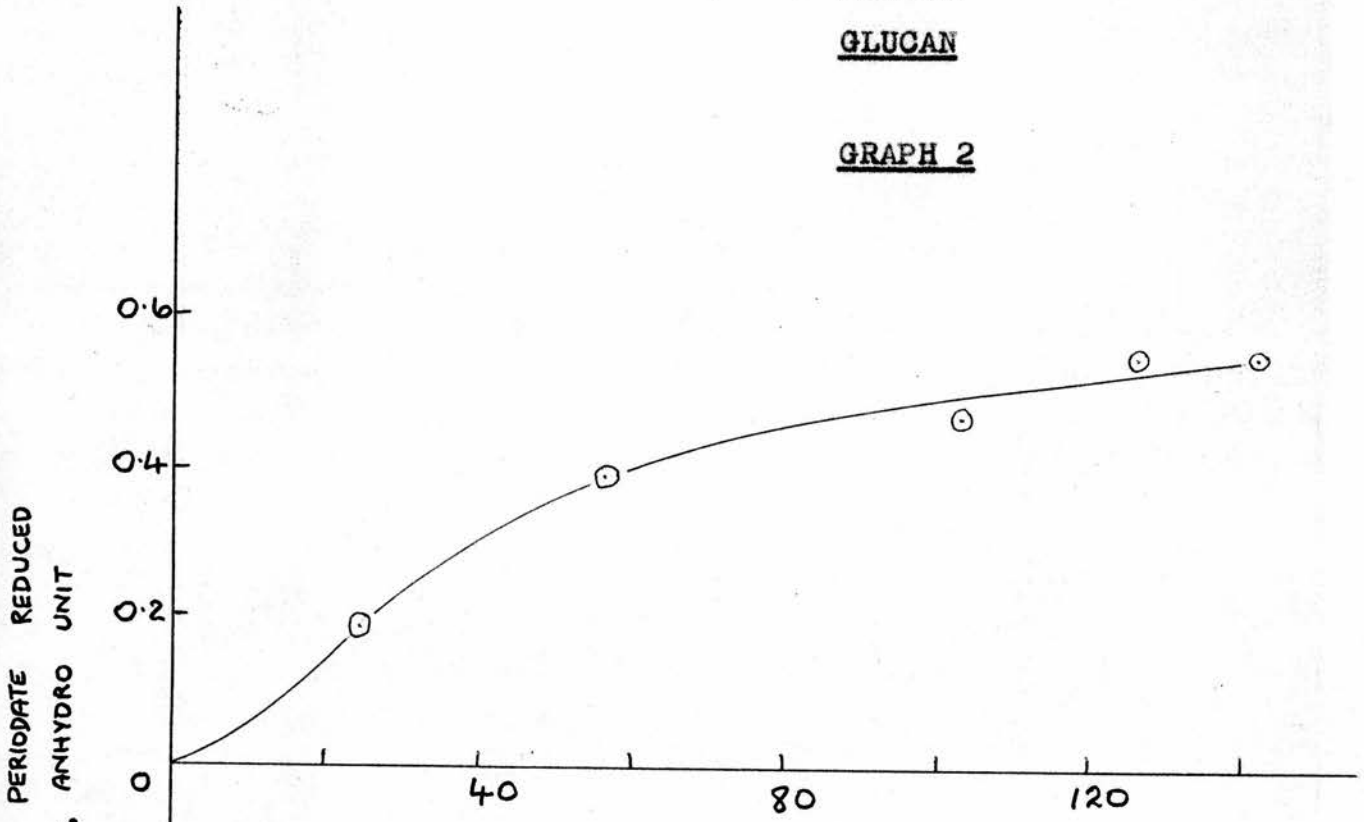


PERIODATE OXIDATION

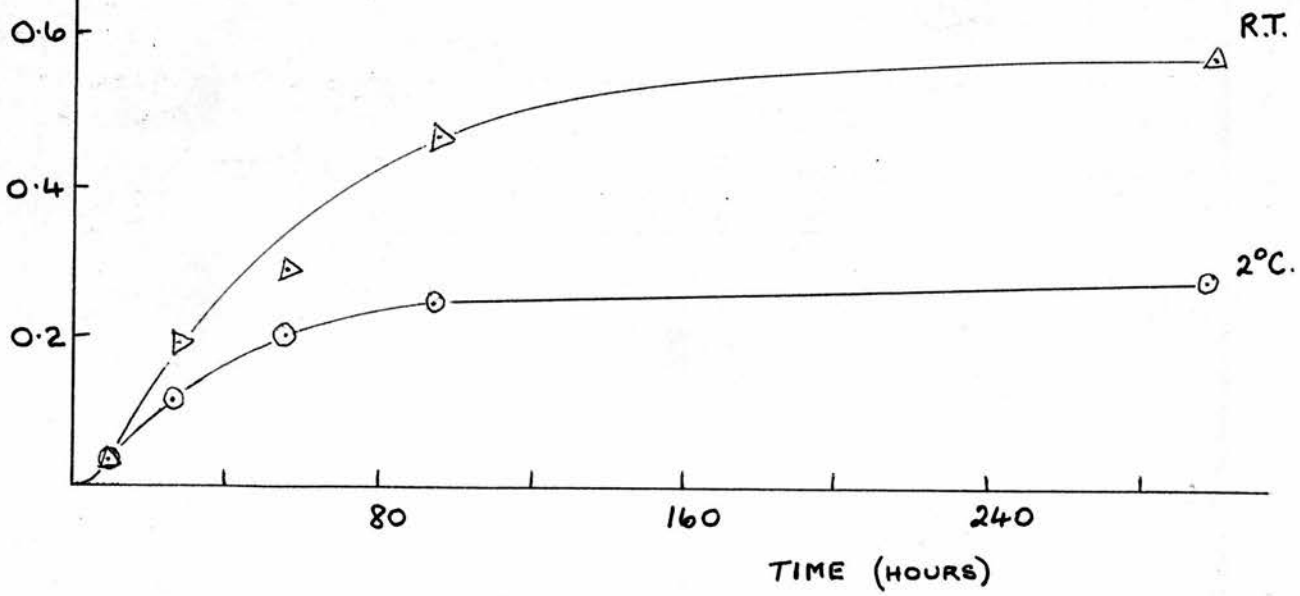
OF THE

GLUCAN

GRAPH 2



GRAPH 3

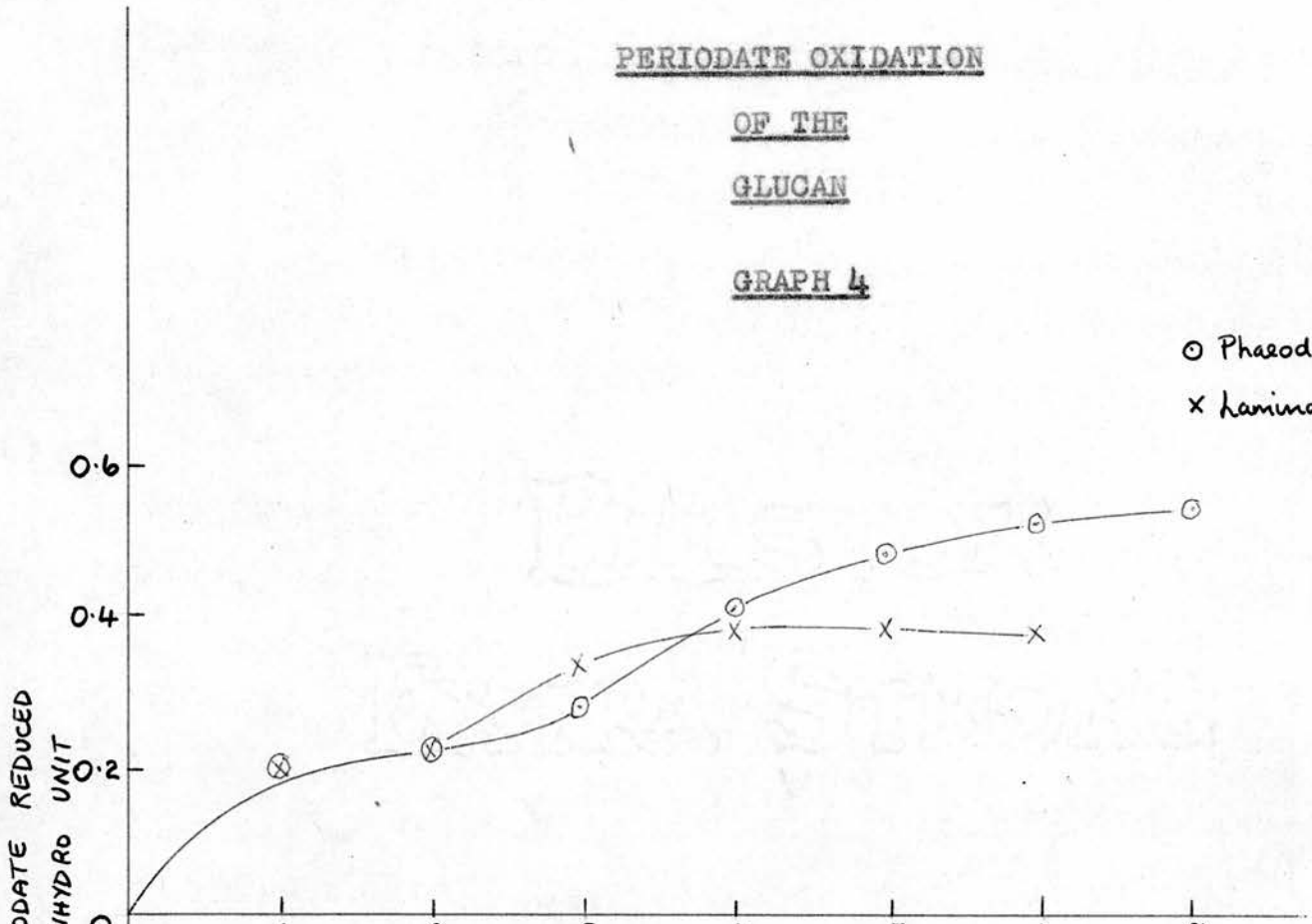


PERIODATE OXIDATION

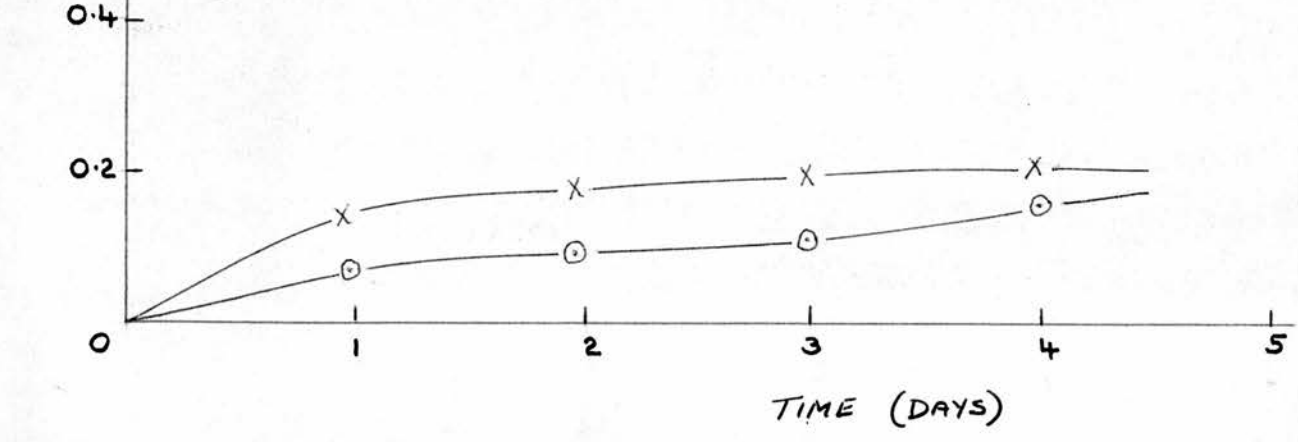
OF THE

GLUCAN

GRAPH 4



GRAPH 5



Exhaustive cold water treatment of the decolourised organism led to the isolation, by ethanol precipitation, of polysaccharide material in 14% yield (based on the dry weight of organism). It had ash 4.2%, protein ca 12.5% and contained glucose as the major constituent along with much smaller amounts of xylose, mannose and rhamnose (p.44).

A number of techniques was investigated for the purification of this material (Expt.5 p.46). The most satisfactory method was found to be gradient elution from a column of DEAE-cellulose with potassium chloride. This method removed the contaminating protein and colouring matter and separated the carbohydrate into two fractions. The major fraction, (340 mg. from 950 mg. of crude material), had a carbohydrate content of 90% and contained only glucose. It had $[\alpha]_D$ +42° and gave no colour with iodine, and was therefore not a starch-type polymer. The smaller fraction (25 mg.) had a carbohydrate content of 65% and contained, in addition to glucose, xylose and mannose.

The tentative chromatographic identification of glucose as the product of hydrolysis of the polysaccharide was confirmed by the glucose specific enzyme, glucose oxidase (Expt.6 p.48). Incubation of the neutralised polysaccharide hydrolysate with the enzyme at 35°C for 24 hours resulted in the complete conversion of the monosaccharide to gluconic acid.

From evidence supplied by Jermyn (105), the successful fractionation of the glucan on DEAE-cellulose suggests a branched molecular structure. (c.f. 'glucan A' from Monodus subterraneus, this thesis, Part II, p.128).

The quantity of sodium periodate reduced by a polysaccharide under controlled conditions can give an indication of the principal linkage in the polymer. For example, a 1,3'-linked polymer will have its main chain undegraded. If monosaccharide residues are revealed in the hydrolysate of the periodate oxidised polymer, then this can indicate either 1,3'- linkages or branch points in the polymer.

Periodate studies (Expt.7 p.49) on Phaeodactylum glucan at 2°C showed a strong resemblance to the periodate oxidation of laminarin carried out under the same conditions (Expt.7(c) p.51). However, at room temperature, the rate of oxidation of Phaeodactylum glucan by periodate increases much quicker than that for laminarin. The former polysaccharide reduces finally ca 0.54 moles. sodium periodate per anhydro C_6 unit, whereas the latter reduces ca 0.39 moles. per anhydro C_6 unit. Oxidation studies in more concentrated periodate solutions (Expts.7(a) and 7(b)(Graphs 2 and 3) carried out at 2°C and room temperature gave the same pattern as Experiment 7(c) (Graph 4). However, in very dilute periodate solution, (Expt.7(d)) the rate of reduction of periodate is slower for Phaeodactylum glucan than for laminarin (Graph 5). The difference is especially evident at 2°C. When oxidation was allowed to proceed at room temperature, Phaeodactylum glucan increased in its rate of reduction of periodate. The initial considerable difference in rates of reduction of periodate by the two polymers may possibly be explained by the mannitol end groups in laminarin being more readily oxidised under

these mild conditions than vic glycols of the glucopyranose end units.

