STRUCTURAL AND METABOLIC STUDIES OF CARBOHYDRATES IN ALGAE

by

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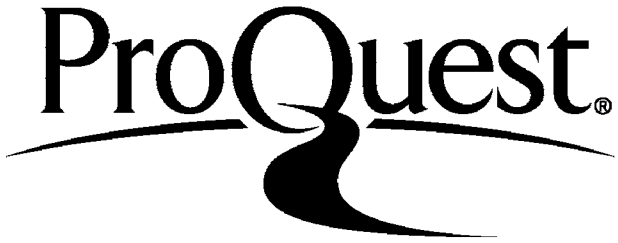

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ABSTRACT

PART I

Investigation of the Carbohydrates synthesised by the marine green alga Acetabularia crenulata.

By means of sequential extractions with different solvents the following carbohydrates were isolated and characterised.

1. From an alcoholic extract D-glucose, D-fructose, allulose, myo-inositol and an alcohol tentatively identified as allo-quercitol. This is only the second time allulose has been found in Nature and the first time in any alga. An homologous series of fructose-containing oligosaccharides were also separated and characterised as 2,1-linked units terminated by a molecule of sucrose at the potential reducing end.

2. Aqueous extraction gave a mixture of a fructan (major) and a sulphated heteropolysaccharide.

a) These two polysaccharides were separated on a column of DEAE-cellulose. Using the classical techniques the fructan was characterised as an inulin type polysaccharide.

b) (i) The sulphated polysaccharide contained D-glucuronic acid, D-galactose, L-rhamnose and small proportions of xylose and 4-O-methylgalactose. Each of the sugars were separated and characterised. The presence of the last sugar has not been reported as a constituent of any green algal polysaccharide before.

(ii) By extraction of stalks and caps separately with cold and hot water and elution from the DEAE-cellulose with 0.5 and 1.0 M potassium chloride similar sulphated polysaccharides with variable proportions of the different sugars and proportions of sulphate were separated.

(iii) Structural studies by partial desulphation, methylation, periodate oxidation and partial hydrolysis established the essential similarity of these fractions and that the main structural features are: highly branched molecules containing 1,3-linked D-galactose, 4-sulphate (major), and 6-sulphate, 1,2-linked L-rhamnose, and glucuronic acid, galactose and rhamnose all present as end groups. Glucuronic acid is linked to both rhamnose and galactose and galactose units are mutually linked in the macromolecule.

3. A β-(1 → 4)linked mannan was extracted with alkali. Methylation, periodate oxidation and gel filtration studies proved this structure and indicated some degree of branching and a higher molecular weight than those of previously reported for mannans from green algae.

PART II

Photosynthetic studies on 1. *Acetabularia mediterranea*, 2. *Fucus vesiculosus* and 3. *Ulva lactuca*.

1. Pulse labelling experiments with $^{14}CO_2$ on *A. mediterranea*

followed by ethanolic extraction led to the separation of labelled sucrose, glucose, fructose and the first three oligosaccharides characterised in Part I (1). A possible biosynthetic interconversion of these carbohydrates is described from the results of these experiments.

2. a) Pulse labelling experiments with $^{14}CO_2$ on Fucus vesiculosus a marine brown alga, show that of the low molecular weight carbohydrates formed by photosynthesis, mannitol is formed first The possible conversion of mannitol into laminaran via mono-and di- glucosylmannitol is discussed.

b) The polysaccharides, laminaran, xylogalactofucoglucuronan (A), xyloglucuronogalactofucan (B), fucoidan (C) and alginic acid were extracted and separated by various fractionation techniques. The radioactivity in each was measured as was the radioactivity in the constituent sugars of the fucose-containing polysaccharides (all of which are sulphated). From the changes in the radioactivity of these polysaccharides in different samples it is postulated that (A) is synthesised first and transformed into (C) via (B). Low molecular weight carbohydrates present in the acid extract are suggested as precursors for the acid polysaccharides. The residual material after acid and alkali extraction was hydrolysed and the radioactivity of the sugars in the hydrolysate was

measured. Glucose was the major radioactive sugar.

3. Ulva lactuca, a marine green alga. Similar experiments on U.lactuca were carried out.

a) Examination of the 80% ethanol extracts showed that sucrose is the first sugar to be synthesised and this is followed by glucose and fructose. Xylose, ribose(?) and myo-inositol also incorporated radioactivity.

b) Starch and a sulphated glucuronoxylorhamnan were extracted and their radioactivities were measured. The former appears to be synthesised most rapidly and to be an active metabolite and the sulphated polysaccharide is laid down as a long term storage product or as part of the skeletal structure of the alga.

c) The residual material after ethanolic and aqueous extractions was examined in the same way. Glucose is again the major sugar and the results indicate that the carbohydrate is laid down in the cell wall after other constituents.

CONTENTS

CONTENTS ..... continued

PART I

STRUCTURAL STUDIES OF CARBOHYDRATES IN ALGAE

Most of the plants growing in the sea belong to the class known as algae. They are one of the most primitive groups in the plant kingdom and evolved early in the earth's history, and morphologically they differ very little from those found as fossils. Although there are some freshwater species, they are mainly found in marine waters. They vary in form from unicellular plants, free-floating in the sea, to species which are several metres long and are fastened to rock with a root-system called a rhizoid. The algae are not differentiated into root, stem and leaves as are the land plants. Instead they have a thallus which varies in form from species to species, some looking almost like flowers with a "stem" and "leaves", and others looking like lettuce, for example, the sea lettuce, Ulva lactuca.

The algae are classified mainly according to their colour, as the difference in pigments generally coincides with important morphological distinctions. They are divided into the following groups: Brown (Phaeophyceae), red (Rhodophyceae), green (Chlorophyceae), and blue-green (Cyanophyceae). They all possess chlorophyll and photosynthesise as do the land plants. Generally the green and brown seaweeds grow nearest to the surface of the sea, while the red ones are found further down, but examples of all types can be found at all levels from the surface.

Brown weeds were early collected for production of iodine,

soda and potash, but now there are other cheaper ways of getting these products. Brown and red weeds have been used as food both for human beings and for animals, particularly in Asian countries. Industries using algae for production of gelling materials such as alginate and agar can be found in several parts of the world. The carbohydrates of the red and brown weeds have received the most attention because of their economic importance. It is only during the last few decades that interest has arisen in the carbohydrates of the green algae. Few chemical studies have been carried out on those of the blue-green algae.

The carbohydrates present in algae can be roughly divided into three groups according to their solubility in various solvents.

I Low molecular weight carbohydrates, (soluble in 80% ethanol)

II Other soluble carbohydrates (mainly water-soluble)
   a) Food reserve material
   b) Other soluble polysaccharides.

III Cellwall material or structural polysaccharides.

I. Low molecular weight carbohydrates.

The algae, like landplants, photosynthesize and it was shown by Bean and Hassid (1955) that the first product of photosynthesis in the red weed Iridaea flaccidum, is 3-phosphoglyceric acid. This is also the first product of photosynthesis in certain landplants (Bassham and Calvin, 1957). Each of the groups of algae contain characteristic low molecular weight carbohydrates.

Most of these compounds are present in very small amounts, except in a few instances where they account for a considerable proportion of the dry weight of the alga.

PHAEOPHYCEAE

D-Mannitol occurs in large amounts in the brown weeds, e.g. up to 25% of the dry weight in some Laminaria species is due to mannitol (Black, 1950), and this is believed to be a storage product and also a substrate forrespiration in these plants (Bidwell 1967).

D-Volemitol has been found in Pelvetia canaliculata and 1-O-D-mannitol-β-D-glucopyranoside, and 1,6-O-D-mannitol-di(-β-D-glucopyranoside), has been reported in several brown algae (Lindberg and Paju, 1954). Laminitol has been found in Laminaria-species (Lindberg and McPherson, 1954) and sucrose, galactose and mannose have been found in Cladostephus species (Fanshawe and Percival, 1958).

RHODOPHYCEAE.

Floridoside, 2-O-glycerol-α-D-galactoside, is present in most species of the Rhodophyceae (Majak et al, 1966), and seems to be an end-product of photosynthesis and a reserve material in the red algae (Bidwell, 1958). In species lacking floridoside, 2-D-glyceric acid-α-D-mannopyranoside seems to be the most important sugar-derivative, e.g. in Polysiphonia (Colin and Augier, 1939). In addition to these, 3-O-floridoside-α-D-mannopyranoside and

iso-floridoside (1-O-glycerol-α-D-galactopyranoside) has been isolated from several red algae (Majak et al, 1966a) and a small quantity of mannitol has also been found (Craigie et al, 1966).

CHLOROPHYCEAE.

The main low molecular carbohydrate of photosynthesis in green algae seems to be sucrose (Craigie et al, 1966b). Sucrose, glucose and fructose have all been found in all the species investigated. In addition small quantities of other sugars, some polyalcohols and myo-inositol have been found in a few species. Recently a new sugar, sucroselactate, has been found in a freshwater Rhizoclonium species and in the marine alga Cladophora laetevirens (Percival and Young, 1971).

II. Soluble, higher molecular weight carbohydrates.

a. Food reserve material.

PHAEOPHYCEAE

Laminaran, a β-(1→3) linked glucan (Bächli and Percival, 1952) is present in most of the brown algae (Quillet, 1958). In the Laminarias the amount present varies from 20-36% of the dry weight of the fronds in autumn and winter - to only a small percentage in spring (Black and Dewar, 1949). Seasonal variations are not so great in the fucans.