From the relatively low reduction of periodate, the presence of 1,3'- linkages is indicated. The fact that the glucan under investigation reduces a greater quantity of sodium periodate than laminarin suggests a possible higher degree of branching in the molecule.

Further similarities to laminarin are provided by partial acid hydrolysis (Expt. 8, p. 52). When the two polysaccharides were heated at 100°C with 0.1N sulphuric acid for 1 hour the same series of oligosaccharides was produced, chromatographically identical with laminaribiose, gentiobiose, and laminaritriose. The presence of gentiobiose indicates the presence of 1,6'- linkages, either in the main chain, or as branch points.

The configuration of the principal interglycosidic linkage (i.e. α or β) can be determined by the action of specific enzymes. Enzymes termed β 1,3-glucanases have even a higher degree of specificity hydrolysing only β linkages which exist between C_1 and C_3 of glucose residues. When an enzyme system of this type was incubated with the glucan in citrate buffer at pH 4.5, a series of oligosaccharides (in addition to free glucose) was produced, chromatographically identical with laminaribiose, gentiobiose and laminaritriose. Simultaneous studies on laminarin gave exactly the same pattern. Prolonged incubation (72 hrs.) converted all the oligosaccharides to glucose, except for the one correspond-

ing to gentiobiose. These results provide strong evidence that the glucan is essentially a β 1,3'-linked polymer.

The complete destruction of laminaribiose and laminaritriose by the enzyme indicates contamination of the latter by glucosidase. This was confirmed by Dr. Reese, to whom we are indebted for the gift of the enzyme.

The glucan was methylated (Expt. 10 p. 53) in the first instance with dimethyl sulphate and sodium hydroxide according to the method of Haworth. Two series of five Haworth methylations gave a partially methylated product which was given four treatments with methyl iodide and silver oxide to complete the substitution. Infra red analysis of the final methylated product (ca 1 mg.) as a nujol mull indicated a large absorption associated with the methoxyl group, with only a trace of free hydroxyl.

The methylated glucan was methanolysed, and the mixture of methyl glycosides was analysed by gas chromatography (Expt. 11). This revealed the presence of:

Methyl 2,3,4,6 tetra-O-methyl glucoside

Methyl 2,4,6 tri-O-methyl glucoside

The methyl glycosides were hydrolysed with acid, and chromatographic examination of the neutralised hydrolysate revealed in addition to 2,3,4,6 tetra-O-methyl glucose and 2,4,6 tri-O-methyl glucose the presence of some material with the mobility of di-O-methyl glucose.

The mixture of methylated sugars were separated on 3MM paper. Two fractions consisting of a) 2,4,6 tri-O-methyl

glucose and b) di-O-methyl glucose were investigated.

The tri-O-methyl glucose was characterised as crystalline material having an identical melting point and mixed melting point when compared with an authentic sample of 2,4,6 tri-O-methyl glucose. The di-O-methyl glucose, present in extremely small quantity, was tentatively characterised as 2,4 di-O-methyl glucose by its ionophoretic mobility in borate buffer, (an authentic sample, and other di-O-methyl glucoses, being run as controls) and by demethylation to glucose. This 2,4 di-O-methyl glucose is not the characteristic product of under-methylation, and its presence therefore confirms the existence of 1,6' branches in the original polysaccharide.

Smith degradation of the glucan showed that the monosaccharide units were largely unattacked by sodium metaperiodate, when large quantities of glucose were recovered after mild hydrolysis. A small quantity of glycerol appeared to be present as well. Glycerol could arise from non-reducing glucose end-units or from inter-glycosidic 1,6'- or 1,2'-linkages. However, methylation studies failed to reveal the tri-O-methyl sugars which would result from linkages of this type. Thus the detectable presence of glycerol adds to the evidence for branching in the molecule.

CONCLUSION

From the experimental evidence obtained, there seems to be little doubt that the water-soluble glucan synthesised by Phaeodactylum tricornutum is essentially a β 1,3'- linked glucan. Smith degradation and methylation studies provide

evidence for branching in the molecule, the latter method indicating C_6 as a possible branch point. This type of glucan (chrysolaminarin), is the typical food reserve of the Chrysophyta, the Phylum to which P. tricornutum belongs, and it differs from the laminarin of the Phaeophyceae in the apparent absence of mannitol terminated chains.

PART 1(b)

THE GLUCURONOSYLMANNAN

FROM

PHAEODACTYLUM TRICORNUTUM

PART 1(b)

INTRODUCTION

MANNANS

Known examples of polysaccharides based solely on mannose as their structural unit are, as yet, very few, especially among the marine algae. The presence of mannans in the crude cellulose of soft woods has frequently been reported, but in most instances the polysaccharide involved is a glucomannoglycan with a high proportion of mannose to glucose. A true mannan (ivory nut mannan) forms about 60% of the endosperm of the seed of the tagua palm (Phytelephas macrocarpa)(112)(113). The D-mannose units are mainly β -D-(1→4)- linked in a linear molecule. Similar mannans are to be found in the tubers of some Orchidaceae spp.(Salep mannan) and in the green coffee bean. These polymers are characterised by their highly insoluble nature.

Polymers of an entirely different structure are the mannans synthesised by yeast and Penicillium charlesii G.Smith. Methylation studies on the former polysaccharide (114) led to the isolation of 2,3,4,6-tetra-, 3,4-di-, 3,4,6-tri-, 2,4,6-tri-, and 2,3,4-tri-O-methyl-D-mannose in the molar proportions 15:14:7:7:1. Fragmentation by acetolysis gave pure 2,- and 6-O-α-D- mannopyranosyl D-mannose, but no indication of 1,3'-linkages was given by this method. No unique structure could be ascribed to yeast mannan, but it is evident that the polymer is highly branched.

The Penicillium polysaccharide, known as mannocarolose,

occurs along with a galactan as an extracellular polymer (115). The pure mannan was isolated by selective hydrolysis of the galactan. Hydrolysis of the methylated mannan gave 2,3,4,6 tetra-O-methyl-D-mannose (2 parts), 3,4,6 tri-O-methyl-D-mannose (5 parts) and 3,4 di-O-methylmannose (1 part). Hence the mannose units are joined together by 1,2'- linkages with a branching point at C_6 of one of these units. Periodate oxidation confirmed these structural features.

The mannan synthesised by the marine alga Porphyra umbilicalis has been studied by Jones (116). Methylation has indicated β-1,4'- linked mannose residues to be present, the β-configuration being deduced from the negative rotation ($[\alpha]_D$-41°) in formic acid. This mannan is highly insoluble, characteristic of a β-1,4'- linked mannan. Periodate oxidation studies confirmed the methylation results, and in addition indicated a branched molecule with an average repeating unit of 12-13 mannose residues.

Preliminary investigations into the cell wall structure of the Green seaweeds Codium, Acetabularia and Halicoryne (117) have revealed that mannose is the major structural unit. Based on periodate oxidation studies and negative rotation, a tentative suggestion that the structures of these cell wall polysaccharides were β-1,4'- linked mannans was forwarded. More recently, Love (118) has made a more detailed investigation into the structure of the cell wall polysaccharide of Codium, and has confirmed the main interglycosidic linkages to be β1,4'. In spite of repeated purification by precipitation

with Fehling's solution, complete removal of glucose units was not achieved and the purified mannan still contained about 5-10% of 1,4'- linked glucose residues. Tentative evidence of chemical linkage was obtained by the isolation of a mannosylglucose from a partial acid hydrolyate, but this was not completely characterised.

Experimental

Experiment 14

Isolation of the Glucuronosylmannan

The residual material, (from 10 g. Phaeodactylum organisms), after extraction with 4% sodium hydroxide at room temperature, (Expt.3(v)), was given further treatment with 4% sodium hydroxide (200 ml.) on a boiling water-bath in an atmosphere of nitrogen for 2 hours. After cooling, the slight undissolved residue was centrifuged down and the alkaline solution neutralised with glacial acetic acid. Polysaccharide material was isolated, after dialysis and concentration, by precipitation with ethanol, drying and freeze-drying. The product, hereafter referred to as polysaccharide (M), (450 mg.) had $[\alpha]_D$ + 34°. Analysis for reducing sugars (98) gave a carbohydrate content of 55% (based on mannose). Chromatographic and ionophoretic examination of the neutralised hydrolysate (Solvents A, B, Fischer and Dorfel,[118A] and phosphate buffer pH 7.2) revealed spots with the mobilities of mannose (major constituent) and glucuronic acid (Sprays 1, 3 and 5). Two other substances had ionophoretic mobilities relative to glucuronic acid of 0.70 and 0.51, and chromatographic mobilities Rmannose 0.4 and 0.2 (Solvent A). Polysaccharide (M) was found to have a uronic acid content (102) of 27% and an ester sulphate content of 7.5%. The nitrogen content (96) was nil. That the sulphate was organically bound, and not contaminating inorganic sulphate, was shown by the negative test for sulphate, on a solution of polysaccharide(M) in water with barium chloride.

Experiment 15

Re-estimation of the Carbohydrate Content of the Polysaccharide

A standard solution containing a mixture of glucuronic acid and mannose in the proportions 27:73 respectively was prepared. Aliquots containing from 10 µg.- 100 µg. of sugars were withdrawn and analysed by the phenol-sulphuric acid method. A graph of µg. sugars against optical density(E) was plotted. A re-assessment of the carbohydrate content of the polysaccharide material using this graph gave a value of ca 70%.

Experiment 16

Attempted Fractionation of the Polysaccharide

(a) Attempted Precipitation with Fehling's Solution

Polysaccharide material (ca 20 mg.) was dissolved in dilute sodium hydroxide (ca 5 ml.). Fehling's solution was added, but did not cause any precipitation.

(b) Attempted Purification on a DEAE-cellulose Column(104)

The column was prepared as previously described (p.47). Polysaccharide(M) (326 mg. 70% carbohydrate)in water (10 ml.) was allowed to drain into the column. Gradient elution with water→ 0.5M potassium chloride (1.1l. of each) was used in the first instance followed by gradient elution with 0.5M potassium chloride to 0.3M potassium hydroxide (1.1l. of each). 25 ml. fractions were collected at a flow rate of 50 ml. an hour. The polysaccharide content of each tube was estimated by the phenol-sulphuric acid method (98). One main fraction was obtained (see graph 6 , p.91)which was isolated by combining the contents of tubes 30-36, dialysing and freeze-drying

(Yield 111 mg.). This material, much improved in appearance, had a carbohydrate content of 70%, and the hydrolysate gave the same chromatographic pattern as the original material.

Experiment 17

Treatment of the Phaeodactylum Residue prior to Alkali Extraction with Sodium Methoxide (119)

The residual material (1 g., 31% carbohydrate, 4% ester sulphate) was suspended in water (40 ml.). A solution of sodium borohydride (10 ml., 1%) was added, and the mixture was left to stand at room temperature for 48 hours. It was dialysed, concentrated and freeze-dried. The derived material (S) was further dried over phosphorus pentoxide in vacuo at 60°C for 48 hours.

Sodium metal (0.5 g.) was weighed into a dry flask fitted with a drying tube containing calcium chloride. Dry methanol (22 ml.) was added in portions through a reflux condenser. When cool, the reduced material (S) was introduced and refluxed for 48 hours in a system enclosed by a calcium chloride tube. The reaction mixture, now a brownish colour, was centrifuged and a brown solid, ca 50% by weight of the initial material, was isolated after washing with dry methanol.

The sodium methoxide treated material (ca 11 mg.) was hydrolysed and compared chromatographically with the hydrolysate of a similar quantity of initial material. The chromatograms (Solvent C, Spray 1) proved to be identical. No material corresponding to a methylated sugar was detected.

Experiment 18

Determination of the Equivalent of Polysaccharide(M)

Polysaccharide material (M) (31 mg.,27% uronic acid, 7.5% sulphate ester), dissolved in water, was passed several times through a column (32 x 1.8 cm.) containing Amberlite IR 120(H^+) resin until the polymer was completely converted into the acid form. Potentiometric titration with carbon dioxide-free $\frac{N}{100}$ sodium hydroxide gave a value of 418 for the equivalent.

Experiment 19

Rate of Sulphate Hydrolysis (120)

Samples of a solution of the polysaccharide (M) in 0.250N-hydrochloric acid (0.250 ml., containing ca 50 µg. of esterified sulphate) were sealed in Pyrex test-tubes (3" x $\frac{3}{8}$") and heated on a boiling-water bath for times ranging from 15 minutes to 7 hours. After cooling, centrifuging, and opening, the contents of each tube were exactly neutralised with sodium hydroxide solution (2.50N, 0.025 ml.). Chloroaminodiphenyl reagent (0.250 ml.) and a trace of cetavlon were added to each tube which were then allowed to stand for several hours. After centrifuging, a portion of the supernatant (0.1 ml.) was diluted to 10 ml. with 0.1N-hydrochloric acid and the optical density (E) of this solution read at 254 mµ.

The graph of log $^a/_{a-x}$ against time was plotted in each case, where log $^a/_{a-x} = \frac{E_{blank} - E_{24}}{E_t \quad E_{24}}$

E_{24} = optical density after 24 hours

E_t = optical density after t hours

E_{blank} = optical density of reagent

The half-life ($t_{\frac{1}{2}}$) was calculated, where $t_{\frac{1}{2}} = \frac{\log 2}{\text{slope}}$. The half-life for this polymer was found to be 1.51.

Experiment 20

Characterisation of the Monosaccharide Constituents

Polysaccharide(M) (175 mg.) was hydrolysed for four hours with N-sulphuric acid at 100°C. Chromatographic examination of the neutralised hydrolysate (Solvent A, Spray 1) revealed spots with mobilities of mannose and glucuronic acid in addition to two apparently incompletely hydrolysed constituents with mobilities relative to mannose of 0.4 and 0.2.

In the first instance, neutral material was separated from acidic material on 3MM paper in Solvent B. Sugars were located by developing side strips with Spray 1), and eluted with water. This separation gave two fractions :

Fraction 1, concentrated to a syrup (35 mg.), did not crystallise, but was chromatographically pure,with the mobility of mannose. It gave a phenylhydrazone with the same m.p.197°C as D-mannose phenylhydrazone, and did not show a depressed melting point when mixed with an authentic sample.

Fraction 2, (42 mg. syrup), was shown to contain three acidic components (ionophoresis in phosphate buffer, Spray 3) with mobilities relative to glucuronic acid of 1.0, 0.70 and 0.51.

This material was rechromatographed on 3MM paper in Solvent A. Three fractions were eluted (2a, 2b, 2c) after

locating the sugars in the usual way.