Laminaran yields glucose as the main reducing sugar present after acid hydrolysis (Beattie et al, 1961) but small proportions (0.2%) of mannose have been reported (Smith and Unrau, 1959). Peat et al (1958) found 1.7% and 2.7% of mannitol

in insoluble (from L.hyperborea)and soluble (from L.digitata) laminaran respectively. From the proportions of mannitol present, it was calculated that 40% of the molecules have mannitol as the terminating carbohydrate at the potential reducing end of the molecule, linked at C-1 or C-6, and the rest of the molecule terminated by a reducing glucose unit linked at C-3. A small quantity of gentiobiose is also obtained when the laminaran is subjected to mild acid hydrolysis, indicating the presence of (1 → 6) links. There is still some dispute as to whether this is due to a true branch point or to (1 → 6) inter-residue linkages. But there is a greater evidence for (1 → 6) branch points than for (1 → 6) inter-residue linkages in the samples of insoluble laminaran from L.hyperborea and the soluble laminaran from L.digitata (Percival and McDowell, 1967).

RHODOPHYCEAE

Floridean starch. This reserve polysaccharide was first isolated from Furcellaria fastigiata by Kylin in 1913, and shown to be a glucan which is related structurally to starch since it was hydrolysed by malt diastase. Floridean starch from Dilsea edulis was studied by Fleming et al (1956) who found an average chainlength of 9 glucose-residues and the presence of (1 → 6) branch-points. This polysaccharide is an amylopectin-glycogen-type polysaccharide. Peat et al (1959) have shown that floridean starch is closely related to amylopectin of land plants. A

small proportion of α (1 → 3) linkages are present as shown by the isolation of nigerose from a partial acid hydrolysate.

Floridean starch is universally present in the red algae.

CHLOROPHYCEAE.

Starch. X-ray diffraction patterns of starches from various species of green algae closely resemble those of starches found in land plants (Meeuse and Kreger 1954). They comprise approximately 2% of the dry weight of the algae. The first to be investigated was the glucan from Caulerpa filiformis (Mackie and Percival, 1960). Fractionation of this starch was unsuccessful, but any amylose present might have been destroyed during the fairly drastic pretreatment of the weed during this extraction of the polysaccharides. Methylation showed the presence of (1 → 4) and (1 → 6) linkages, and an average chainlength of 23 units. These data show that the glucan is probably an amylopectin-type polysaccharide. Love et al (1963) isolated starches from a number of green seaweeds by precipitation as their starch-iodine complexes from the aquous extracts of these weeds. All except that from Caulerpa were separated into amylose and amylopectin. There seems to be a great similarity between the starch from green seaweeds and that from land plants, although starches from the Chlorophyceae are more readily hydrolysed than those of land plants (Meeuse and Smith, 1962) and appear to have a lower molecular weight.

Fructans, thought to be of the inulin-type, i.e. (2 → 1) linked, have been found in various green algae, (du Merac, 1955, 1956; Percival and Young, 1971). As the study of fructans represent a part of this thesis, they will be discussed in more detail later.

b. Other soluble polysaccharides.

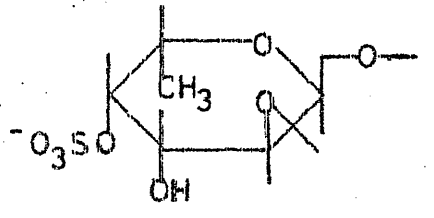
According to Preston (1958), these polysaccharides are mainly constituents of the continuous amorphous phase of the cellwalls and comprise 30-70% of the dry weight in different algae (Myers and Preston, 1959).

PHAEOPHYCEAE

Fucoidan, found in all brown seaweeds, was first isolated by Kylin (1913). It is thought to occur in the intercellular tissues or mucilaginous matrix (Black, 1954) and has been shown to be present in the exudate from the fronds of Laminaria digitata (Lunde and Heen, 1937), Ascophyllum nodosum (Dillon et al, 1953) and Macrocystis (Schweiger, 1962). Hydrolysis of fucoidan gives mainly fucose, but galactose, xylose and uronic acid have also been found. In a highly purified sample from Himanthalia lorea, the fucose content was 56.7%, 36% sulphate, 9% galactose, 1.5% xylose and ca. 3% uronic acid (Percival and Ross, 1950). Fucoidan extracted from Fucus vesiculosus and Ascophyllum nodosum when subjected to free boundary electrophoresis (Larsen and Haug, 1963), gave rise to three different boundaries, indicating that this fraction comprises more than one polysaccharide. A fucan, free

from any other sugar units has so far never been isolated.

The fucoidan from F.vesiculosus, with fucose-content 38% and sulphate content 32.8%, has been studied (Percival and Ross, 1950; Conchie and Percival, 1952). This high sulphate content corresponds to about one sulphate group per fucose unit. Methylation studies combined with the fact that most of the sulphate is stable to alkali, indicate that the main structural feature of this polysaccharide is (1 → 2) linked L-fucose-units, sulphated on C_4:



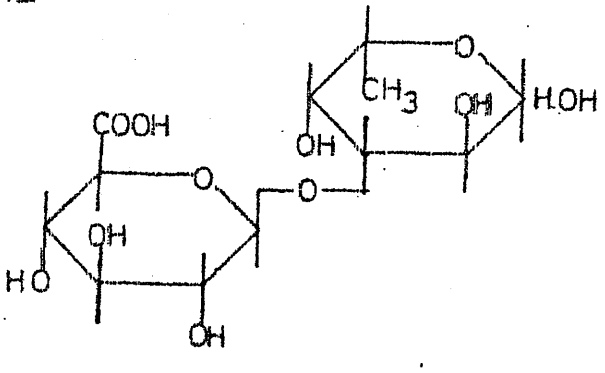
After complete methylation of the fucoidan, free fucose was found in the hydrolysate indicating the presence of either a disulphated residue or a branchpoint with one sulphate.

"Glucuronoxylofucans" Extraction of brown algae with dilute alkali, following the removal of acid-soluble carbohydrates, yields a mixture of alginic acid and a sulphated polysaccharide. This polysaccharide fraction from Ascophyllum nodosum (Haug and Larsen, 1963, Larsen et al, 1966) was called "Ascophyllan" and contained ca.25% fucose, 26% xylose, 19% sodiumuronate, 13% sulphate and 12% protein. Attempts to remove the protein moiety from this polysaccharide were unsuccessful, and it was thought that a chemical linkage

must exist between it and the carbohydrate.

When this polysaccharide was subjected to 0.5N-oxalic acid hydrolysis at 100°C - and dialysed - there remained inside the dialysis tube a soluble polysaccharide which contained almost all of the uronic acid present in the original material. The authors therefore concluded that the backbone of the polysaccharide consists of a glucuronan, with relatively long sidechains of sulphated fucose and xylose residues.

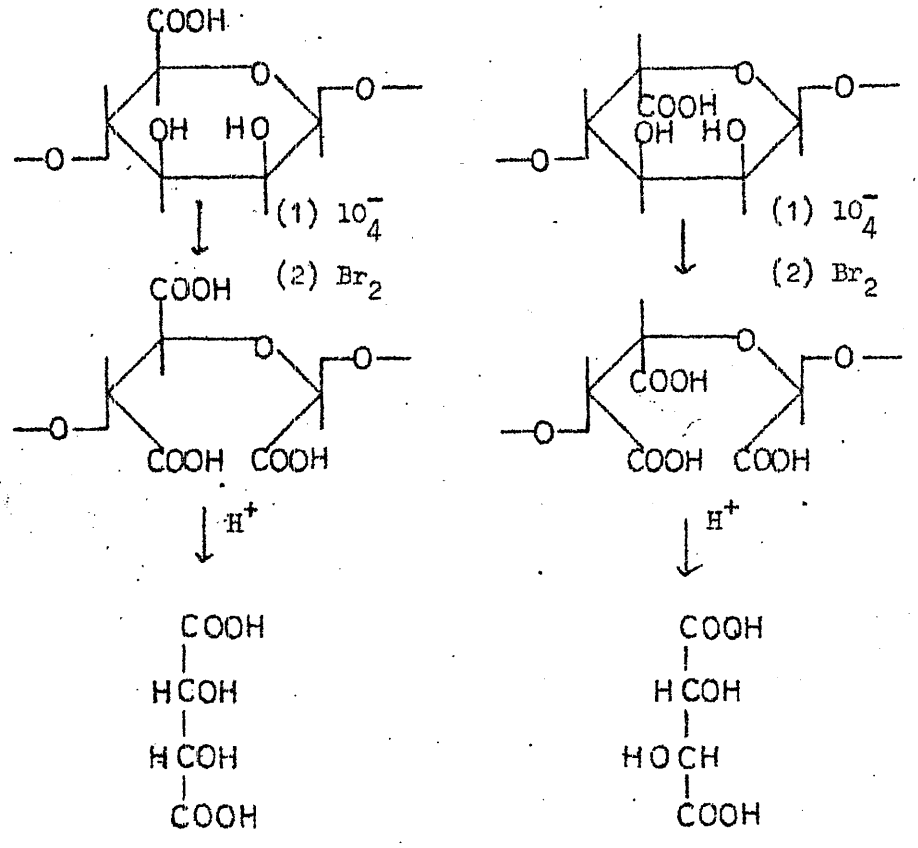
When Ascophyllum nodosum was extracted with ammonium oxalate/oxalic acid at pH 2.8, after acid and alkali extractions, polysaccharides consisting of 17-20% alginic acid and a "glucuronoxylofucan" which contained fucose, xylose and glucuronic acid, in molar proportions 5:1:1, 20% sulphate and 3.8% protein were obtained (Percival, 1968). As auto-hydrolysis of the latter in a dialysis tube resulted in a degraded polysaccharide containing fucose, xylose and glucuronic acid in the molar proportions of 3.5:1:2.5, it was concluded that this polysaccharide does not have a glucuronic acid-backbone. A major oligosaccharide present in a partial acid hydrolysate of this polysaccharide was characterised 3-O-(β-D-glucopyruronosyl)-L-fucose:



Recent studies in this laboratory indicate that "glucuronoxylofucans" comprise a whole family of polysaccharides containing various proportions of fucose, xylose, galactose, glucuronic acid and sulphate, ranging from those with high uronic acid, low fucose and sulphate--contents to low uronic acid and high fucose and sulphate contents (Mian, 1971).

Alginic acid is located mainly in the middle lamella and in the primary cell wall of most brown algae (Kylin, 1915, Anderson, 1956). The alginate is believed to act as a cation exchanger and as such quickly comes into equilibrium with the salts in the surrounding seawater (Wassermann, 1948, 1949). The alginate is present as the salt of a mixture of cations, but the calcium-content is high enough to render it insoluble. The alginic acid content varies with the season (Black, 1950) and seems to be smaller when the algae undergo rapid growth and vice versa. Alginic acid is normally extracted with dilute sodium carbonate at pH8 and purified by precipitation as the calcium-salt.

Alginic acid consists of mannuronic acid (Nelson and Cretcher, 1929, 1930) and guluronic acid units (Fischer and Dörfel, 1955, Drummond et al, 1958 and 1962). Periodate and bromine oxidation of alginic acid followed by hydrolysis gave both threaric and erythraric acid, which shows that both uronic acid residues are (1 → 4) linked.(ص٣I).



Erythraric acid Threaric acid

Fig 1.

When alginic acid was subjected to hydrolysis with 1,M--oxalic acid for 10 hours at 100°C (Haug et al, 1966) part of the polysaccharide was readily hydrolysed to oligosaccharides leaving an insoluble residue. The hydrolysis was repeated, and the residue from this hydrolysed a third time. The first hydrolysis gave a more rapid depolymerisation than the other two. From this the authors concluded that the polysaccharide had been split randomly in the first hydrolysis, and that the two later hydrolyses

attacked at the ends of the chains. The resistant polysaccharide was separated into two fractions by precipitation of one containing mainly guluronic residues at pH 2.85 and the other one that remained soluble consisted mainly of mannuronic acid residues.

In the supernatant from the first hydrolysis guluronic and mannuronic acid were found in addition to two diuronides. The same diuronides were probably also obtained after the other hydrolyses. Based on these observations, the structure of alginic acid is thought to be a molecule built up of blocks of mannuronic acid and guluronic acid units, separated by sections of alternating guluronic and mannuronic acid residues. The homogenous sections are thought to be protected from hydrolysis by their crystalline character.

The ratio of mannuronic acid to guluronic acid in alginic acid varies in different species of brown algae (Percival and McDowell, 1967).

RHODOPHYCEAE

The water soluble polysaccharides from the red algae are mainly sulphated galactans. They are essentially linear polymers of alternating (1 → 3) and (1 → 4) linked galactose units. This simple pattern is often masked by other features, depending on the alga from which the polysaccharide has been isolated. The differences are due to the presence of both D and L-galactose, 3,6-anhydro-D and L-galactose, monomethylgalactoses and various

degrees of sulphation (Peat and Turvey, 1965, Rees, 1965).

The sulphated galactans can be divided into three distinct types of polymers:-

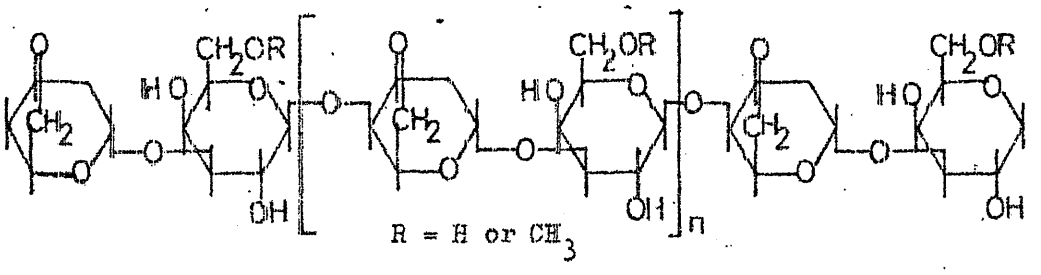
1. the agar-type
2. the carrageenan-type
3. the porphyran-type

although recent studies have revealed polysaccharides related to more than one type, e.g. the galactan from Polysiphonia fastigiata (Peat and Turvey, 1965).

Agar is synthesised by a wide variety of species of the red algae, generally called agarophytes. It is the common name for a mixture of polysaccharides, which originally were thought to consist of two polysaccharides only, namely the neutral polysaccharide agarose and the sulphated polymer agaropectin (Araki, 1937, Araki and Hirase, 1960). The structure of agarose has been studied extensively, both chemically and enzymically, and these results show that the polysaccharide is mainly composed of alternating β-(1 → 3) linked D-galactose and α-(1 → 4) linked 3,6 anhydro-L-galactose units. 6-O-Methyl-D--galactose has also been found.

Enzymic hydrolysis (Araki and Arai, 1956) of the polysaccharide gave the two oligosaccharides, neo-agarobiose (O-3,6-anhydro--α-L-galactopyranosyl-(1 → 3)-galactose and the corresponding tetrasaccharide. No free D- or L- galactose could be detected, which indicates that the 3,6-anhydro-L-galactose is present at the

non-reducing end of the polysaccharide chains. Methylation did not give rise to 2,3,4,6-tetra-O-methyl-galactose, further confirming these findings. The general formula for agarose is believed to be:



Agaropectin. This polysaccharide is sulphated and contains mainly D-galactose and 3,6-anhydro-L-galactose, as in agarose, but some of the 3,6 anhydro-L-galactose residues are thought to be replaced with L-galactose-sulphate(Araki, 1966) and some of the D-galactose in Gelidium species is replaced with the pyruvic acid acetal, 4,6-O-(1-carboxyethylidene)-D-galactose (Hiraae, 1957). Arai (1961) reported the presence of arabinose and glucuronic acid in the agaropectin from Ahnfeltia plicata.

Enzymic studies have shown that agaropectin has some features in common with agarose, as neo-agarose, tetraose and hexaose can be obtained on cleavage of the polysaccharide. The site of the sulphate group is not yet known.

Recent fractionation studies on DEAE-Sephadex A50 (Duckworth and Yaphe, 1971) have indicated that agar is not made up of one neutral and one charged polysaccharide, but is composed of a complex series of related polysaccharides ranging from an almost neutral

polysaccharide to a highly charged galactan. It is found that 4,6-O(1-carboxyethylidene)D-galactose is present in that part of the molecules where there is low sulphate content. Further evidence of heterogeneity was obtained by fractionation on Dowex 1 x 2 ion exchange columns (Izumi, 1971).

Carrageenan. The red algae, Chondrus and Gigartina are the species mostly utilized for preparation of carrageenan. It differs from agar mainly in that 3,6-anhydro-D-galactose replaces the 3,6 anhydro-L-galactose of agar (Percival, 1954, O'Neill, 1955) and in that it has a higher content of, mainly alkali-stable, ester-sulphates.

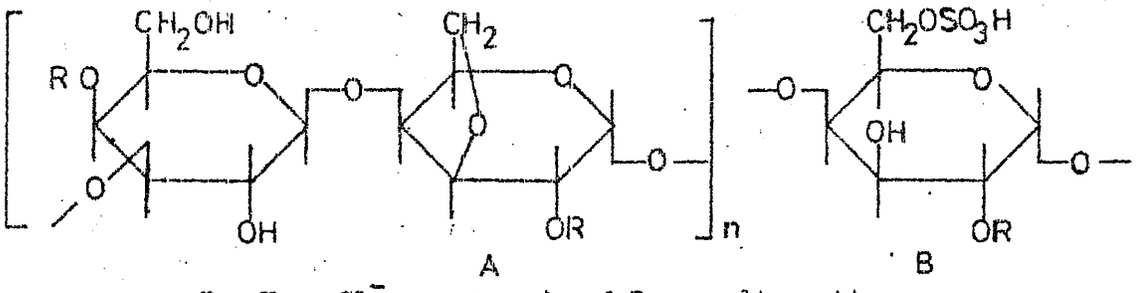
Smith and Cook (1953) separated the carrageenans by fractional precipitation with potassium chloride into an insoluble fraction, K-carrageenan, and a soluble fraction, λ -carrageenan.

K-carrageenan is mainly a linear polymer, of alternating β(1 → 3) linked D-galactose, and α-(1 → 4) linked 3,6 anhydro-D-galactose, some of the units of which are sulphated. The disaccharides carrabiose, O-β-D-galactopyranosyl (1 → 4) -3,6-anhydro-D-galactose, and neo-carrabiose-sulphate, O-α-3,6-anhydrogalactopyranosyl-(1 → 3) D-galactose-4-sulphate, have been isolated by mercaptolysis (O'Neill, 1955) and enzymic hydrolysis respectively (Weigl et al, 1965)

Alkali treatment of K-carrageenan removed some sulphate and more 3,6-anhydro-D-galactose was formed (Rees, 1961) indicating the presence of (1 → 4) linked D-galactose sulphated at C_6 in the original material. The 1 → 3 linked units are thought to have

4-sulphate and the (1 → 4) linked 3,6-anhydro sugars are associated with 2-linked sulphate (Anderson et al, 1968). The (1 → 4) linked D-galactose is probably sulphated at C-6 or C-2 and C-6.