(i) Fraction 2a (15 mg.) crystallised from glacial acetic acid, had a melting point of 175°C and chromatographic examination revealed two spots with the mobilities of glucurone and glucuronic acid. No depression was observed when a mixed melting point was taken with authentic D-glucurone. X-ray powder photographs of the two substances, kindly provided by Dr. Beevers, were also identical.

(ii) Fraction 2b (4.2 mg.) ionophoretically pure, had a mobility relative to glucuronic acid of 0.70 and a chromatographic mobility (Solvent A, Spray 1) of 0.4 relative to mannose.

(iii) Fraction 2c (3.7 mg.) was also ionophoretically pure, with a mobility, relative to glucuronic acid, of 0.51 (Phosphate buffer). Chromatographically, (Solvent A, Spray 1), it had $R_{mannose}$ 0.2.

Experiment 21

i) Reduction of Fraction 2b

The acidic substance ($R_{mannose}$ 0.4), dried over phosphorus pentoxide, was suspended in tetrahydrofuran (5 ml. freshly distilled over sodium metal followed by lithium aluminium hydride). Lithium aluminium hydride (enough to cover a spatula tip) in tetrahydrofuran (5 ml.) was added in small portions and the suspension was refluxed for 1.5 hours. Excess lithium aluminium hydride was destroyed by the addition of water (ca 30 ml.) and the white residue was centrifuged down. The supernatant, which had pH 10, was treated with Amberlite

IR 120(H^+) resin, concentrated to a syrup, and dried in vacuo over phosphorus pentoxide.

ii) Methylation of Reduced Fraction 2b

The reduced product was dissolved in dimethylformamide (2 ml.) and treated with methyl iodide (2 ml.) and silver oxide (300 mg.) on an ice bath in the dark. The mixture was shaken in the ice for an hour, and then at room temperature for 20 hours. After filtration, the residue was washed thoroughly with dry chloroform, and the combined filtrate and washings allowed to stand in contact with calcium sulphate for 18 hours. After removal of the calcium sulphate, the solution was concentrated and methanolysed.

Gas liquid chromatographic analysis of the methyl glycosides gave small peaks with the retention times of 2,3,4,-6-tetra-O-methylglucose, and several unidentified peaks.

Experiment 22

Reduction and Hydrolysis of Fraction 2c

The completely dried substance ($R_{mannose}$ 0.2) was in the first instance ester glycosided (G.P.13,p.33). The derived methyl ester methyl glycoside was dissolved in 0.05M boric acid (1 ml.) at pH 5.2. Sodium borohydride (5 mg.) dissolved in water (1 ml.) was added, giving a final pH of 9, and the solution allowed to stand at room temperature overnight. Excess sodium borohydride was decomposed by neutralising the solution with glacial acetic acid. The solution was then treated with IR 120(H^+) resin, evaporated several times with methanol, and finally concentrated to a syrup.

The reduced product was hydrolysed in the usual way, and

chromatographic examination of the neutralised hydrolysate revealed spots with the mobilities of glucose and mannose.

Incubation of the hydrolysate with the enzyme glucose oxidase followed by chromatographic analysis revealed the complete removal of material with the speed of glucose. Still present was a spot level with mannose, and in addition a substance with the mobility of gluconic acid, run as control, was detected (Spray 3).

Experiment 23

i) Oxidation of Polysaccharide(M) with Sodium Metaperiodate (106)

Two portions of polysaccharide (17.1 mg. and 12.5 mg.) were oxidised with sodium metaperiodate. The first portion was treated with 0.015M periodate (20 ml.) at 2°C in the dark, while the second was oxidised at room temperature with 0.1M periodate (5 ml.), also in the dark. Aliquots were removed from each reaction solution at various intervals, and after the appropriate dilution, their optical densities were measured at 223 mμ.

Table 6

2°C		Room temperature	
Time (hrs.)	Moles IO_4^- reduced per C_6 anhydro unit	Time (hrs.)	Moles IO_4^- reduced per C_6 anhydro unit
6	0.14	3	0.57
18	0.20	8	0.67
30	0.32	22	0.94
42	0.34	28	0.94
66	0.38	33	0.94
96	0.45	48	0.94
120	0.45		

ii) Reduction of the Oxopolysaccharide

Excess sodium metaperiodate was destroyed by passing sulphur dioxide gas through the combined solutions for several minutes. The oxopolysaccharide was isolated by precipitation with ethanol, and then reduced with sodium borohydride (60 mg.) in 0.05M boric acid (ca 5 ml.) at room temperature for 24 hours. Excess borohydride was decomposed with glacial acetic acid, borate was removed by distillation with methanol, and the polyalcohol hydrolysed with $\bar{N}$-sulphuric acid at 100°C for four hours. Chromatographic examination of the neutralised hydrolysate revealed a strong reducing spot with the mobility of mannose and a trace of material with the speed of glycerol (Solvent B, Sprays 1 and 3). Ionophoresis in phosphate buffer tentatively identified a trace of glyceric acid M_{GA} 1.72 and showed the complete absence of glucuronic acid.

Experiment 24

Determination of the Formic Acid Released on Periodate Oxidation of Polysaccharide(M)

Polysaccharide(M) (123.2 mg.) was dissolved in distilled water (35 ml.) and the pH was adjusted to 5.8. Sodium metaperiodate was added, making a final concentration of 0.1M with respect to the periodate. The solution was kept in the dark at a constant temperature (2°C). When the oxidation was complete (ca 4 days), an aliquot (10 ml.) was removed from the reaction solution, mixed with ethylene glycol (0.5 ml.) and left in an atmosphere of nitrogen for five minutes. The formic acid liberated was titrated potentiometrically with carbon

dioxide-free 0.005$\bar{N}$ sodium hydroxide. The end point of the titration was taken when the pH of the solution returned to the initial value of 5.8.

The amount of formic acid released was found to be 0.38 moles. per C_6 anhydro unit.

The oxopolysaccharide(P) was isolated by precipitation with ethanol (10 volumes), centrifuging, and drying by evaporation. Yield ca 80 mg.

Experiment 25

Hydrolysis of Oxopolysaccharide(P) Followed by Reduction with Borohydride

Oxopolysaccharide(P) was hydrolysed with $\bar{N}$-sulphuric acid at 100°C for four hours. Chromatographic examination of the neutralised solution revealed a strong spot with the mobility of mannose. No material corresponding to glucuronic acid could be detected.

The hydrolysate was then reduced with sodium borohydride (400 mg.) in 0.05M boric acid (10 ml.). After destroying any excess borohydride with glacial acetic acid, the solution was concentrated several times with methanol, and then examined chromatographically (Solvent B, Spray 3). Two strong spots with the mobilities of glycerol and mannitol along with traces of material with slower speeds were detected. Material crystallised which was chromatographically identical with mannitol and gave a melting point and mixed melting point identical with an authentic sample of mannitol.

Experiment 26

Smith Degradation of Polysaccharide(M)

(i) Periodate Oxidation

Polysaccharide(M) (340 mg.) was oxidised with 0.1M sodium metaperiodate (50 ml.) at pH 4.9 in acetate buffer. Aliquots (0.1 ml.) were withdrawn from time to time, and after 60 hours, the oxidation was found to be complete. The reaction solution was divided into two portions, a) 10 ml. and b) 39.5 ml.

(a) Excess periodate was destroyed by the addition of excess ethylene glycol. The solution was dialysed and concentrated.

(b) In this case sulphur dioxide gas was passed through the solution to destroy excess oxidant. The solution was treated with ethanol (10 volumes), and the precipitated oxopolysaccharide isolated by centrifugation.

(ii) Reduction with Sodium Borohydride

The oxopolysaccharides from (a) and (b) were dissolved in 0.05M boric acid (10 ml. and 50 ml. respectively) and separately treated with sodium borohydride (100 mg. and 400 mg. respectively). The reductions were allowed to proceed at room temperature for 24 hours in an atmosphere of nitrogen. Both solutions were neutralised with glacial acetic acid, dialysed and distilled several times with methanol.

(iii) Mild Hydrolysis

After a final concentration, both polyalcohols were dissolved in $\bar{N}$-sulphuric acid (ca 1 ml. and 4 ml. respectively). The acid solutions were left at room temperature for two days. Addition of ethanol (10 volumes) to each solution produced

white precipitates of carbohydrate material. The degraded polymers (22.4 mg. and 89.7 mg. respectively) both gave only mannose on hydrolysis, and each had an ester sulphate content of ca 10%.

Experiment 27

Oxidation of the Degraded Polymer with Sodium Metaperiodate

Degraded polysaccharide (16.3 mg.) from the Smith Degradation (Experiment 26) was oxidised with 0.017M sodium periodate (10 ml.) in the dark at 2°C. Aliquots were removed and their optical densities read at 223 mμ.

Table 7

Time (hours)	4	7	26	58
Moles. 10_4^- reduced per C_6 anhydro unit	0	0.102	0.195	0.195

Experiment 28

Methylation of the Degraded Polysaccharide

The remaining degraded polymer (ca 85 mg.) was shaken with dimethyl sulphoxide (7 ml.) and dimethylformamide (3 ml.). Sodium hydroxide pellets (5 g.) were added, and dimethyl sulphate (4 ml.) dropped into the mixture, with stirring, over eight hours. The reaction was carried out in an atmosphere of nitrogen for the first two hours, and after 18 hours the mixture was heated on a boiling water bath to decompose the residual dimethyl sulphate. Water (10 ml.) was added to dissolve any solid sodium hydroxide and, after cooling to 5°C, the mixture was neutralised with 10$\bar{N}$ sulphuric acid. The aqueous solution was extracted exhaustively with chloroform in a liquid-liquid extractor for twelve hours. The chloroform

extract was concentrated and was found to contain very little material. The aqueous solution still contained the polysaccharide.

The aqueous solution was dialysed, concentrated, and then treated with sodium hydroxide (15 ml., 30%) solution in an atmosphere of nitrogen. Dimethyl sulphate (6 ml.) was added, with stirring, over 6 hours, and the reaction was allowed to continue overnight at room temperature. This procedure, using sodium hydroxide and dimethyl sulphate, was repeated four more times. The partly methylated material, isolated after dialysis and freeze-drying, was extracted with dry chloroform. The small amount of chloroform-soluble material was redissolved in methyl iodide (ca 3 ml.). Silver oxide (500 mg.) was added in 6 half-hourly portions while the methyl iodide was under gentle reflux. The reaction was allowed to continue overnight. The mixture, on cooling, was filtered, the solid residue extracted with hot chloroform and the combined filtrate and washings concentrated and given two further methylations in this way with methyl iodide and silver oxide and re-extracted with chloroform.

Experiment 29

Examination of the Methylated Polymer by Gas Liquid Chromatography

The chloroform soluble methylated material was methanolysed in the usual way. Examination of the derived methyl glycosides of the methylated sugars by gas liquid chromatography tentatively identified the following sugar derivatives :-

Methyl 2,3,4,6 tetra-O-methylmannoside

Methyl 2,4,6 tri-O-methylmannoside

A slight peak with the retention time of 4,6-di-O-methylmannose was also observed.

Experiment 30

Hydrolysis and Examination of the Methylated Sugars

The methyl glycosides (Experiment 29) were hydrolysed with N-sulphuric acid for four hours at 100°C. Chromatographic examination (Solvent C, Spray 2) revealed spots with the mobilities of 2,3,4,6 tetra-O-methylmannose, tri-O-methylmannose and di-O-methylmannose. The intensity of the trimethyl derivative was easily the strongest, while the other two constituents were approximately in equal concentration. The trimethylmannose gave a pink spot (Spray 1) identical with 2,4,6 tri-O-methylmannose and different from the brown spot given with 3,4,6 tri-O-methylmannose run for comparison.

Experiment 31

Partial Acid Hydrolysis of Polysaccharide(M)

Polysaccharide(M) (1.8 g.) was hydrolysed in a sealed tube with N-sulphuric acid at 100°C for one hour. Ethanol (10 volumes) was added to the cooled acid solution, and the precipitated polysaccharide was rehydrolysed under the same conditions. This procedure of hydrolysis and alcohol treatment was continued until no alcohol insoluble material remained. The acid supernatant solutions were combined and neutralised in the usual way with barium carbonate. Chromatographic examination (Solvent A, Spray 1) revealed spots with Rmannose 1.0,

0.4, 0.2, 0.05, with a trace at the speed of glucuronic acid.

Experiment 32

Fractionation of the Partial Acid Hydrolysate on a Cellulose Column.

(i) Preparation of the Column

Cellulose, as a slurry in acetone, was carefully poured into a glass column (diameter 34 mm.) to prevent the formation of air bubbles. The cellulose was allowed to settle under gravity with acetone constantly flowing through the column, which was then washed with distilled water (2 litres). The packed cellulose finally had a height of 460 mm.

(ii) Elution of the Partial Acid Hydrolysate

The column was first of all washed with Solvent E (2 litres). The partial hydrolysis syrup (ca 1.4 g.), in which the acid constituents were present as the barium salts, was eluted with the Solvent E(121) at a rate of ca 60 ml. per hour, and collected in 10 ml. fractions. Fractions were analysed by concentrating portions (1 ml.) to dryness, and examining the residue chromatographically. Four fractions were collected, none of which comprised a single entity.

Fraction 1 contained substances with chromatographic mobilities identical to mannose and glucuronic acid (faint), and a spot with the speed Rmannose 0.4 (Solvent A).

Fraction 2 comprised mainly the compound with a mobility of Rmannose 0.4 along with lesser amounts of a second substance with Rmannose 0.2.

Fraction 3 gave two substances with mobilities Rmannose 0.2 and 0.05, the former being present in greater quantity.

Fraction 4 showed a small quantity of a substance with mobility Rmannose 0.2, and contained largely the slower constituent at Rmannose 0.05.

(iii) Refractionation of Fraction 2

A second, and smaller cellulose column (20 x 330 mm.) was prepared as previously described. Fraction 2 syrup (160 mg.) was applied to the top of the washed cellulose and eluted, again with Solvent E. Chromatographic examination of the eluent showed the presence of both constituents of the mixture.

Finally, the mixture was applied to Whatman 3MM paper, and chromatographed in Solvent A. The individual components were detected by developing side strips of the chromatograms (Spray 1), and the appropriate areas cut out and eluted with water. This procedure gave two chromatographically and ionophoretically pure fractions. Fraction (A) had Rmannose = 0.4 (Solvent A) and an ionophoretic mobility of 0.70 relative to glucuronic acid (Phosphate buffer pH 7.2). Fraction B had Rmannose = 0.2, and ionophoretic mobility relative to glucuronic acid of 0.51.

Fraction 3, from the first cellulose column, was rechromatographed on Whatman 3MM paper and gave material identical with Fraction B, in addition to a small quantity of a substance with a chromatographic mobility (Solvent A) of Rmannose 0.05.