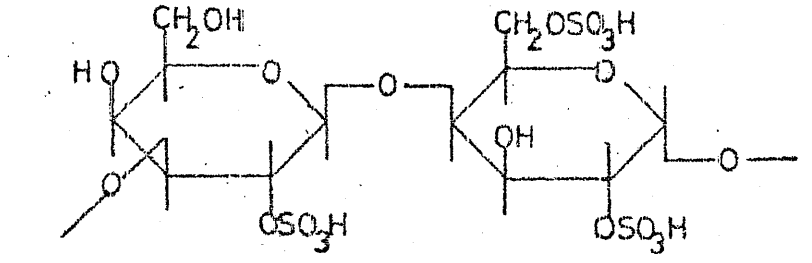
General formula for K-carrageenan:



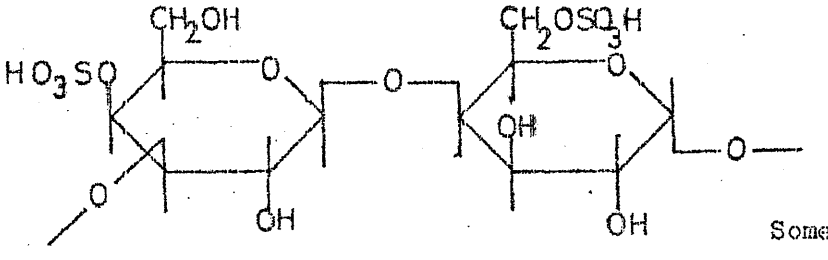
R = H or SO_3^- A and B are alternatives.

λ-carrageenan, the soluble fraction obtained by potassium chloride fractionation, represents a very complex system. It is highly sulphated, contains mainly D-galactose, but also some 3,6-anhydro-D-galactose. The polymers may be fractionated further by precipitation with ethanol, thus giving a polymer containing only D-galactose (Smith et al, 1955). This consists of highly sulphated alternating (1 → 3) and (1 → 4) linked galactose units. Some of the sulphate is alkali-labile, indicating some sulphate on C-3 or C-6. When subjected to precipitation with potassium chloride (Anderson et al, 1968) this gave after alkali treatment an insoluble μ-carrageenan, containing all the (1 → 3) linked D-galactose-4-sulphate units and most of the 3,6-anhydro-D-galactose units, which probably were the D-galactose-6-sulphate units in the unmodified form. The soluble polysaccharide, from modified λ-carregeenan, had all the 3-linked D-galactose-2-sulphate units and a high proportion of the 4-linked 3,6-anhydro-D-galactose-2-sulphate units. The latter is probably the 4-linked D-galactose-

2,6-disulphate unit in the original material.



λ-carrageenan



μ-carrageenan

Some SO_3^- may be replaced by H.

Porphyran This type of polysaccharide is mainly found in Porphyra and Laurentia spp. Porphyran resembles agarose in containing 3,6-anhydro-L-galactose and 6-O-methyl-galactose and resembles carrageenan in containing galactose-6-sulphate (Turvey and Rees, 1961, Su and Hassid, 1962). The polymer consists mainly of alt β-(1 → 3) linked D-galactose or 6-O-methyl-D-galactose residues alternating with α-(1 → 4) linked L-galactose-6-sulphate or 3,6-anhydro galactose, (Turvey and Williams, 1961 and 1964) the proportions of the sugars varying from season to season and in different environment (Rees and Conway, 1962). The sum of L-sugars seems always to be equal to the sum of D-sugars and the galactose-6-sulphate accounts for the total sulphate content of the polysaccharide.

When an alkali-modified porphyran was methylated, a molecule with the same constitution as methylated agarose was produced, and this on methanolysis gave methylated agarobiose (Anderson and Rees, 1965).

There seems to be a great similarity between agarose and porphyran, the main difference being the degree of sulphation of L-galactose and methylation of the D-galactose.

CHLOROPHYCEAE

Sulphated watersoluble polysaccharides are found in all the green marine algae which have been investigated. They can roughly be divided into groups:

1. Containing mainly galactose, arabinose and xylose.

2. Containing rhamnose, xylose and glucuronic acid.

The following species belong to Group 1; Cladophora (Fisher and Percival, 1957) Chaetomorpha (Hirst et al, 1965), Caulerpa (Mackie and Percival, 1961) Codium (Love and Percival, 1964) and Rhizoclonium (Percival and Young, 1971). The polysaccharides have a sulphate ester content of 15-20% and arabinose and galactose are in most samples the major sugars in the polysaccharide. Although D-galactose, L-arabinose, D-xylose and traces of L-rhamnose have been found in these polysaccharides, all attempts to fractionate them into homo-polysaccharides have been unsuccessful(Fisher and Percival, 1957, Johnson and Percival, 1969). Cladophora rupestris was the first seaweed in this group to be studied chemically (Fisher and Percival, 1957). The sulphate esters were found to be stable to alkali and

methylation and periodate oxidation studies gave evidence for xylose and galactose as non-reducing end groups. The rest of the xylose seemed to be present either as (1 → 4) linked units or as 4-sulphated end groups, and galactose as (1 → 3) linked galactopyranose and 1,6 linked galactofuranose. There are also indications that arabinose and rhamnose are present as (1 → 3) linked pyranose residues. After Barry-degradation which removed all the xylose (O'Donnell and Percival, 1959) all the sulphate is believed to be located on arabinose and galactose. Later studies (Hirst et al, 1965) gave an increase in xylose content after alkaline desulphation, indicating sulphate at C-2 or C-3 of arabinose. Treatment with sodium-methoxide gave 3-O-methyl-L-arabinose and 2-O-methyl-L-xylose, indicating sulphate on C-3 of arabinose units. Recent studies (Bourne et al, 1970) have shown that the polysaccharide from C.rupestris is comprised to a considerable extent of blocks of at least eight (1 → 4) linked arabinose units, some sulphated at C-3 and linked together by single galactose units.

Studies on the water soluble polysaccharide from Chaetomorpha (Hirst et al, 1965) indicate that its structure is very similar to that of Cladophora. The main difference is a lower galactose content in Chaetomorpha. The polysaccharide from Codium fragile is also similar to the above mentioned polysaccharides (Love and Percival, 1964). It contains galactose and arabinose as the major sugars, with smaller amounts of mannose, xylose and glucose. The mannose is probably due to contamination with some of the cell-wall mannan.

When fractionated on a DEAE-cellulose column, a small amount of pure sulphated arabinogalactan was obtained as one of the fractions. After alkali treatment of the polysaccharide, an increase in 3,6-anhydro-galactose occurred, indicating sulphate-ester on C-3 or C-6 of galactose. Partial acid hydrolysis gave two disaccharides, galactose (1 $\xrightarrow{\beta}$ 3) galactose and arabinose (1 $\xrightarrow{\beta}$ 3) arabinose. Galactose-6 sulphate and galactose-4-sulphate were also isolated. No arabinose-sulphate was found.

The water soluble polysaccharide from Caulerpa (Mackie and Percival, 1961) has not been studied extensively. Apart from that from Codium, it differs from the other polysaccharides in containing D-mannose and having a very small proportion of arabinose. In fact in one sample arabinose was absent, the xylose present is probably due to a low molecular weight β-(1 → 3) linked xylase which is known to be present in the alga.

No structural studies have been reported on the polysaccharides from Rhizoclonium sp.

Group II The following green algae have been shown to synthesize sulphated polyuronides: Ulva lactuca (Brading et al, 1954) Enteromorpha compressa (McKinnel and Percival, 1962) and Acrosiphonia arcta (O'Donnell and Percival, 1959).

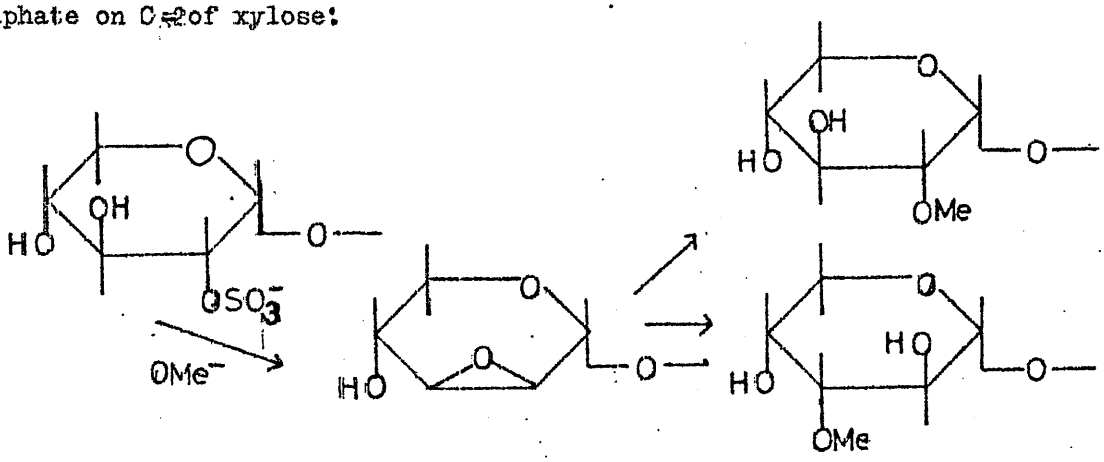
The watersoluble polysaccharides from these three species contain the same sugars and have many similar properties. The uronic acid content is 18-20%, sulphate content 16-17.5% in Ulva and

Enteromorpha and 7.8% in Acrosiphonia. Enteromorpha and Ulva polysaccharides contain similar ratios of L-rhamnose, D-xylose and D-glucose, while that from Acrosiphonia contains relatively less rhamnose. No fractionation into homo-polysaccharides has been successful, but a fractionation of the polysaccharide from Ulva (Percival and Wold, 1963) on DEAE-cellulose gave three fractions, which on hydrolysis were shown to contain the same sugars but a different proportion of sulphate. Ultra centrifugation of one of the fractions and of the mixture, revealed one band for the first and indications of heterogeneity in the latter, suggesting that the mucilage from Ulva lactuca consists of poly-disperse heteropolysaccharides. All these polysaccharides give a high proportion of 4-O-β-D-glucopyruronosyl-L-rhamnose (O'Donnell and Percival, 1959, McKinnel and Percival, 1962), on partial hydrolysis. Methylation studies (Brading et al, 1957, O'Donnell and Percival, 1959) confirmed (1 → 4) linked rhamnose and indicated (1 → 4) linked xylose as well as xylose end groups.

The polysaccharide from Ulva lactuca has been most extensively studied. The position of sulphate ester has been deduced from the following experiments to be at C-2 of rhamnose and C-2 of xylose (Percival and Wold, 1963). When the polysaccharide was subjected to periodate oxidation before and after desulphation, more rhamnose was cleaved in the latter case. When periodate oxidation was performed on the desulphated material at +2°C, rhamnose

was still cleaved, indicating free cis vicinal hydroxyl groups confirming C-4 linkages. An infrared spectrum of the polysaccharide gave a peak at 850 cm^{-1} indicating sulphate on an axial hydroxyl group. When L-rhamnose is present in a polysaccharide it is thought to be more stable in the IC conformation, where C-2 is axial. The sulphate is therefore thought to be on the C-2 position of rhamnose.

After alkaline desulphation, arabinose and lyxose were found in the hydrolysate of the desulphated polysaccharide, and 2-O-methyl-xylose was formed after treatment with sodium methoxide, thus indicating sulphate on C-2 of xylose:

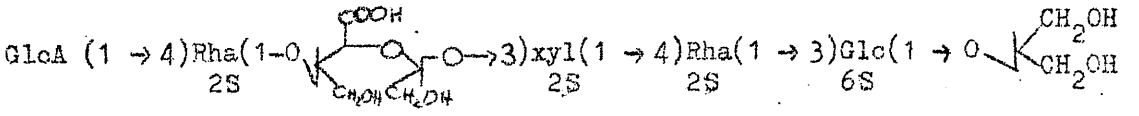


Partial acid hydrolysis (Haug and Percival, 1966) gave two glucuronoxyloses thought to be glucuronic acid (1 → 3) xylose and glucuronic acid (1 → 4) xylose. Hydrolysis of the desulphated reduced polysaccharide from Ulva gave the following oligosaccharide:

rhamnosyl (1 → 4) xylosyl (1 → 3) glucose. (glucose being derived from the glucuronic acid).

An acidic oligosaccharide (A) was isolated after a Smith degradation of

the polysaccharide (Haq and Percival, 1966b).



Methylation studies and gas liquid chromatography (g.l.c.) on this together with the site of sulphate esters indicate the following structure for the oligosaccharide.

GlcA(1 → 4)Rha(1 → 4)GlcA(1 → 3)xyl(1 → 4)Rha(1 → 3)Glc(1 → 4)xyl
2S 2S 2S 6S

This must be a major structural feature of the polysaccharide.

Although the Ulva polysaccharide is the one that has been most extensively studied in this group, it is believed that the others have a similar structure. They are all highly branched, sulphated, contain the same sugars, and give glucuronic acid (1 → 4) rhamnose on a partial acid hydrolysis.

III Cell wall material or structural polysaccharides.

The main organic substances in cell walls are polysaccharides, but it has been proved that proteins and lipids are also present (Northcote et al, 1958, 1960). The carbohydrate part of the cell wall in algae has normally a less complicated structure than the soluble polysaccharide. They can be divided into three groups, according to their constituent sugar:

glucan (cellulose), xylan and mannan.

Cellulose has been found in a number of algae. Its presence in brown algae was postulated as early as 1915 by Kylin,

and structural studies have been carried out on samples from Laminaria hyperborea, L.digitata and Fucus vesiculosus. (Percival and Ross, 1949). Evidence for $\beta(1 \rightarrow 4)$ linkages was obtained and the average chainlength found to be about 160 units.

Cellulose has been postulated in various species of red algae (Ross, 1953) but that from Gelidium amansii (Araki and Hashi, 1948) is the only one whose structure has been studied. This cellulose was found to be similar to that of cotton except for its colloidal properties.

X-ray diffraction studies suggest that cellulose is present in various families of the Chlorophyceae, but no structural studies have been carried out as it has not been possible to isolate pure cellulose.

Xylans have been found mainly in red and green algae, but evidence for the presence of a xylan in brown algae has been obtained (Lloyd, 1960). The xylan of green algae is composed of $\beta(1 \rightarrow 3)$ linked D-xylose units, as found in Caulerpa filiformis (Mackie and Percival, 1959). This was proved by methylation studies, negative rotation and a 90% recovery of the starting material after periodate oxidation. The average chainlength was found to be 42-43 units. The xylan of the red alga Rhodymenia palmata has been extensively studied and found to contain both $\beta(1 \rightarrow 3)$ and $\beta(1 \rightarrow 4)$ linked xylose, mainly the latter (Percival and Chanda, 1950, , Manners and Mitchell, 1963). Recently it

has been shown that this xylan can be separated into two polysaccharides by extraction with water and dilute acid. The essential structures of the two xylans are similar, but the proportions of $\beta(1 \rightarrow 3)$ and $\beta(1 \rightarrow 4)$ linkages differ. (Bjørndal et al, 1965).

Mannans have been found in the cellwall of Rhodophycean and Chlorophyceaæn algae only. All mannans so far investigated comprise $\beta(1 \rightarrow 4)$ linked D-mannose-units, with an average chainlength between 12 and 16 units. Small proportions of glucose do occur in some mannans, but only once has there been found evidence for a true glucomannan (Love and Percival, 1964).

As studies of a mannan comprise a part of this thesis, they will be discussed in more details later.

General methods

Evaporation was carried out under reduced pressure at ca.40°C.

Specific rotations were measured in a 10 cm. long polarimeter tube in a Perkin Elmer 141 polarimeter. Unless otherwise stated, water was used as solvent.

Dialysis was carried out in cellophane tubes against distilled water. Toluene was added inside the dialysis tube to prevent microbial growth.

Carbohydrate content was determined by the phenol-sulphuric acid method (Dubois et al, 1956) Standard graphs were made either of a monosaccharide, or of a synthetic mixture of sugars resembling the polysaccharide being investigated.

Ketose content was determined by the method of Arni and Percival, (1951).

Sulphate content was measured by the method of Scott (1960).

Uronic acid content was determined either by the method of Scott,(cpc), (1960) or the modified carbazole method (Bitter and Muir, 1962).

Melting point was determined on a Gallenkamp micro melting point apparatus.

Water used was either distilled or deionised.

Periodate oxidation was carried out according to the spectrophotometric method of Aspinall and Ferrier (1957).

Degree of polymerisation (DP). This was determined by the method of Timell (1960).

Acid hydrolysis

1. Formic acid hydrolysis

The sample (5-10 mg) was dissolved in 90% formic acid (1 ml) and heated in a sealed tube in a carbondioxide atmosphere at 100°C for 6 hrs. The hydrolysate was diluted with water (5 vols.) and heated for another two hours at 100°C to hydrolyse the formylesters. The solution was evaporated to dryness and all formic acid was removed by repeated evaporation after addition of methanol.

2. Oxalic acid hydrolysis

The sample was dissolved in 2% oxalic acid solution, heated at 80°C for 4 hours and neutralised with calcium carbonate. The neutral hydrolysate was filtered, and evaporated to dryness. Paperchromatography was normally carried out on Whatman No.1 paper. Preparative paper chromatography was carried out on Whatman No.3 MM paper.

Solvent systems:

A. Ethylacetate: Acetic acid: Formic acid: Water - 18:3:1:4

B. n.-Butanol: Ethanol: Water: 40:11:19

C. Ethylacetate Pyridine: Acetic acid: Water - 5:5:1:3

D. n-Butanol: Pyridine: Water - 6:4:3

E. Ethyl-methyl-ketone, half saturated with water + 1% NH_3

F. Chloroform: Acetone: 5 M-NH_3 - 1:8 : 1

G. Benzene: Ethanol - 20:3 or 9: 1.

Sugars and alcohols were located by means of one of the following reagents:

1. Aniline oxalate, saturated in 50% ethanol. After spraying, the paper was heated at 105°C for 5-10 min. (Hough et al, 1950).
2. Urea-hydrochloride. After spraying, heated at 105°C for 5-10 min. (Dedonder, 1952).
3. 2 parts 2% sodium meta-periodate and 1 part 1% potassium permanganate were mixed prior to spraying. The papers were allowed to dry at room temperature (Lemieux and Bauer, 1959)
4. Glucose oxidase (Salton, 1960).
5. Silver nitrate, sodium hydroxide dip. (Trevelyan et al, 1950)
6. Ninhydrin.
7. Periodate-benzidine. (Gordon et al, 1956)

Thin-layer chromatography was carried out on silica gel plates, 0.25 mm thick, in either solvent system F or G.

$$R_x \text{ values} = \frac{\text{Distance travelled by "unknown" compound}}{\text{Distance travelled by reference sugar X}}$$

$$R_f \text{ values} = \frac{\text{Distance travelled by compound}}{\text{Distance travelled by solvent front.}}$$

Electrophoresis was performed in a Shandon High Voltage Electrophoresis Apparatus, model L24 on Whatman No.3 MM paper.

Buffers:

(a) Pyridine acetic acid buffer, pH 6.7
(6 ml glacial acetic acid + 923 ml pyridine), at
3000 V for 2 hours.