Experiment 33

a) Estimation of the Degrees of Polymerisation (DP) of Fractions A and B (122)(123)

To samples of Fraction A and Fraction B (1 ml. portions containing 51 µg. and 87.5 µg. respectively) was added 1%

aqueous sodium borohydride (1 ml.). To a second series of 1 ml. samples of Fractions A and B, containing the same quantities of material, was added 1% sodium borohydride in $\bar{N}$-sulphuric acid (1 ml.), "inactive borohydride". The two sets of solutions were allowed to stand at room temperature for 2 hours. Aqueous phenol, (5%, 1 ml.), followed by concentrated sulphuric acid (5 ml.) from a fast flowing pipette, were then added to each solution. After 30 minutes at room temperature the optical density of each solution was read at 487 mμ. against a water blank prepared in the same way as the test solutions. The optical densities of the reduced and non-reduced solutions are given below.

Table 8

		Optical density
Fraction A	reduced	0.144
	non-reduced	0.431
Fraction B	reduced	0.492
	non-reduced	0.841

Calculations

Fraction A. The non-reduced reading 0.431 corresponds to 51.0 μg. of an aldobiouronic acid consisting of glucuronic acid and mannose. The optical density of the reduced substance corresponds to 28.4 μg. of glucuronic acid. The calculated weight of glucuronic acid from 51.0 μg. of aldobiouronic acid is 27.8 μg. Hence, allowing for experimental error, the optical densities of reduced and non-reduced Fraction A correspond to an aldobiouronic acid. No other arrangement of

the two sugar constituents gives any correlation between the measured optical densities.

Similar reasoning, and estimation of weights of aldotriouronic and aldobiouronic acids indicated that Fraction B was an aldotriouronic acid. The non-reduced reading corresponds to 87.5 µg. of an aldotriouronic acid comprising glucuronic acid and mannose (1:2). The reduced reading corresponds to a weight of aldobiouronic acid of 58.0 µg. The weight of aldobiouronic acid calculated from 87.5 µg. of an aldotriouronic acid is 60.1 µg. No other structure gives any correlation between the optical densities of the reduced and non-reduced solutions. Hence, Fraction B must be an aldotriouronic acid comprising glucuronosylmannosylmannose.

The weight of each specie was calculated from standard graphs of mixtures of glucuronic acid and mannose in the ratios 1:0, 1:1, 1:2, and 1:3.

b) Partial Hydrolysis of Fraction B. Fraction B (3 mg.) was heated with N-sulphuric acid for 6 hours at 100°C and after neutralisation the derived syrup on chromatographic examination revealed spots with mobility of the starting material, the aldobiouronic acid (Fraction A) glucuronic acid (very faint) and mannose.

Experiment 34

Conversion of Fractions A and B to Neutral Compounds, and their Subsequent Methylation

Fraction A (15 mg.) and Fraction B (12 mg.) were converted into their methyl ester methyl glycosides by refluxing with 3% methanolic hydrogen chloride (1.25 ml.) for 6 hours. The

solutions were neutralised with silver carbonate and filtered. The salts were extracted with dry methanol, and the combined filtrate and washings taken to dryness. The residues were then taken up in 0.05M boric acid (3 ml.), cooled to 0°C and treated with sodium borohydride (20 mg.) in water (1 ml.). The reaction was allowed to take place in the refrigerator for 24 hours. Excess borohydride was decomposed by neutralising the reduction solutions with glacial acetic acid, and cations were removed by shaking with IR120(H^+) resin. Borate was removed as volatile methyl borate by evaporating the solutions several times to near dryness with methanol (7 x 3 ml.). Finally, the reduced residues were dried by evaporation with a mixture of methanol and benzene.

The derived neutral derivatives of Fractions A and B were then separately dissolved in redistilled dimethylformamide (0.5 ml.) and placed in a cold room at 4°C. Redistilled methyl iodide (0.5 ml.), also at 4°C, was slowly spotted into each solution, and silver oxide (0.5 g.) added (124). The mixtures were shaken in the dark for 1 hour in the cold room, and then for 60 hours at room temperature, also in the dark. After filtering, the solid residue was washed several times with dry chloroform. The combined filtrate and washings were taken to dryness on a rotary evaporator, and then on a high vacuum pump for five minutes.

Experiment 35

Examination of Methylated Fractions A and B

The derived methylated materials were methanolysed in the normal way (G.P. 13). Examination of the derived methyl

glycosides of the methylated sugars by gas liquid chromatography indicated the following sugar derivatives :-

Fraction A. Methyl 2,3,4,6 tetra-O-methylglucoside
Methyl 2,4,6 tri-O-methylmannoside

Fraction B. Methyl 2,3,4,6 tetra-O-methylglucoside
Methyl 2,4,6 tri-O-methylmannoside
Methyl 3,4,6 tri-O-methylmannoside

Experiment 36

Hydrolysis and Examination of the Methylated Sugars of Fractions A and B

The methyl glycosides of each Fraction (Expt.35) were hydrolysed at 100°C with N-sulphuric acid for four hours. Chromatographic examination of the neutralised hydrolysates (Solvent C, Spray 1) revealed spots corresponding to tetra-O-methylglucose and tri-O-methylmannose in both cases. The R_G (0.88) values of 2,4,6 tri-O-methylmannose and 3,4,6 tri-O-methylmannose are the same. Ionophoretic examination of the methylated sugars of Fractions A and B revealed a single spot M_G 0.0 for Fraction A and two spots M_G 0.0 and 0.33 for Fraction B. Authentic samples of 2,4,6 and 3,4,6 tri-O-methylmannoses had M_G 0.0 and 0.33 (borate buffer, pH 10, Spray 3) respectively.

The methylated sugars of Fraction B were separated on Whatman No.1 paper in Solvent C. The individual sugars were located in the usual way by developing side-strips (Spray 1), and the appropriate areas eluted. Two fractions were eluted.

Fraction 1 had chromatographic and ionophoretic mobility

of tetra-O-methylglucose. Demethylation (100) and chromatographic examination revealed glucose only.

Fraction 2 had a chromatographic mobility of tri-O-methylmannose and from its colour,(Spray 1) a mixture of pink and brown, it seemed to consist of more than one substance.

Ionophoresis in borate split the material into two spots with M_G values 0.33, and 0.0, the former value being identical to 3,4,6 tri-O-methylmannose. Chromatographic examination of the demethylated material revealed only mannose.

Experiment 37

Oxidation of Fractions A and B with Sodium Metaperiodate

Each fraction (7.0 mg. and 13.3 mg. respectively) was first of all refluxed with 3% methanolic hydrogen chloride for 4 hours. Each solution was neutralised with silver carbonate and filtered. The residue was thoroughly extracted with methanol, and these washings combined with the filtrate were concentrated to dryness. The derived methyl ester methyl glycosides were taken up in acetate buffer (ca 40 ml.) at pH 4.5. Sodium metaperiodate (5 ml., 0.1 M)was added, and each solution was made up to 50 ml. with acetate buffer. The amount of periodate consumed was measured by removing aliquots (1 ml.) and diluting 250 times with water before reading the absorptions at 223 mμ. against a water blank. The fraction of periodate reduced at any particular time was obtained by knowing the difference in absorbance between the original sodium periodate solution and sodium iodate solution of the same molarity under the same conditions.

Table 9

Fraction A	Time (hours)	12	20	132	214	312
	Moles IO_4^- reduced per C_6 anhydro unit	0.99	1.89	2.12	2.12	2.12

Table 10

Fraction B	Time (hours)	12	24	92	144
	Moles IO_4^- reduced per C_6 anhydro unit	1.07	1.07	1.25	1.25

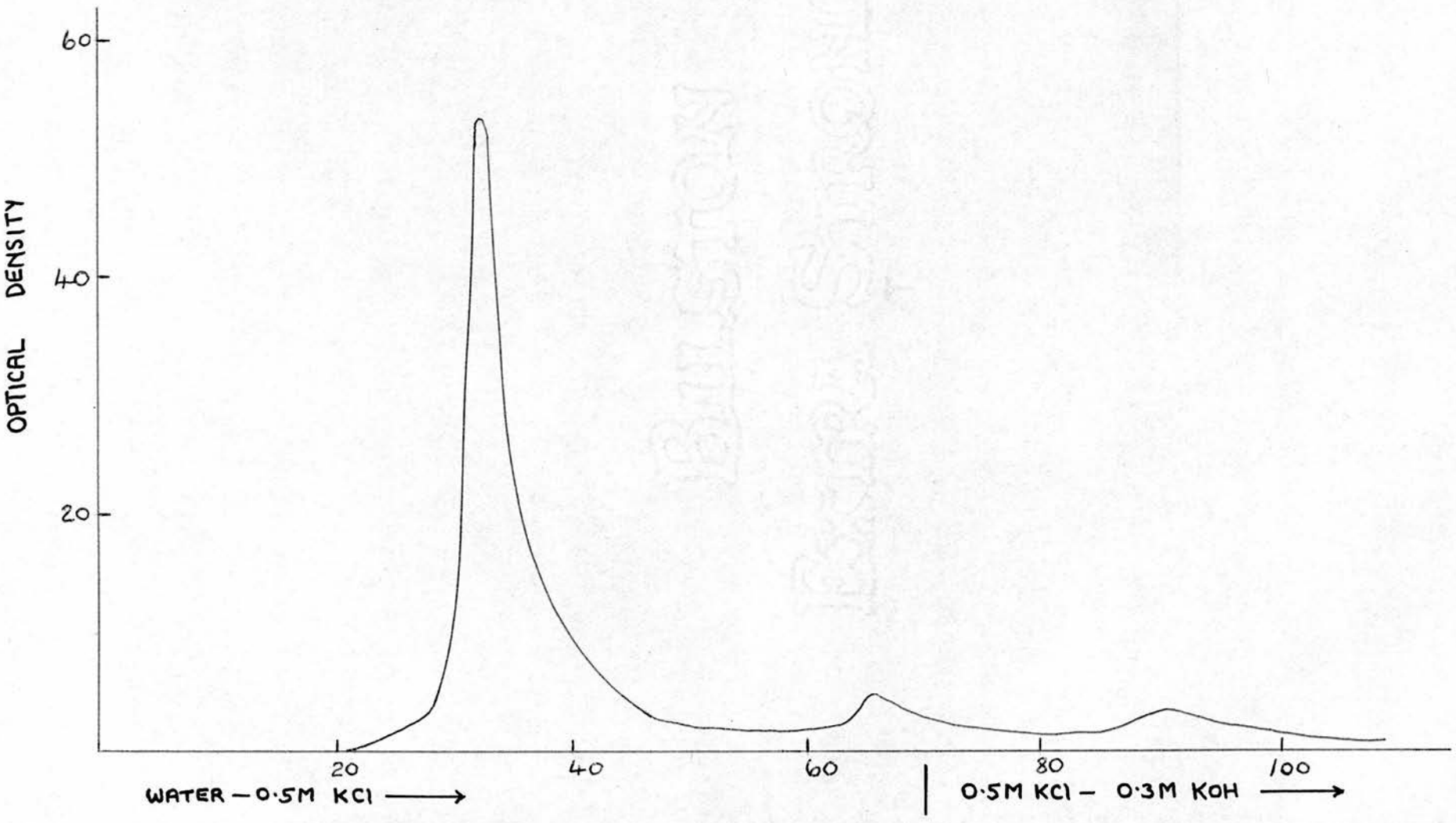
DISCUSSION

It was found that the residual material from Phaeodactylum tricornutum after cold and hot water extraction, chlorite treatment, and subsequent cold 4% sodium hydroxide extraction (p.43) comprised mainly carbohydrate material. When treated with hot 4% sodium hydroxide solution under nitrogen (Expt. 14) it almost completely dissolved, forming a brown solution. Precipitation of polysaccharide material (M) (5% of the Phaeodactylum organism) was achieved by the addition of excess ethanol, after neutralisation, dialysis, and concentration of this solution. It had $[\alpha]_D$ + 34° and comprised mainly mannose. With the exception of ca 8% it was readily soluble in water, in contrast to the highly insoluble β -1,4-linked mannan of ivory nut and coffee bean. Chromatographic examination of an acid hydrolysate revealed, in addition to mannose, smaller quantities of acidic material with the chromatographic and ionophoretic mobility of glucuronic acid, and two slower acidic spots $R_{mannose}$ 0.4 and 0.2, and $M_{glucuronic\ acid}$ 0.70 and 0.51 respectively. Analysis of polysaccharide(M) showed a uronic acid content of 27% (102), a sulphate content (95) of 7.5% and the complete absence of nitrogen (96). The equivalent of a polysaccharide with this percentage of uronic acid and no sulphate or ash would be 176/27 x 100 = 652. Polysaccharide(M) has a sulphate ester content of 7.5%, which corresponds to ca 48 g. of sulphate (i.e. 0.5 equivalents) in 652 g. of polysaccharide. Therefore, 652 g. of polysaccharide(M) corresponds not to 1 but to 1.5 equivalents. Hence the equivalent weight

GRAPH 6

FRACTIONATION OF POLYSACCHARIDE (M)

ON DEAE-CELLULOSE



of polysaccharide(M) should be 652/1.5 = 434. Polysaccharide(M) was converted completely into the acid form by passing through a column of IR 120(H^+) resin (Expt.18). Titration of the acidic residues with carbondioxide-free sodium hydroxide gave an equivalent of 418, in reasonable agreement with the calculated value.

The initial estimation of the carbohydrate content of material (M) by the phenol sulphuric acid method (98) based on mannose, gave a value of 55%. But it was found that the optical densities of solutions of glucuronic acid analysed by this method were considerably less than those of mannose of the same concentrations, and re-estimation based on a 73% mannose and 27% glucuronic acid content gave a value of 70%. As a result of the method of extraction, the uronic acid and sulphate ester residues are present as sodium salts; allowing for this, for 7.5% sulphate, and for about 10% of moisture this is a reasonable figure for a pure glucuronosylmannan.

Attempts to fractionate this glucuronosylmannan by precipitation with Fehling's solution(Expt.12a) or elution from a DEAE-cellulose column (Expt.16b) were unsuccessful. No copper complex was formed with the Fehling's solution, another point of difference from β-1,4-linked mannans which complex strongly with copper. The polysaccharide material which was eluted as a single peak from the DEAE-cellulose column had the same chemical composition as the starting material.