(b) Borate buffer pH10 [17.8 g boric acid + 8 g sodium hydroxide in 2 litre water]

$$M_y \text{ values} = \frac{\text{Distance travelled by "unknown" compound}}{\text{Distance travelled by reference compound y)}}$$

Preparation of DEAE-cellulose column

Diethylaminoethyl cellulose (DE 52 microgranular, Whatman) (50 g.) was suspended in 1 l 0.5 N HCl and de-aerated for 20 minutes. The supernatant was decanted off, the cellulose filtered and washed with water till neutral. The cellulose was then suspended in 1 l 0.5 N potassium hydroxide and treated as above. This was repeated three times, and cellulose was then transferred to a column and washed with water until free of chloride ions.

The polysaccharide solution was applied to the top of the column and gradient elution was carried out with potassium chloride (0 - 1.0 M). Fractions of 20 ml were collected and tested for carbohydrate content, and thus the strength of the potassium chloride solution which was required for elution of the various fractions of the polysaccharides was determined.

Gas liquid chromatography (g.l.c.)

A Pye Argon gas chromatograph with an Argon ionisation detector using dry argon as carrier gas was used. The gas flow and temperature was adjusted to get the desired conditions according to the samples being analysed.

Columns used were:

1. Butane-diol-succinate polyester 15%
2. Polyphenyl ether [m-bis-(m-phenoxy-phenoxy) benzene] 10%
3. Apiezon K 7.5% Chromosorb W
4. S.E. 30 3%
5. ECNSS-M 3% Chromosorb W
6. Ethyleneglycol adipate polyester 10%

The liquid phases were coated on Celite or Chromosorb W which had been acid and alkali washed and precoated with dimethyl dichlorosilane.

Preparation of Trimethylsilyl ethers (TMS derivatives) (Sweeley et al, 1963).

The material (5-10 mg) was dried, dissolved in 1 ml pyridine, and to the solution was added 0.1 ml trimethylsilylchloride and 0.2 ml hexamethyl disilazane. After shaking for several minutes, the mixture was evaporated to dryness. This residue was then dissolved in n-hexane before analysis by g.l.c. on columns 3 and 4. Retention times T, for sugars and their alcohols, is relative to that of the trimethylsilyl derivative of xylitol.

Methylation of polysaccharides was carried out after Björndal and Lindberg's (1969) modification of the Hakomori method (1964). The

methylsulphinylcarbanion was prepared as described by Sandford and Conrad (1966).

Methylglycosidation.

The dried hydrolysate was refluxed with dry methanol using methanol treated Amberlite 1 R 120 (H^+) resin as catalyst (Bollenback, 1963). After filtration the mixture was evaporated to dryness. The syrup was dissolved in chloroform and analysed by g.l.c., using columns 1,2 and 6. Retention values T, are relative to that of methyl-2,3,4,6-tetra-O-methyl β-D-glucopyranoside.

Alditol acetates were prepared by the method of Bjørndal et al (1967). After dissolving in chloroform, the mixture was analysed by g.l.c. on column 5.

The retention values T are given relative to the acetate of 2,3,4,6-tetra-O-methylglucitol.

Demethylation. The method of Bonner et al (1960) was used.

Proportions of sugars present in polysaccharides were determined by g.l.c. After hydrolysis of the polysaccharides, the sugars were reduced to their respective alcohols with potassium boro-hydride. The reaction mixture was left over-night and excess borohydride was destroyed by adding Amberlite 1R 120 (H^+) resin which would also remove potassium ions. Borate was then removed by evaporation after addition of methanol. The derived alcohols were analysed by g.l.c. as their trimethylsilyl ethers on column 3. The areas under each peak were measured, and the amount present was read from appropriate standard graphs.

Measurement of radioactivity (i) Liquid scintillation counting.

A measured aliquot of the sample was placed on a filter paper - 2 x 4 cm; after drying, the paper was placed in a counting vial and ca. 12 ml of a scintillant was added. Counting was carried out to an accuracy of ± 3% in a Tracerlab machine. Scintillants were either

A. PPO (2.5-diphenyl-oxazole) 5 g.
DimethylPOPOP (1,4-di-[2(4-methyl-5-phenyl-oxazolyl)]benzene 0.3 g.
Toluene 1 l.

or B. p-Terphenyl 6 g.
DimethylPOPOP 0.3 g.
Toluene 1 l.

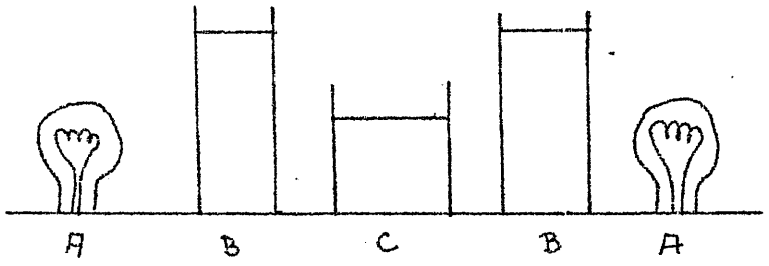
To measure the radioactivity in the different sugars of a polysaccharide, the hydrolysate was chromatographed on paper in appropriate solvent systems. Guide strips were used for the location of the monosaccharides and the appropriate areas were cut out and counted as described above.

(ii) Scanning of paperchromatograms and electrophoretograms was carried out in a Panak thinlayer Radiochromatogram Scanner, after cutting the paper to the right size to fit on top of a thinlayer plate.

(iii) Radioautography was carried out by placing a Kodak Microtex X-ray film on the paperchromatogram and leaving it for ca. 1 week. After development of the film the radioactive components could be

located on the paper chromatogram.

Conditions for the culture experiments.



A. 4 200W. bulbs, incandescent.

B. Waterscreen with constantly changing water

C. Culture vessel.

The culturing experiments were carried out in the system shown in the figure. The light intensity at the centre of the vessel was 1800 footcandles. The water screens were used to prevent heating of the seawater in the culture vessel.

CARBOHYDRATES OF ACETABULARIA sp.

Acetabularia is described by Prescott (1969) as a "gracefully stalked, saucer-like disc with the romantic common name of Mermaid's Wineglass". The alga can be up to 10 cm. tall, grows on shells and corals and is calcified when growing in the sea. It is relatively easy to culture Acetabularia, but then it is not calcified. It is a giant unicellular alga, and because of its abnormal size and the fact that the alga survive for long periods following such operations as enucleation and transplantation, it is probably one of the algae which has been most extensively studied both from a biological and a physiological viewpoint.

Acetabularia is regarded as a sub-family of the Dasycladaceae, order Siphonocladales and class Chlorophyceae (Sweiger, 1969; Keck , 1964). The mature cell consists of rhizoid, stalk and cap. The rhizoid is a ball of entangled branches, one of which contains the cell nucleus. The cap can be up to 10 mm in diameter and is the reproductive organ.

In the main phase of its life cycle, Acetabularia is mononucleate and diploid. After formation of the zygote the alga starts growing. At one end, the rhizoid is formed, which holds the nucleus, and at the other end a stalk starts growing. As the stalk is growing, whorls of hairs develop, which fall off after a short time leaving a ring of small scars on the stalk.

When this reaches a certain stage, fertile "whorls", i.e. the cap, start forming. At this stage, the nucleus starts dividing in the rhizoid and a cytoplasmic streaming takes place, transporting the secondary nuclei and the stalk cytoplasm into the now fully-grown cap. After a certain period the spores (cysts) are formed. After a resting period, gametes are produced which fuse and form the zygote.

The important finding by Hämmerling (1931) that the alga is uninuclear and that the nucleus is located in the rhizoid during the vegetative phase, made it possible to develop techniques for amputation and other operations on the alga. It was found that on a 2 cm. long sample at the time of enucleation it was capable of continuing the growth of the stalk, forming whorls and finally producing a cap (Hämmerling, 1932). Various people have reviewed this type of work and other manipulations carried out on the alga (Schweiger, 1969; Keck, 1964; Brachet and Bonotto, 1970).

Acetabularia has been studied biochemically, mainly to see whether or not there are any differences between nucleate and enucleate cells, and if there are, which parts of the metabolism are concerned. Very few chemical studies on the carbohydrates present in Acetabularia have been done. Claass and Keck (1959) found that the ethanol extract of Acetabularia mediterranea contained fructose, glucose, sucrose and six other ketose-containing

oligosaccharides which they postulated were fructans, because du Merac (1953, 1955) had shown that the reserve polysaccharide was a fructan. X-ray powder photographs and a rotation of -39° indicated that the fructan was probably of the Inulin type.

Studies of the growth zone of A.mediterranea, combined with histochemical studies, indicated that part of the cellwall was composed of anionic polysaccharides, probably of the polyuronide type (Wertz, 1960). Culture experiments involving incorporation of ^{35}S into the alga suggested that sulphate esters were present, in the cellwall, probably as sulphate-containing polysaccharides (Clauss, 1961).

Zetsche (1967) studied the composition of the cellwall of caps and stalks separately. These were extracted first with acetone, followed by 0.1M sodium desoxycholate. The residue was hydrolysed and analysed by paper chromatography. It was found that cap and stalk contained various proportions of the carbohydrates. Galactose, glucose and mannose were the main sugars in both caps and stalks, but rhamnose and traces of xylose were only found in the hydrolysate from the caps.

The cellwall itself, from A.calyculus was thought by Iriki and Miwa (1960) to be a mannan with $[\alpha]_D = -50.6$ (C = 1.54 in 50% $ZnCl_2$). Periodate oxidation suggested a (1 → 4) linked mannan with an average chainlength of 16. No further studies concerning the structure of the carbohydrates in Acetabularia sp. have to the author's knowledge been carried out.