Sulphate ester groups which are adjacent and trans to a free hydroxyl group in a polysaccharide have been shown to be labile to alkali (125). Any such groups present in the native

glucuronosylmannan would have been lost during extraction. It is possible to locate such groups by treatment with sodium methoxide since this reagent cleaves the sulphate residue and causes the intermediate formation of epoxide rings. Attack by the methoxide ion can then occur with the formation of monomethyl sugars. This method has been successfully applied to the acid polysaccharide of the green seaweed Ulva lactuca (54) as shown below,(Fig. 15). Attack in this instance occurred

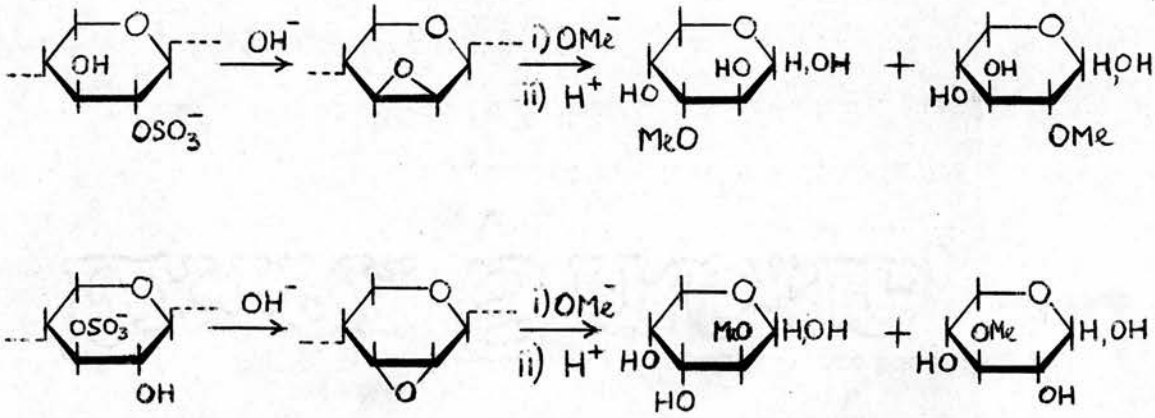
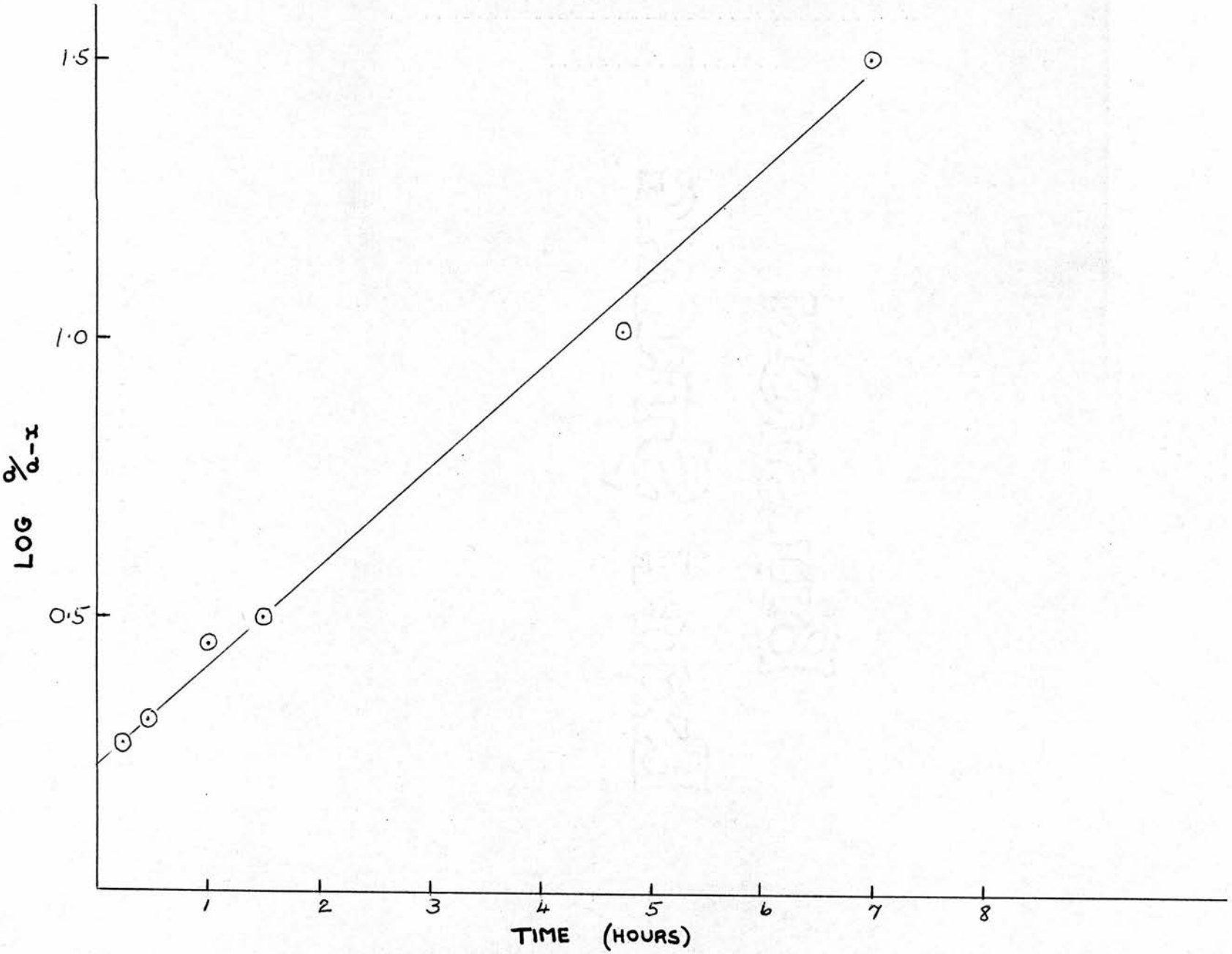


Fig. 15

preferentially at C-2 and after hydrolysis of the resulting polysaccharide 2-O-methyl-D-xylose was isolated and characterised. From this it was deduced that some of the D-xylose units in the Ulva polysaccharide were sulphated at C-2. Application of this procedure to Phaeodactylum tricornutum (Expt. 17) before treatment with alkali failed to yield any methylated sugars, from which it may be concluded that all the ester sulphate is still linked to the extracted glucuronosylmannan(M) and is stable to alkali.

GRAPH 7

RATE OF SULPHATE ESTER HYDROLYSIS IN POLYSACCHARIDE (M)



Studies on the rates of hydrolysis of the sulphate esters of monosulphated glucose and galactose have shown significant differences which can indicate whether the sulphate is linked axially, equatorially or to a primary hydroxyl group on the sugar moiety, (General Introduction, p.6). It was, therefore, of interest to study the rates of hydrolysis of the sulphate ester in the present polysaccharide material. Samples of polysaccharide (M), hydrolysed for various times, released sulphate at a rate which corresponded to a half-life of 1.5h. Although this is approximately the rate of hydrolysis of the primary sulphate of the two sugars previously studied, it would be premature to deduce anything conclusively in the present studies until more is known of the rates of hydrolysis of authentic mannose and glucuronic acid sulphates.

The presence of uronic anhydride and mannose residues in polysaccharide (M), tentatively identified by chromatography, was confirmed by their separation from an acid hydrolysate and the formation of crystalline glucurone and mannose phenylhydrazone respectively (Expt.20). The former derivative had m.pt. 175°, gave no depression when mixed with an authentic sample of glucurone, and gave an X-ray powder photograph identical to the authentic material. The latter had m.pt.197°C and gave no depression when mixed with an authentic sample of mannose phenylhydrazone.

Two other acidic substances, Fractions 2b and 2c, were also separated from the acidic hydrolysate of polysaccharide (M) (Expt.20). These two will be described in more detail later (p.98) when the partial hydrolysis products of poly-

saccharide (M) are discussed.

When polysaccharide (M) was oxidised with sodium metaperiodate at 2°C and at room temperature in the dark (Expt.23), the amount of periodate reduced was 0.45 and 0.94 moles. per C_6 anhydro unit respectively. The low reduction of periodate at 2°C suggests an appreciable proportion of 1,3'- linkages in the polysaccharide. The apparent rapid overoxidation at room temperature may be explained by the presence of non-reducing glucuronic acid end groups which are considered to overoxidise as shown below (Fig.16)(126),through the formation of a malondialdehyde grouping.

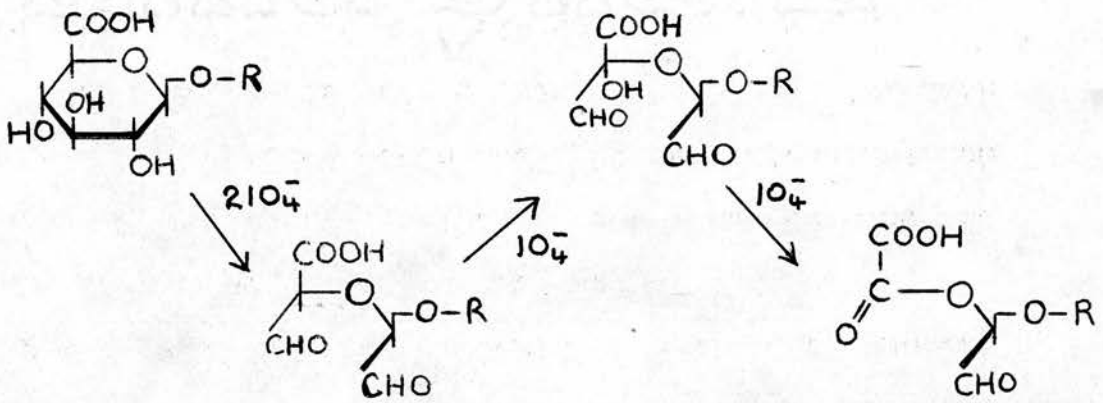


Fig.16

The combined solution of oxopolysaccharides was reduced with sodium borohydride and then hydrolysed with sulphuric acid. Chromatographic examination of the neutralised hydrolysate tentatively identified mannose as the main constituent, and traces of glycerol and glyceric acid. Ionophoresis in phosphate buffer also indicated the presence of a small quantity

of glyceric acid. The presence of this acidic fragment provides evidence for the existence of glucuronic acid residues at the non-reducing ends of the polysaccharide molecules. Glycerol is derived from mannose terminated chains, or from interglycosidic 1,2'- or 1,6'- linkages. The absence of glucuronic acid in the hydrolysate shows that all these residues must be vulnerable to periodate oxidation.

Further periodate oxidation studies on polysaccharide (M) revealed, on complete oxidation, a formic acid release of 0.38 moles. per C_6 anhydro unit (Expt.24). This could be derived from end group mannose and/or glucuronic acid units.

The oxopolysaccharide from this last experiment was first of all hydrolysed, and then treated with sodium borohydride (Expt.25). Chromatographic examination of the reduced hydrolysate revealed strong spots with the mobilities of mannitol and glycerol. The former crystallised and was identified by its melting point and mixed melting point with authentic mannitol, confirming again the immunity of a high proportion of the mannose units to periodate oxidation.

It was found that after Smith degradation of polysaccharide (M), a degraded polymer, (ca. 33% of the starting material), could be precipitated from the acidic solution with alcohol. This gave only mannose on hydrolysis and had a sulphate ester content of ca. 10% (Expt.26). The possibility of sulphate contamination from the sulphur dioxide used to destroy the excess periodate was eliminated by treating a portion of the oxidation solution with ethylene glycol, (Expt.26).

The degraded polymers eventually isolated from both treatments had the same sulphate content. On further oxidation with sodium periodate (Expt.27), the degraded polymer reduced only 0.195 moles. of periodate per C_6 anhydro unit.

Since the glucuronic acid residues appear to be completely cleaved by periodate oxidation, and yet unattacked polysaccharide material remains, it seems likely that the uronic anhydride units occur in side chains in polysaccharide (M).

The degraded polymer was initially methylated (Expt.28) by a modification of the Kuhn procedure. This, however, did not give any significant quantity of chloroform soluble methylated material. Methylation was continued, therefore, using dimethyl sulphate and sodium hydroxide according to the method of Haworth. After five Haworth methylations, the partly methylated material was given three Purdie treatments with methyl iodide and silver oxide.

Examination of the methanolysed methylated polysaccharide by gas liquid chromatography (Expt.29) revealed peaks with the retention times of methyl 2,3,4,6 tetra-O-methylmannoside, methyl 2,4,6 tri-O-methylmannoside and a slight peak tentatively identified as a methyl di-O-methylmannoside.

Acid hydrolysis of the methyl glycosides (Expt.30) and chromatographic examination of the derived methylated sugars revealed spots with the mobilities of 2,3,4,6 tetra-O-methylmannose, tri-O-methylmannose, and di-O-methylmannose. The colour of the tri-O-methylmannose spot with Spray 1 indicated it to be 2,4,6 tri-O-methylmannose, and its intensity was far greater than the other methylated sugars produced. It appears

that this degraded polymer is the backbone of polysaccharide (M) and consists of 1,3'-linked mannopyranose units, with occasional units carrying sulphate groups. Under the conditions used in the periodate oxidation, only ca.3 moles. of periodate should be reduced per molecule and therefore an average chain length of ca. 15 units is indicated for this backbone.

It is evident from the methylation results on the degraded polymer that the Smith degradation stripped the side chains completely from the backbone of polysaccharide (M). If any fragments had remained attached to the degraded polymer, a much greater proportion of dimethylmannose would have been present in the hydrolysate of the methylated material. Small amounts of dimethylmannose that were detected could arise from the presence of the sulphate ester residues.

Relative rates of acid hydrolysis of methyl glycopyranosides have been shown to differ considerably (127), and it can be argued that,if several types of linkage are present within a polysaccharide, they will be hydrolysed at different rates. Oligosaccharides formed by partial acid hydrolysis of polysaccharides will therefore contain primarily the most stable linkages. Uronic anhydride residues stabilise the linkage through C_1 of the uronic anhydride unit. Consequently, partial acid hydrolysis of polysaccharides containing uronic anhydride residues usually give aldobio- and aldotriouronic acids.

Partial acid hydrolysis of polysaccharide (M) (Expt.31), on chromatographic examination of the hydrolysate, was shown to give four substances with $R_{mannose}$ 1.0, 0.4, 0.2, 0.05 in Solvent A. Only a trace of material with the mobility of

glucuronic acid could be detected.

Attempts to fractionate the partial hydrolysate on a cellulose column (Expt.32) were only partially successful. Substances with mobilities $R_{mannose}$ 0.4 and 0.2 (identical with Fractions 2a and 2b (p.73)) were finally separated on 3MM paper in Solvent A into chromatographically and ionophoretically pure compounds.

The first of these ($R_{mannose}$ 0.4) was shown to have a DP of 2.1 and to be an aldobiouronic acid. After reduction with lithium aluminium hydride (p.73), and methylation by the Kuhn technique (124), it was methanolysed and examined by gas liquid chromatography. Peaks with the retention times of methyl 2,3,4,6-tetra-O-methylglucosides and several unidentified peaks were revealed. The presence of glucuronic acid as the non-reducing residue was thus established, and the unidentified peaks were presumed to be due to a methylated mannitol (see later) derived from the reducing end of the aldobiouronic acid. A further portion of the aldobiouronic acid was converted into the methyl ester methyl glycoside and reduced with borohydride (Expt.34). The derived neutral disaccharide, after methylation and methanolysis, was examined by gas liquid chromatography. Peaks with the retention times of methyl 2,3,4,6-tetra-O-methylglucosides and methyl 2,4,6-tri-O-methylmannosides were obtained.

Acid hydrolysis of the glycosidic mixture (Expt.36) gave tetra-O-methylglucose and 2,4,6-tri-O-methylmannose (paper chromatography and ionophoresis in borate buffer). These results indicate that the aldobiouronic acid is O-(D-gluco-

pyranosyluronic acid)(1→3)-D-mannose. (Fig. 17)

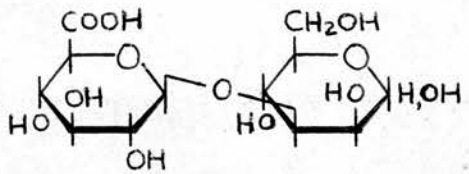


Fig. 17

Confirmation was obtained by the rapid reduction of 1.9 mole. of periodate by the methyl ester methyl glycoside. This increased to 4.2 moles on prolonged oxidation, in keeping with the ready over-oxidation of end group glucuronic acid (see p. 94).

The slower substance $R_{mannose}$ 0.2 had a DP of 3.1 (Expt. 33) and was characterised as an aldotriouronic acid. After conversion into the methyl ester methyl glycoside (Expt. 20 iii) and reduction with sodium borohydride, it gave on hydrolysis a mole of glucose and two moles of mannose (paper chromatography, visual examination). Incubation with glucose oxidase confirmed the presence of glucose. Methanolysis of the methylated neutral disaccharide glycoside and gas liquid chromatographic examination revealed peaks with the retention times of 2,3,4,6-tetra-O-methylglycosides and 2,4,6- and 3,4,6-tri-O-methylmannosides. Paper chromatography of an acid hydrolysate showed tetramethylglucose and a single spot for the tri-O-methylmannose. These were separated and the tri-O-methylmannose fraction shown to consist of the 2,4,6- and 3,4,6-tri-O-methyl derivatives on ionophoresis in borate buffer. The aldotriouronic acid is therefore a glucuronosylmannosylmannose.

Partial hydrolysis and chromatographic examination of the

products revealed, in addition to unhydrolysed material, the aldobiouronic acid, characterised on p.99, and mannose, indicating the aldotriouronic acid to be O-(D-glucopyranosyluronic acid)(1→3)O-D-mannopyranosyl(1→2)-D-mannose,(Fig.18).

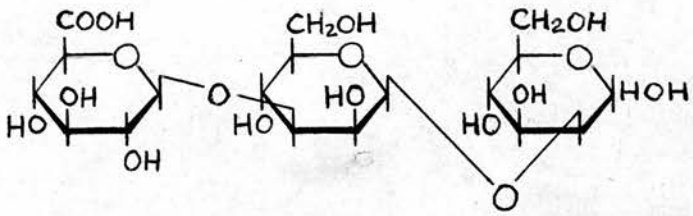


Fig.18

Periodate oxidation of the methyl ester methyl glycoside rapidly reduced 3.0 moles. of periodate per trisaccharide molecule, in keeping with proposed structure, but this only rose to 3.75 moles. on prolonged oxidation and there is no obvious explanation for the comparatively small amount of over-oxidation in this instance.

CONCLUSION

After exhaustive extraction of the *Phaeodactylum tricornutum* organism, the residue, with the exception of *ca.* 10% of non-carbohydrate material, comprises a sulphated glucuronosylmannan. This appears to constitute the cell wall of the organism and is the first example of this type of polymer to be found in nature.

Structural studies of the present glucuronosylmannan have shown that it contains a backbone of 1,3'- linked mannose units to which are attached other mannose and glucuronic acid units as side chains. Partial hydrolysis studies have shown that O-(D-glucopyranosyluronic acid)(1→3)-D-mannose and O-(D-gluco-

pyranosyluronic acid)(1→3) O-D-mannopyranosyl (1→2)-D-mannose are structural units in the side chains.

Periodate oxidation and Smith degradation experiments also indicate that there is considerable branching in the molecule. All the glucuronic acid residues are vulnerable to periodate and from the formic acid released it appears probable that many are present as end units.

The ester sulphate is equivalent to ca. 1 in every 5 of the sugar residues, but no unequivocal evidence for its exact site has been obtained, although the presence of sulphate residues in the Smith degraded polymer proves that some at least of these groupings must be linked to mannose. The stability of the ester sulphate to alkali eliminates any possibility of it being linked to end group glucuronic acid. That the majority of the linkages have a β - configuration is deduced from the low rotation of the polysaccharide. Further studies will be necessary before it is possible to advance a formula for this complex polymer.

It is of interest to note that since this work was completed, a mannan containing ca. 6% uronic acid has been fractionated from the mannan of the Huacra Pona palm, (Iriartea ventricosa)(128). However, although few structural studies were carried out, it appears from its source most probably to be a β 1,4-linked mannan of the ivory nut type.

PART 1(c)

THE LOW-MOLECULAR CARBOHYDRATES

FROM

PHAEODACTYLUM TRICORNUTUM

PART 1(c)

INTRODUCTION

Low-molecular Carbohydrates in Algae

Several species of brown algae have been investigated with respect to their low-molecular carbohydrates. Fucus vesiculosus (129), Pelvetia canaliculata (130), Laminaria cloustoni (131), Fucus spiralis and Desmarestia aculeata (132) all contain mannitol, 1-mannitol β -glucoside, and 1,6-mannitol di-(β -glucoside). 1-Mannitol acetate was obtained only from F. vesiculosus, L-cloustoni and F. spiralis, the Fucus spp. having the higher amounts. P. canaliculata has been the only specie of brown alga, so far studied, to synthesise the heptitol, D-volemitol (133)(134).

A new C- methyl inositol, now named laminitol, was isolated, first from L. cloustoni (134), and then from F. spiralis and D. aculeata (132). Two C-methyl inositols had previously been described; one, mytilitol, isolated from the mussel Mytilis edulis (135), the other, isomytilitol, a synthetic product prepared by Posternak (136). Laminitol proved to be different from either of these inositols in giving a hexa-acetate with a much lower melting point, and also in having a greater chromatographic mobility. It has now been characterised as either 4-C-methylmyoinositol or its antipode (137).

Only four of the nine possible inositols (cyclohexane-hexols) are naturally occurring, (+) and (-)-inositols, myoinositol (also called mesoinositol), and scyllitol (scyllo-inositol). Of these, myoinositol is the most widespread.

In addition to the carbohydrates previously mentioned, L. cloustoni has been found to synthesise the β-1,3'-linked di- and tri-saccharides laminaribiose and laminaritriose (131).

Studies on the alcohol soluble fraction of the red seaweed Furcellaria fastigiata (138) have revealed the presence of myoinositol, mannitol, floridoside (2-glycerol α-D-galactoside) and 3-floridoside-α-D-mannopyranoside. Porphyra umbilicalis has been shown to contain scyllitol, laminitol, mannitol, volemitol, glycerol, galactose, floridoside, and 1-glycerol-α-D-galactopyranoside (139). A third specie of red alga, Gelidium cartilagineum synthesises floridoside, myoinositol, and laminitol.

Two of the green seaweeds have been investigated (140). Mannitol and sucrose have been isolated from Enteromorpha compressa. The presence of myoinositol was demonstrated chromatographically. Studies on Chlorella resulted in the isolation of sucrose, maltose, maltotriose and myoinositol.

A di- and trisaccharide, containing only glucose, have been reported from studies on the alcohol soluble carbohydrates of a mixture of species of diatoms (69), but no attempt was made to characterise these carbohydrates.

EXPERIMENTAL

Experiment 38(a)

Isolation of the Low-molecular Carbohydrates

Cold water extraction (Expt.4(v)(a) p.44) of Phaeodactylum tricornutum organisms (10 g.), led to the isolation of a glucan (1.43 g.) by alcoholic precipitation. The supernatant aqueous ethanolic solution (B) was concentrated to dryness, dissolved as far as possible in water, centrifuged, and freeze-dried to a greenish, rather sticky solid (2.01 g.) which had a carbohydrate content of ca.20% (97). Direct chromatographic examination tentatively identified glucose, along with a considerable amount of other material with lower chromatographic mobilities. The supernatant solutions B from four extractions of P.tricornutum were combined and examined.

Experiment 38(b)

Decolourisation and Deionisation of Solution B

The aqueous ethanolic Solution B (10% v/v of ethanol) of the low-molecular carbohydrates was shaken for ca.5 minutes at 40°C with activated charcoal. The mixture was filtered, and the charcoal washed with aqueous ethanol.

The now completely colourless Solution B was concentrated to a small volume (ca.5 ml.). On standing, the solution deposited crystals, which on centrifugation and examination proved to be inorganic salts. Repeated concentration, in this manner, removed a considerable proportion of the inorganic content of Solution B. When no more salts were deposited by this method, the solution was concentrated to a syrup. A further quantity of crystals was deposited which were isolated by washing with

50% aqueous ethanol. When all adhering syrup was removed, the crystals were dried with ethanol and ether. Yield ca. 10 mg. Chromatographic examination revealed two non-reducing spots with Rg values of 0.67 and 0.45 in Solvent A.

The syrup from Solution B, still containing some inorganic salts, was dissolved in water and passed repeatedly through columns of IR 120(H^+) and IR 45(OH^-) resins till the solution was completely neutral.

The final eluent, now completely decolourised and deionised, was concentrated to a colourless syrup (ca. 1.5 g.). Chromatographic examination (Solvent A, Sprays 1 and 3) revealed spots with Rg values of 1.15, 1.0, 0.51, 0.22 and 0.13 (Spray 1; all reducing), and 0.67 and 0.45 (Spray 3; non-reducing).

Experiment 39

Fractionation of Solution B on a Cellulose Column

A cellulose column (20 mm. x 335 mm.) was prepared as previously described (Expt.32). The column was washed with Solvent G (1.5 litres). The syrup (750 mg.), dissolved in a minimum of water, was applied to the top of the cellulose and eluted with Solvent G at a rate of ca. 60 ml. per hour. The eluent was collected in 10 ml.fractions and analysed by concentrating aliquots (1 ml.) to dryness and examining the residue chromatographically. No separation of the mixture was achieved.

The column was rewashed with Solvent A (1.5 litres), Solution B reapplied to the top of the cellulose, and the column eluted at a rate of ca.60 ml. per hour. Aliquots (1 ml.) from the 10 ml.fractions were, as before, concentrated to dryness,

and examined chromatographically. Only one of the fractions (7) contained a single entity, and partial fractionation was achieved. Seven fractions were collected as listed below. The components of each are characterised by their Rg values in Solvent A with Spray 3. Solvent A was used in preference to any other because it gave much more efficient separations of the components of each mixture.

Fraction 1 (Tubes 45-64) Rg 1.15 (v.strong), 1.0 (faint)

Fraction 2 (Tubes 65-95) Rg 1.15 (strong), 1.0 (strong)

Fraction 3 (Tubes 96-99) Rg 1.15 (moderate), 1.0 (v.strong) 0.65 (trace)

Fraction 4 (Tubes 100-115) Rg 1.15 (faint), 1.0 (moderate), 0.65 (strong)

Fraction 5 (Tubes 116-132) Rg 0.65 (strong), 1.0 and 0.51 (traces)

Fraction 6 (Tubes 133-183) Rg 0.51 (strong), 0.45 (strong), 1.0 (trace)

Tubes 184 to 249 contained a negligible amount of carbohydrate.

Fraction 7(Tubes 250-285) Rg 0.22

Each of the Fractions 1-6 was rechromatographed on 3MM paper in Solvent A. The components of each mixture were located in the normal way by developing side strips of each chromatogram with Spray 3. The appropriate areas of each paper were cut out, and corresponding fractions combined and eluted with distilled water. Six different and apparently chromatographically pure compounds were isolated, and investigated.

i) Fraction A, (52 mg.) Rg 0.22. ii) Fraction B,(4 mg.) Rg 0.51.

iii) Fraction C, (3.5 mg.) Rg 0.67. iv) Fraction D,(20 mg.)Rg 1.15. v) Fraction E,(10 mg.) Rg 0.45. vi) Fraction F,(42 mg.) Rg 1.0

The weights of Fractions A, B and F were calculated by the phenol-sulphuric method (98), and based on glucose. Fraction C was weighed as crystalline material. Fractions D and E were weighed as syrups.

Experiment 40

Examination of Fraction A

i) Chromatography

Fraction A in Solvent A had an Rg 0.22 and gave a brown colour when heated with aniline oxalate. In borate buffer ionophoresis it had M_G 0.53.

A portion (ca. 5 mg.) was completely hydrolysed with N-sulphuric acid. Chromatographic examination of the neutralised hydrolysate (Solvents A and F, Sprays 1 and 3) revealed spots with the mobilities of glucose and galactose, the former being the major constituent. Paper chromatograms impregnated with tungstate buffer at pH8 were used with Solvent F to give an improved separation of glucose and galactose (141).

ii) Enzymic Hydrolysis of Fraction A

Fraction A (ca. 5 mg.) was incubated with a specific β 1,3 glucanase at 36°. Chromatographic examination of the hydrolysate revealed spots with mobilities Rg 1.0 and 0.5. Prolonged incubation (70 hrs.) did not remove the spot with Rg 0.5.

The hydrolysate was reduced with sodium borohydride and examined ionophoretically (molybdate buffer pH5). Spots with $M_{sorbitol}$ 1.0 and 0.0 were revealed (Spray 3).

iii) Reduction of Fraction A

Fraction A (5 mg.) was dissolved in 0.05M boric acid (2 ml.) and treated with sodium borohydride (10 mg.) in water (1 ml.). The reduced product had Rg 0.23 in Solvent A.