EXPERIMENTAL

Acetabularia crenulata (identified by the Marine Biological Station at Discovery Bay, Jamaica, West Indies) was obtained from various shores on Jamaica. Batch I was collected around Christmas 1968, Batch II in March 1969 and Batch III in September 1969. The alga was contaminated with other algae and sand, and after their removal and separation of the alga into caps and stalks, the following amounts were available for analysis:

TABLE I

	Stalk	Cap
Batch I	2.1 g	6.9 g
Batch II	9.5 g	24.5 g
Batch III	8 g	11 g

As Zetsche (1967) had found a difference in the carbohydrates present in the cellwall of caps and stalks, it was decided to investigate the two parts of the alga separately.

Extraction of the alga. The caps and stalks were separately ground to a fine powder in a mortar in liquid nitrogen, transferred to a Soxhlet apparatus and successively extracted with I Light petroleum (bp 60-80°), II Benzene, III Chloroform. Each extraction was repeated until the extract was colourless. The purpose of this pretreatment was to remove chlorophyll, lipids and other coloured material, which in a preliminary experiment had been found to interfere in paper chromatography of components

soluble in 80% ethanol. All these extracts were discarded.

The pretreated alga was than extracted as follows:

(a) With 80% ethanol, refluxing for 3 periods of 60 minutes each.

(b) The residue from (a) was exhaustively extracted with cold water for several hours.

(c) This was followed by exhaustive hot water extractions (90°C).

(d) The residue from (c) was extracted with 4% sodium hydroxide at room temperature under a nitrogen atmosphere for four hours.*

(e) This was followed by an extraction with 20% sodium hydroxide at 70°C under a nitrogen atmosphere for nine hours.

(f) After washing free from alkali, part of the residue was hydrolysed with formic acid.

Extraction (a) was carried out on all batches of Acetabularia separately, but batch I and II were combined for extractions (b) to (f). Extraction (b) was omitted for batch III, which was extracted first with hot 80% ethanol and then with hot water.

Determination of the carbohydrate and ketose content in the various fractions

The carbohydrate and ketose content of the combined 80% ethanol extracts were measured. Preliminary studies showed that the water-soluble carbohydrates could be separated into neutral and charged materials which after separation were dialysed, freeze-dried and weighed. Extract (e) was purified and weighed. The amounts of

* Hydrolysis showed the presence of galactose, mannose and rhamnose. No further studies were carried out on this fraction.

the various types of carbohydrates in the alga are summarised in Table II.

The samples of alga collected in Jamaica were highly calcified and the extent of calcification was estimated by two methods.

(a) 5.00 ml 1.0 N-H_2SO_4 was added to 211.9 mg of calcified whole Acetabularia cells and the mixture set aside until evolution of carbon dioxide had ceased. The amount of sulphuric acid left was then determined by titration with N-NaOH (3.58 ml) i.e. 1.42 ml 1.0 N-H_2SO_4 was used to release carbon dioxide from the calcium carbonate deposited on the alga. This is equivalent to 140 mg calcium carbonate. Therefore the amount of calcium carbonate deposited on tne alga is ca.65% of the total dry weight.

(b) The calcified whole alga, 133.3 mg, was covered with 4.274 g N-sulphuric acid. This was left in the refrigerator and weighed at intervals. When the weight was constant, carbon dioxide, equivalent to 66 mg calcium carbonate had evolved. A control to correct for any evaporation of water containing the same amount of acid was set up. The content of calcium carbonate was found to be 50% by this method.

TABLE II

Total carbohydrate and ketose content present in the different fractions of the alga. (as percentage of the dry weight of the alga*)

Fraction	Stalk	Cap
80% Ethanol extract		
Carbohydrate content	0.8%	2.6%
Ketose content	0.7%	0.9%
Cold water extract		
Neutral polysaccharide	0.08%	0.7%
Sulphated polysaccharide	0.46%	1.5%
Hot water extract		
Neutral polysaccharide	0.4%	10.3%
Sulphated polysaccharide	0.8%	1.6%
20% NaOH extract	0.95%	1.1%

* If based on non-calcified alga, these percentages would be at least doubled.

Low molecular weight fraction

Preliminary studies involving paper chromatography in solvents A,B and D, and location with reagents 1,2,5 and 6,/p.27 indicated, in addition to sugars and alcohols, the presence of inorganic salts and amino acids, and also the presence of oligo-ketoses. The charged compounds were removed by passing the concentrated extracts successively through columns consisting of Amberlite IR 120(H^+) (17 x 2 cm) and

Amberlite IR 45($HCOO^-$) (20 x 2 cm), and elution with water. The carbohydrate and ketose contents of these derived neutral extracts were measured (see Table II).

The neutral extracts were separated into monosaccharides and oligosaccharides by preparative paper chromatography on Whatman No.3MM paper, using solvent A.

The following components were separated and characterised.

D-Glucose. This had the same chromatographic mobility as authentic glucose in several solvent systems and was confirmed with the glucose-oxidase spray.

D-Fructose. The paperchromatographic mobility was the same as that of authentic fructose, and it gave a blue colour with spray 2. The crystalline 2,3;4,5-di-O-isopropylidene derivative was prepared and had mp and mixed mp 93-95°C (Cadette et al, 1952).(Major component).

Allulose This sugar had $R_{glucose}$ values of 1.45 (solvent B), 1.74 (solvent A) and 1.31 (solvent D). $M_{glucose}$ in borate buffer, 3000V for 1 hour was 0.76. These values were identical to those of an authentic sample of allulose. This allulose was isolated from the leaves of Itea virginiana (kindly supplied by the University of London's Botanical Supply Unit), and was purified by the method of Hough and Stacey,(1963). This is the only known natural source of allulose.

G.l.c. of the trimethylsilyl derivatives of the unknown and of the authentic material both gave three peaks with

identical retention times on column 3 at 175°C; 1.74 (strong) 1.93 (weak) 2.0 (strong). On column 4, under the same conditions, they each gave a single peak with retention time 1.46.

Myo-Inositol An alcohol with the same chromatographic mobility as myo-inositol in solvents A,B and D, was separated. When analysed by g.l.c. as the trimethylsilyl derivative on column 4, both the unknown and the myo-inositol gave a single peak, T = 4.55.

Unknown alcohol When the total neutral extract was left, crystals formed. These had a mp. of 262-263°C and $R_{glucose}$ 1.40 (solvent A). The melting point was not depressed by mixing with authentic allo-quercitol (1,2,3,4/5). Due to lack of more weed, further studies on this were not possible.

Ketose-containing oligomers.

Of these, the fastest moving compound, ran like authentic sucrose in all paper chromatography solvents. The syrup obtained, after separation and purification, was non-reducing and gave on hydrolysis with oxalic acid glucose and fructose in equal quantities.

The lower oligo-ketoses were separated by preparative paper chromatography in solvent B. Their respective $R_{sucrose}$ values were (see Table III):

TABLE III

Solvent	A	B	D
Compound:			
F_3*	0.47	0.61	0.72
F_4	0.22	0.40	0.53
F_5	0.10	0.26	0.39
F_6	0.04	0.14	0.30

* The oligosaccharides are given the symbols F_3, F_4, etc, according to the number of sugar units present.

A slower moving fraction contained higher homologues of the same type of oligosaccharides. This mixture was chromatographed in solvent B with F_5 as reference compound. After 19 days the paper chromatogram was taken out of the tank and oligomers up to F_{15} had separated. As F_5 had been used as a reference compound and the R_f value for this had been found previously, the R_f values of the higher oligomers could be calculated.

The formula $R_m = \log (1/R_f - 1)$ was applied to this series (Bate-Smith and Westall, 1950). When R_m was plotted against the assumed DP, a straight line was obtained (Fig. 2) indicating that the compounds form a true homologous series.