The reduced material was hydrolysed with N-sulphuric acid. Chromatographic examination on tungstate impregnated paper at pH8 in Solvent F of the neutralised hydrolysate revealed spots with mobilities of glucose, galactose and sorbitol. Ionophoresis in molybdate buffer at pH5 gave a spot with $M_{sorbitol}$ 1.0, along with material which did not complex at all with the buffer.

iv) Degree of Polymerisation

Fraction A was found to have a degree of polymerisation of 3.1 by the method previously described (Expt.33, p.83).

v) Methylation of Fraction A

Fraction A (5 mg.) was dissolved in dimethyl formamide (1 ml.) and placed in the cold room. Cold redistilled methyl iodide (1 ml.) was added slowly, dropwise, and silver oxide (0.5 g.) introduced. The mixture was shaken for 1 hour in the cold, in darkness, and then for 60 hours at room temperature, also in the dark. The mixture was filtered, the residue washed with dry chloroform, and the combined filtrate and washings concentrated to a syrup. The derived methylated material was methanolysed in the usual way with 3% methanolic hydrogen chloride for 6 hours under reflux. After neutralising with silver carbonate, the mixture was filtered, the salts washed with methanol, and filtrate and washings taken to dryness. Examination of the derived methylglycosides by gas liquid

chromatography gave peaks with the retention times of methyl 2,3,4,6 tetra-O-methyl glucoside, 2,3,4,6 tetra-O-methyl galactoside and 2,4,6 tri-O-methyl glucoside.

vi) Hydrolysis and Examination of the Methylated Sugars

The mixture of methyl glycosides was hydrolysed with N-sulphuric acid for 4 hours at 100°C, neutralised with barium carbonate, and concentrated to a syrup. Chromatographic examination of the neutralised hydrolysate (Solvent C, Spray 1) revealed pink spots identical in colour and mobility with 2,3,4,6 tetra-O-methylglucose, 2,3,4,6 tetra-O-methylgalactose, and 2,4,6 tri-O-methylglucose. In addition, traces of spots with R_G values of 0.77 (pink) and 0.66 were also detected.

The mixture of methylated sugars was applied to No.1 paper and eluted in Solvent C for 14 hours. Four bands were located, in the normal way, and eluted with methanol.

Band 1 had R_G 1.0. Chromatographic examination of the demethylated (100) material revealed only glucose.

Band 2 had R_G 0.92 chromatographically identical with 2,3,4,6 tetra-O-methylgalactose, an authentic sample being run as control. Demethylation and chromatographic examination revealed a trace of a spot with the mobility of galactose.

Band 3 was chromatographically identical with 2,4,6 tri-O-methylglucose, run as control, having an R_G 0.85 (c.f. 2,3,6 tri-O-methylglucose R_G 0.88). It had M_G 0.0 on ionophoresis in borate buffer, and demethylation gave only glucose.

Band 4 had R_G 0.77. 2,4,6 tri-O-methylgalactose, run as control, was just a shade faster with R_G 0.78, and had the same colour. The quantity of material isolated was not

sufficient even to give a chromatogram of the demethylated material.

Experiment 41

Examination of Fraction B

Fraction B had an Rg in Solvent A 0.51 and an M_G in borate of 0.57, identical with laminaribiose run as a control in both experiments.

(i) Hydrolysis with Acid

Fraction B (ca. 1 mg.) was hydrolysed with N-sulphuric acid, neutralised with barium carbonate, and examined chromatographically in Solvent A, and ionophoretically in borate buffer at pH10. In both cases only a spot corresponding to glucose could be detected.

(ii) Enzymic Hydrolysis

Fraction B (ca. 2 mg.) was incubated in water with a β-1,3 glucanase at 36° for 24 hours. Chromatographic examination of the enzymic hydrolysate revealed only glucose.

(iii) Estimation of the Degree of Polymerisation

The degree of polymerisation of Fraction B was estimated by the method previously described (Expt. 33, p. 83), and found to be 2.1.

Experiment 42

Examination of Fraction C

(i) Isolation

Fraction C crystallised from 50% aqueous ethanol as colourless prisms, with a melting point of 260-262°C (slight decomp.).

(ii) Chromatography

Fraction C had Rg in Solvent A of 0.67, and reacted

readily with Spray 3, but not at all with Spray 1. In borate buffer at pH10 it had an M_G of 0.82. A sample of the C-methyl inositol, laminitol, kindly donated by Dr. Lindberg, was run as control and had exactly the same chromatographic and ionophoretic mobilities.

(iii) Characterisation

The melting point is similar to that of laminitol (266-269°C), and a mixed melting point did not give a depression.

Experiment 43

Examination of Fraction D

(i) Chromatography

Fraction D had an Rg of 1.15 in Solvents A, B and F, and gave a brown colour when heated with Spray 1. When tungstate impregnated chromatographic paper was used at pH8 in Solvent F, Fraction D had an Rg 0.33. Ionophoresis in borate buffer at pH10 showed this material to have an M_G 0.82.

(ii) Hydrolysis

Fraction D (ca. 10 mg.) was hydrolysed with $\bar{N}$-sulphuric acid at 100°C for four hours. After neutralisation, chromatographic examination revealed (Solvents A and B, Sprays 1 and 3) two spots with the mobilities of xylose and glucose.

(iii) Reduction

Fraction D (ca. 10 mg.) was treated with sodium borohydride (10 mg.) in 0.05M boric acid (ca. 1 ml.) according

to the conditions described on page 108.

After concentration to a syrup, the reduced material was hydrolysed with N-sulphuric acid. Chromatographic, (Solvents A and B, Sprays 1 and 3), and ionophoretic, (borate buffer pH10, Spray 3), examination of the neutralised hydrolysate revealed xylose and an unidentified non-reducing substance with Rg 1.0 (Solvent A), 0.81 (Solvent B), M_G 0.86.

Experiment 44

Examination of Fraction E

Chromatography

In Solvent A, this fraction had an Rg value of 0.45 (Spray 3). It did not give any colour with Spray 1. In Solvent F it had Rg 0.35 on ordinary No.1 paper, and on tungstate impregnated paper at pH8 it had Rg 0.30.

Ionophoresis in borate buffer at pH10 revealed two components with M_G values 0.04 and 0.46 (elongated spot), (Spray 3). In arsenite buffer (0.2M) at pH9.6 (142) only one spot was detected with the same mobility as myoinositol. In this case, the substances on the chromatogram were detected by (a) dipping the dried ionophoretogram in a mixture of concentrated nitric acid and ethanol (1:9) and heating at 95°C for 10 minutes, and (b) spraying with a solution of calcium chloride (5%) in 50% aqueous ethanol and heating at 95°C for 15 minutes. Fraction E showed up as a pink spot, as did the controls, myoinositol and (+)-inositol.

Experiment 45

Examination of Fraction F

(i) Chromatography

In Solvents A, B and F, this fraction had an Rg value

of 1.0. Ionophoresis in borate buffer at pH10 revealed a single spot at M_G 1.0.

(ii) Characterisation of Glucose

a) Fraction F (ca. 5 mg.) was incubated in water at 36°C with the enzyme glucose oxidase (ca. 0.5 mg.). Chromatographic, (Solvents A and B, Spray 3), and ionophoretic, (phosphate buffer, pH7.2), examination revealed complete conversion of Fraction F to gluconic acid, an authentic sample being run as control.

b) Fraction F (ca. 5 mg.) was reduced with sodium borohydride (ca. 10 mg.). Chromatographic examination of the reduced material (Solvent F, Spray 3), using tungstate impregnated paper, revealed a strong spot with the mobility of sorbitol. Ionophoresis in sodium molybdate at pH5 gave a spot with $M_{sorbitol}$ 1.0.

DISCUSSION

The cold water extract, after removal of polysaccharide material by alcohol precipitation (Expt.4, p.44), still contained carbohydrate in solution. This aqueous ethanolic solution (B), on concentration and freeze-drying, gave a greenish coloured solid with a reducing sugar content of 20%, (Expt.37). Chromatographic examination of Solution (B) at this stage did not give good separations of the constituents, due to the considerable quantity of inorganic salts present, and to contaminating colouring matter. The inorganic material (10% of the original weight) was derived from the substrate on which P. tricornutum was grown.

Colouring matter was removed (Expt.38) with activated animal charcoal, and the Solution (B) partially deionised by concentrating to small volume and centrifuging off insoluble salts which were precipitated. Complete deionisation was effected by passing Solution (B) several times through columns of IR 120(H^+) and IR 45(OH^-) resins. Chromatographic examination then revealed at least seven substances, only two of which were non-reducing.

Attempts to fractionate the mixture into individual compounds on a cellulose column were only partly successful (Expt. 39). Elution with Solvent G failed to separate the constituents, but Solvent A, which gave the best results with paper chromatograms, gave a partial fractionation. Final separation into chromatographically pure substances was achieved on 3MM paper, previously exhaustively washed with distilled water. Six

different fractions (A-F) were isolated and investigated individually.

Fraction A was a reducing compound with a chromatographic mobility Rg 0.22, which on acid hydrolysis gave glucose and galactose (Expt.40(i)). Enzymic hydrolysis (Expt.40(ii)) by a β-1,3 glucanase gave glucose along with a substance with Rg 0.5 which was not hydrolysed on prolonged incubation with the enzyme. The fact that glucose was produced indicates that there must be at least two adjacent β-1,3 linked glucose residues in the molecule. When Fraction A was reduced with sodium borohydride and hydrolysed with acid, chromatographic examination revealed glucose, galactose and sorbitol. Since this Fraction was found to have a degree of polymerisation of 3.1 (Expt.40(iv)), it would seem from these results that the molecule comprises galactose, as the non-reducing end unit, linked to laminaribiose.

Fraction A was methylated (124), and then methanolysed. Gas liquid chromatography examination of the derived methyl glycosides revealed peaks with the retention times of methyl 2,3,4,6 tetra-O-methylglucoside, methyl 2,3,4,6 tetra-O-methyl-galactoside and methyl 2,4,6 tri-O-methylglucoside. After acid hydrolysis of the methyl glycosides, chromatographic examination revealed spots with the mobilities of 2,3,4,6 tetra-O-methylglucose, 2,3,4,6 tetra-O-methylgalactose and 2,4,6 tri-O-methylglucose. In addition, fainter spots with R_G values of 0.77 and 0.66 were detected, the former comparing closely to 2,4,6 tri-O-methylgalactose.

The mixture of methylated sugars was applied to No.1 paper and four bands were eluted.

Band 1 was identified as 2,3,4,6 tetra-O-methylglucose by its chromatographic mobility and demethylation (100) to glucose.

Band 2 had the exact chromatographic mobility of 2,3,4,6 tetra-O-methylgalactose. Demethylation gave a trace of material with a chromatographic mobility of galactose.

Band 3. This material had R_G 0.85 and M_G 0.0 matching exactly authentic 2,4,6 tri-O-methylglucose run as control. 2,4,6-Tri-O-methylglucose is distinguishable chromatographically from the 2,3,6 derivative (R_G 0.85 and 0.88 respectively). Demethylation gave only glucose.

Band 4 had R_G 0.77. 2,4,6 tri-O-methylgalactose, run as control, had R_G 0.78. Demethylation gave no positive results, probably due to insufficient material.

These latter results indicate that Fraction A is probably a mixture of two trisaccharides, differing in the non-reducing end unit, being galactose in one and glucose in the other. The other two residues are glucose units linked β 1,3' as in laminaribiose. The non-reducing end units are probably linked 1,3' to the second glucose unit since no 2,3,6 tri-O-methylglucose was detected in the methylated products. In addition, ionophoretic examination (Molybdate buffer) of the reduced enzymic hydrolysate (Expt.40(ii)) revealed that the reduced disaccharide had $M_{sorbitol}$ 0.0, characteristic of a 1,3' linkage (141). The possible presence of tri-O-methylgalactose could

arise from slight undermethylation.

Fraction B (Expt.41) was chromatographically and ionophoretically identical to laminaribiose. Acid hydrolysis liberated only glucose, and the material was completely converted to glucose by a β-1,3 glucosidase. Fraction B had a DP of 2.1.

These results thus provide strong evidence that Fraction B is the β-1,3'- linked disaccharide laminaribiose. The presence of this disaccharide is interesting since it has been reported to be synthesised by the brown alga L.cloustoni and because it may be an intermediate product in the biosynthesis of chrysolaminarin, the β-1,3'- linked glucan, already found to be present in P.tricornutum (Part 1(a))

Fraction C was isolated as crystalline material which was chromatographically and ionophoretically identical to the C-methyl inositol laminitol (Expt.42). Although its m.p. was slightly lower than that of laminitol, a mixture of the two compounds did not give a depression in the m.p.

Preliminary chromatographic examination of Fraction D (Expt.43) revealed a spot which, although it gave a brown colour like a hexose when heated with aniline oxalate, did not fluoresce under UV light, a characteristic of other reducing hexoses. Its slower mobility in solvent F on tungstate-impregnated paper, and on ionophoresis,(borate buffer) suggested a possible compound sugar. This was confirmed by

acid hydrolysis. Chromatographic examination of the hydrolysate tentatively identified xylose and glucose. However, when Fraction D was reduced and then hydrolysed, xylose was identified chromatographically along with a non-reducing substance which did not, however, correspond to sorbitol in ionophoretic mobility.

Fraction E (Expt.44), an apparently chromatographically pure substance, gave two spots on ionophoresis, neither of which were reducing; being revealed only slowly with the silver nitrate reagent. The faster of the two had exactly the same chromatographic and ionophoretic mobility as myoinositol run as control, and, furthermore, gave the characteristic red colour of an inositol when heated with concentrated nitric acid and calcium chloride (143). The slower substance had the same M_G as scyllitol, and failed to give a red colour with the concentrated nitric acid reagent, an idiosyncrasy of this particular inositol (142).

In view of the wide occurrence of myoinositol in algae, its presence in this diatom is of considerable interest. Recent work by Loewus (144) has revealed also its biological importance in the metabolism of the uronic acid and pentose containing polysaccharides of the cell wall of the higher plants. In view of the presence of a glucuronosylmannan in P. tricornutum it may well be that it fulfills a similar function in this diatom and in the uronic acid and pentose containing polysaccharides of other algae.

Fraction F (Expt.45), with chromatographic and ionophoretic mobilities identical to glucose, was characterised as

such by its complete conversion to gluconic acid with the enzyme glucose oxidase. Using labelled glucose, Loewus (144) also showed how this sugar was utilized in polysaccharide synthesis through the sugar phosphates and nucleotides.

CONCLUSION

Phaeodactylum tricornutum has been shown to metabolise a wide and biologically important variety of low molecular weight carbohydrates. In addition to glucose, laminaritriose and laminaribiose, a second trisaccharide, O-D-galactopyranosyl-(1→3)-O-D-glucopyranosyl-(1→3)-β-D-glucopyranose was partly characterised. A second disaccharide comprising xylose as the non-reducing entity was also separated, but the identity of the reducing moiety was not established. Three non-reducing substances tentatively identified as laminitol, myoinositol and scyllitol were also separated.

In view of the wide occurrence of mannitol in the Phaeophyceae, its apparent absence in this and other diatoms is somewhat surprising. However, in keeping with this, it must be borne in mind that whereas the β 1,3- linked glucan laminarin of the Phaeophyceae contains a proportion of mannitol terminated chains, the chrysolaminarin isolated from diatoms (82) appears to be devoid of mannitol.

PART II

SMITH DEGRADATION STUDIES ON THE GLUCAN FROM MONODUS SUBTERRANEUS

Part II

Introduction

Monodus Subterraneus Petersen

M. Subterraneus is a non-motile unicellular member of the Xanthophyceae (Fig. 1). This class of alga is quantitatively important in the microfloras of freshwater and soils (145). Monodus subterraneus was first described from a sandy agricultural soil in Denmark (146). The sample used in the following investigation was isolated from wet rocks in a stream near Marion, Connecticut, by Dr. R. A. Lewin, and grown in bacteria-free culture by Dr. Fogg and Dr. Miller in London.

Previous investigations (147) have shown the presence of an essentially β-1-4'-linked glucan. The presence of a small proportion of 1,3' linkages was suggested from the interpretation of a gas-liquid chromatogram of the methanolysed methylated polymer.

It is the purpose of the following investigations to confirm the presence of this minor linkage.

Experimental

Experiment 46

Extraction of M.Subterraneus

METHOD (A)

The organism was extracted in batches of 3 g. (a total of 9 g.), as this was considered to be the most suitable quantity for efficient extraction.

(1) Cold Water Extraction

The colouring matter was removed from the cells (3 g.) by stirring overnight with dimethyl sulphoxide (240 ml.) in an atmosphere of nitrogen. The cell residue was isolated by centrifugation, freed from dimethyl sulphoxide by rapid washing with cold water, and then ground with sand and processed in a homogeniser with water in order to rupture the cell walls. The disintegrated material was centrifuged off.

(2) Hot Aqueous Extraction

The cell residue from the cold water extraction was treated with water (500 ml.) for 3 hours on a boiling water-bath under nitrogen. The hot mixture was centrifuged, and the colourless supernatant liquid set aside.

(3) 50% Aqueous Alcoholic Extract

The residual Monodus was refluxed with 50% aqueous ethanol (500 ml.) for 3 hours on a boiling water-bath under nitrogen. The cell material was separated and the supernatant concentrated under reduced pressure and set aside.

(4) Chlorite Treatment of the Residual Monodus (103)

The residual material in aqueous acetic acid

(0.25 ml. glacial acetic acid in 70 ml. water) was treated with solid sodium chlorite (3 x 400 mg.), added at hourly intervals, on a water-bath at 70°C under nitrogen. This treatment bleached the cell residue.

(5) Alkaline Extraction

The colourless residual material was treated with 4% sodium hydroxide (100 ml.) in an atmosphere of nitrogen for five minutes at room temperature. Undissolved cell material was removed and the supernatant neutralised with glacial acetic acid. The white precipitate deposited was removed, and found to contain negligible carbohydrate (97). The supernatant, concentrated at 40°C under reduced pressure to ca. 15 ml., was treated with ethanol (10 volumes), causing the deposition of a white precipitate. This was separated, dried, dissolved in water, and freeze-dried. (Yield ca. 400 mg.) The glucose content of this material, called hereafter glucan 'A', (determined cuprimetrically) (97) varied in the three extractions from 40%-70%.

Experiment 47

Purification of Glucan 'A'

(1) Dialysis

Glucan 'A' (100 mg., 40% carbohydrate) was dissolved in water and dialysed against running water for two days, and then against distilled water for twelve hours. The solution, after concentration, was freeze-dried. Glucan 'A' recovered, weighed ca. 90 mg., carbohydrate content 45%.

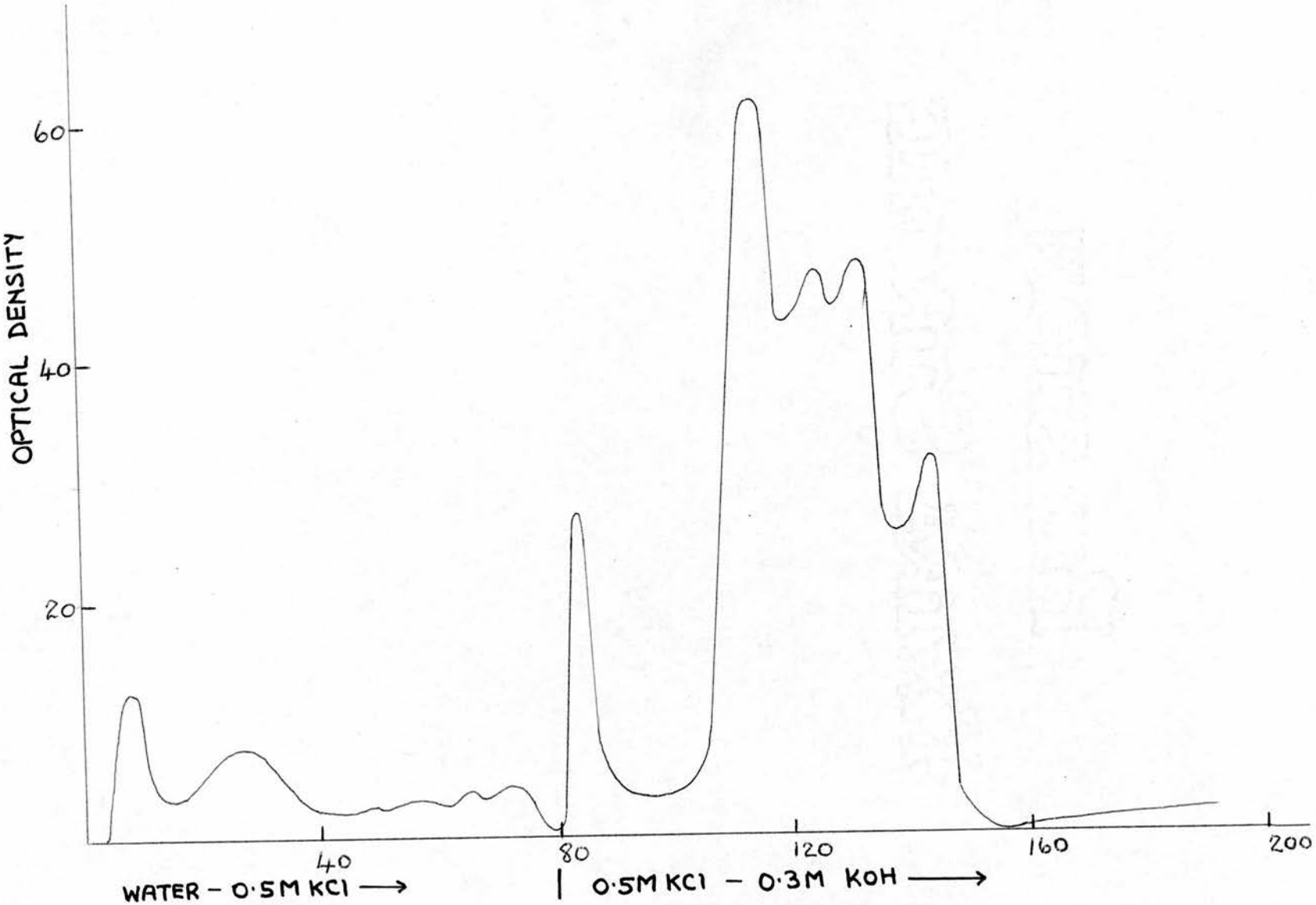
(2) Reprecipitation

Glucan 'A' (ca. 90 mg., 40% carbohydrate), was

GRAPH 8

FRACTIONATION OF GLUCAN A

ON DEAE-CELLULOSE



dissolved in water, and reprecipated with ethanol (10 volumes). A white precipitate of Glucan 'A' was obtained with unchanged carbohydrate content. After redissolving in water, addition of a few drops of $\bar{N}$-hydrochloric acid gave a white precipitate of non-carbohydrate material. Treatment of the supernatant with ethanol (10 volumes) gave a white precipitate of Glucan 'A' (ca. 60 mg.) with a carbohydrate content of 50%.

(3) Attempted Purification with DEAE-Cellulose (104)(105)

A column containing DEAE cellulose was prepared as previously described (p.47). Glucan 'A' (570 mg., ca. 50% carbohydrate) was suspended in water (20 c.c.), applied to the top of the column, and allowed to sink in before elution commenced. A gradient system was used. Initially, the eluent used was from water up to 0.5$\bar{M}$ KCl. (1.1l. of each solution). This system failed to elute any appreciable carbohydrate material (see graph 8). A second gradient system of 0.5$\bar{M}$ KCl. up to 0.3$\bar{M}$ KOH (1 l. of each solution) eluted polysaccharide material of a purity less than that of the material applied to the column. Aliquots (1 ml.) of the eluent were analysed by the phenol/sulphuric acid method (98), and the polysaccharide isolated after dialysis and concentration by freeze-drying.

Four fractions were collected :

Fraction	Tube Nos.	Wt. of p/sac. (mg.)	%age carbohydrate
1	109-120	106	26
2	121-132	78	45
3	133-140	62	23
4	141-148	67	12

Isolation of the material from the neutral eluent gave a white solid (81 mg.),of carbohydrate content 10%.

Experiment 48

Extraction of Monodus Subterraneus

METHOD (B)

(1) Decolourisation

Monodus subterraneus (ca. 6 g.)was frozen with liquid nitrogen, then ground thoroughly. The pulverised cells were extracted exhaustively with n-butanol (6 x 100 ml.), till no more colour was removed by the solvent.

(2) Cold Water Extraction

The decolourised material was extracted thoroughly with water (3 x 200 ml.) for two six-hour periods, then finally for twelve hours. The residual material was then sequentially extracted with (3) hot water and (4) chlorite as before (p.121).

(5) Alkaline Extraction

The bleached residue was stirred with 4% sodium hydroxide at room temperature under an atmosphere of nitrogen. The residual material was centrifuged down, and the supernatant neutralised with glacial acetic acid. The white precipitate of non-carbohydrate material was separated from the solution, which on concentration and treatment with ethanol (10 volumes) yielded a white precipitate (770 mg.) (Glucan 'A') of carbohydrate content 40%.

Experiment 49

Purification of Glucan 'A' using Phenol

Glucan 'A' (770 mg.) was dissolved in water (19 ml.). An

aqueous solution of phenol (90%,19 ml.) was added, giving final concentrations of 2% w.r.t. Glucan 'A', and 45% w.r.t. phenol. The opaque solution was shaken vigorously for thirty minutes before storing in the refrigerator overnight to allow the two layers to separate. Emulsion at the interface of the two phases was dispersed by warming with butyric acid (ca. 1 ml.) The aqueous layer containing the polysaccharide was run off. Phenol contaminating the solution was removed by shaking with ether. Glucan 'A' was finally isolated as a freeze-dried, white, solid (163 mg. 80% carbohydrate). Overall yield = 42%. Hydrolysis, and chromatographic investigation (Solvent A, Spray 1 and 3) revealed only glucose.

Experiment 50

Smith Degradation (109) of Glucan 'A'

(i) Periodate Oxidation of Glucan 'A'

Glucan 'A' (135.5 mg.,80%) was dissolved in sodium metaperiodate (20 ml.,0.14 $\bar{M}$) at room temperature, in the dark. Aliquots (1 ml.) were withdrawn and their optical densities measured at 223 mμ.

Time (hours)	Moles. periodate reduced per C_6 anhydro Unit
6	0.60
12	0.85
24	0.99
48	0.99

Excess periodate was destroyed by bubbling sulphur dioxide gas through the solution for several minutes. The oxopoly-

saccharide was precipitated by addition of ethanol (10 volumes), washed several times with ethanol, and dried.

(ii) Reduction of Glucan 'A' Oxopolysaccharide

The oxopolysaccharide was dissolved in 0.05M boric acid (10 ml.). Sodium borohydride (200 mg.) in water (5 ml.) was added to the polysaccharide solution, and the reduction was allowed to proceed at room temperature for twenty-four hours. Excess sodium borohydride was destroyed by dropping glacial acetic acid into the solution until it was just neutral.

(iii) Mild Hydrolysis

Sulphuric acid was added to the solution to give a concentration of 1$\bar{N}$ w.r.t. the sulphuric acid. After allowing the mixture to stand at room temperature for two days, addition of ethanol (10 volumes) failed to precipitate any polysaccharide. The solution was neutralised with barium carbonate, followed by shaking with IR 120H$^+$,(borate was removed by repeated evaporation with methanol (six times)),before concentrating to small volume (ca. 0.5 ml.) and investigating the carbohydrate mixture chromatographically (Solvents A and B, Sprays 1 and 3). Substances detected were glycollic aldehyde, erythritol, and a slower moving spot with Rg = 0.89 (Solvent B, Spray 3), M_G = 0.2 (borate, pH 10, 750 v.).

The hydrolysate was separated on large sheets of Whatman No.1 paper (Solvent B, Spray 3). Side strips were cut and sprayed, and the appropriate areas of the chromatograms eluted with water. Hydrolysis and chromatographic investigation of the material R_G 0.89, showed spots with mobilities of glucose and erythritol. Treatment with the specific enzyme glucose

oxidase gave spots with mobilities of gluconic acid and erythritol (Solvent B, Spray 3 and phosphate buffer (b),Spray 3). No residual glucose could be detected.

Discussion

Two methods were investigated for the preliminary preparation of the Monodus cells for extraction of the polysaccharide, and extraction of colouring matter. Method (A) (Expt.46) (148) involved decolourising the organism with dimethyl sulphoxide followed by processing the cells with silver sand and water in a homogeniser. The ruptured cells were then sequentially extracted with hot water, hot 50% aqueous ethanol and sodium chlorite. Subsequent treatment of the bleached residual material with 4% sodium hydroxide at room temperature led to the isolation of impure polysaccharide material.

Method (B) (Expt.48) involved freezing the Monodus organisms in liquid nitrogen, grinding thoroughly and decolourising the ruptured cells with n-butanol. After cold water, hot water and sodium chlorite extractions, treatment of the residual material with dilute alkali, as above, led to the isolation of polysaccharide material of similar purity and amount.

Several methods of purification were investigated (Expt.47). In this case DEAE-cellulose, successful in the fractionation of the glucan from P. tricornutum (Part 1(a)), gave no improvement whatsoever. This is in keeping with the evidence that Glucan 'A' is a linear polymer (105). It was eventually purified with phenol (Expt.49).

When Glucan 'A' was oxidised with sodium periodate (Expt. 50), 0.99 moles. of periodate were reduced per C_6 anhydro unit, indicating that a high proportion of the glucose residues had been cleaved. Reduction of the derived oxopolysaccharide

followed by mild hydrolysis and chromatographic examination revealed the presence of erythritol, glycollic aldehyde and a small proportion of glucosylerythritol. The mixture was separated, and the latter material isolated and hydrolysed to glucose and erythritol. Glucose was confirmed by glucose oxidase, showing the presence of uncleaved glucose, (i.e. 1,3' links), in the polysaccharide.

Conclusion

Glucan 'A', an essentially β-1,4'- linked glucan, has thus been shown to contain a small proportion of 1,3' links.

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