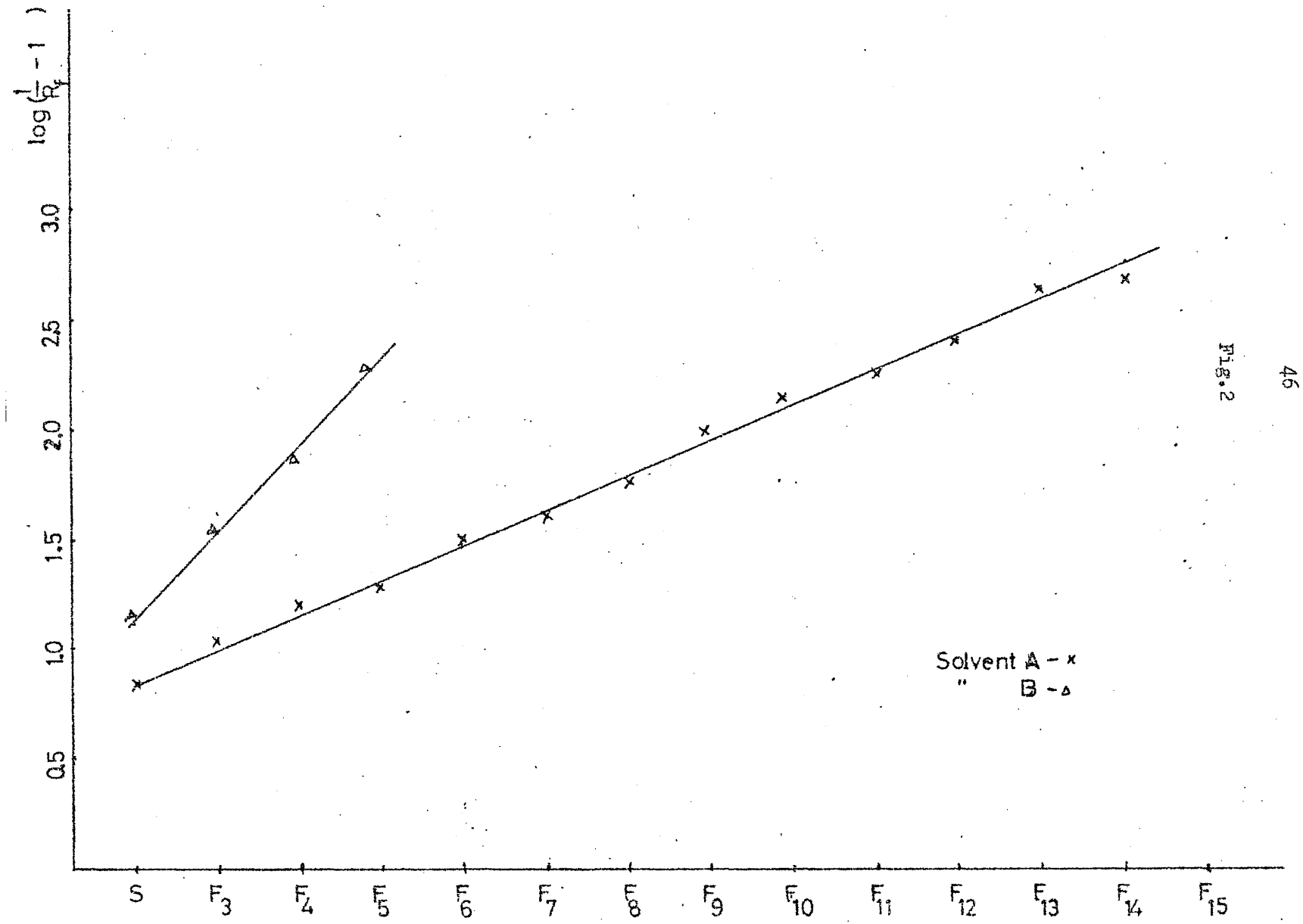

log (1/R_f - 1)
0.5
1.0
1.5
2.0
2.5
3.0
S
F3
F4
F5
F6
F7
F8
F9
F10
F11
F12
F13
F14
F15
Solvent A - x
" B - Δ

Fig.2

Aliquots of F_3, F_4, F_5 and F_6 were hydrolysed with oxalic acid. The syrups gave glucose and fructose upon paper chromatography. The amount of fructose relative to glucose increased from F_3 to F_6.

Methylation of F_4 and F_5.

These two oligosaccharides were separately methylated by one Haworth and two Purdie procedures (Hirst and Percival, 1963, a) and b)). After hydrolysis with oxalic acid, the resultant syrup when chromatographed in solvent E spray 2, revealed two ketose-containing components. R_{fg}-0.62 (A) and 0.86 (B). These compounds were separated by preparative paper chromatography and an aliquot of each was converted into the methyl glycosides and analysed by g.l.c. Column 1 and 2 were used at 175°C. Compound (A) gave peaks with the retention time of those given by methyl 3,4,6-tri-O-methyl-fructofuranosides and compound (B) peaks corresponding to those given by methyl 1,3,4,6-tetra-O-methyl-fructofuranosides and methyl 2,3,4,6-tetra-O-methyl-glucopyranosides (Table IV).

TABLE IV

Compound	T^x Column 1		Column 2	
Methyl 3,4,6-tri-O-methyl-fructofuranoside	0.99	1.49	0.92	1.07
	1.88	2.13	1.27	1.53
Methyl 1,3,4,6-tetra-O-methyl-fructofuranoside	(1.00)*	1.27	(1.00)	1.19
Methyl 2,3,4,6-tetra-O-methyl-glucopyranoside (TMG)	(1.00)	1.40	(1.00)	1.35

x T-values are relative to methyl-2,3,4,6-tetra-O-methyl-β-D-glucopyranoside (TMG)

* Retention times in parenthesis correspond to incompletely resolved peaks.

Another aliquot of compound (A) was subjected to electrophoresis in borate buffer at 3000 V for 2 hours. 1,3,4-Tri-O-methylfructofuranose and 3,4,6-tri-O-methylfructofuranose were run at the same time. (Tetramethylglucose and glucose were used as markers). The 1,3,4-trimethyl derivative does not move, as no complexing with the borate is possible, whereas the 3,4,6-derivatives have free hydroxyl groups at C_1 and C_2 available for complex-formation and therefore moves under the described conditions. of electrophoresis. The tri-O-methyl fructose isolated, moved to the same extent as the authentic 3,4,6-tri-O-methylfructofuranose,

thus confirming the results from the g.l.c. analysis, that the tri-O-methyl-fructose is 3,4,6-tri-O-methyl-fructofuranose (Mg = 0.52).

Discussion

Ethanolic extracts from both caps and stalk of Acetabularia crenulates were separately investigated. Neutral fractions, obtained after removing amino acids, inorganic salts and other charged compounds by ion-exchangers, contained monosaccharides, alcohols and oligosaccharides.

The following monosaccharides were identified: Allulose, D-glucose and D-fructose (p. 43). This is the second natural source where allulose has been detected, the first being in Itea sp. (Hough and Stacey, 1963). The alcohol myo-inositol, was shown to be present and another alcohol was tentatively identified as allo-quercitol.

A homologous series of non-reducing oligofructans of the Inulin-type was also present in both caps and stalks. The presence of 3,4,6-tri-O-methylfructose, 1,3,4,6-tetra-O-methylfructose and 2,3,4,6 tetra-O-methylglucose after methylation of F_4 and F_5 (p. 47) proved that the fructofuranose residues were (2 → 1) linked and a glucopyranose residue was present at the potential reducing end. When $\log \left(\frac{1}{R_f} - 1 \right)$ was plotted against DP of the oligofructans, a straight line was obtained (fig. 2 p. 46) confirming the presence of a true homologous series.

THE FRUCTAN

Introduction

Fructans are widely distributed throughout the plant kingdom. Among land plants they are mainly found in the Graminaceae and Compositae families, where they are known to act as reserve carbohydrates either alone or with starch. Among the algae, fructan has been found only in the Chlorophyceae.

All known fructans contain D-fructofuranose residues linked either (2 → 1) or (2 → 6) to other D-fructofuranose residues. Most of them terminate with (2 → 1) linkage to D-glucopyranose at the potential reducing end, and are non-reducing polysaccharides.

Three main types can be found:

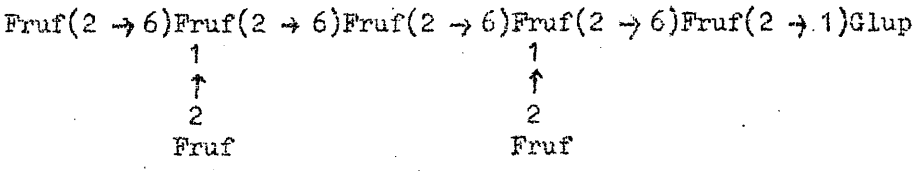
1. Inulin - containing (2 → 1) linked β-D-fructofuranose residues.
2. Levan - containing (2 → 6) linked β-D-fructofuranose residues.
3. Highly branched fructans - containing both types of linkages.

Inulin has been found in Dahlia and Jerusalem artichoke tubers (Hirst et al,1950). Methylation of the dahlia Inulin gave 3,4,6-tri-O-methyl-D-fructose as the main product, showing that this fructan is (2 → 1) linked. Du Merac (1953) reported high molecular weight Inulin in the algae, Acetabularia mediterranea and Batophora oerstedi, whereas lower molecular weight "Inulin" was found in other species of the order Dasycladales (du Merac, 1956). Her characterisation was based on the optical rotation of this "Inulin" found to be -39°, and the presence of fructose in a hydrolysate of the polysaccharide.

Recently fructans of the Inulin type have been found in various species of the Cladophorales and in some Rhizoclonium species (Percival and Young, 1971). All these algae contain a homologous series of linear oligofructans containing (2 → 1) linked fructofuranose residues attached to the fructose moiety of sucrose.

The levan type is mainly found in grasses. It consists mainly of linear chains of 20-30 units terminating in sucrose molecules. It is possible that some grass levans contain single branch points (Bell and Palmer, 1952). The levans from bacteria are probably far more branched than those of plants, and have a much higher molecular weight (Bell and Dedonder, 1954). The branch point was found to be at the 1-position.

Highly branched fructans are mainly found in cereals and plants (Schlubach, 1961). They also terminate in a sucrose unit. Montgomery and Smith (1957) studied the fructan from the wheat endosperm and proposed the following structure. (The distribution between(2 → 1) and (2 → 6) linkages has so far not been determined):



Experimental

The mixture of polysaccharides obtained after dialysis and freezedrying of the cold and hot water extracts of the stalks and caps of Acetabularia (see p. 40) contained both neutral and acidic polysaccharides.

TABLE V.

Total weight of water soluble polysaccharides (crude) from 11.6 g and 31.4 g respectively.

	Stalks	Caps
Cold water soluble	100 mg	1180 mg
Hot water soluble	225 mg	6100 mg

The dialysable fraction

During dialysis of the water extract, carbohydrates passed through the dialysis sac. After the dialysate had been deionised with biodeminrolit-resin in carbonate form, the carbohydrate fraction obtained after precipitation with ethanol, gave on hydrolysis mainly fructose with a trace of glucose. The ratio between glucose and fructose was determined by measuring the total carbohydrate content and the total fructose content and was found to be 1:33.

Attempted separation of the polysaccharides using Sephadex G.75.

A column of Sephadex G.75 (2 x 58 cm.) in 0.5 M sodium sulphate was made. The void volume was 55 ml, determined by passing Blue Dextrandown the column. The total exclusion volume

was 185 ml determined by passing sucrose through the column.

10 mg of the cap polysaccharide was applied to the top of the column and eluted with 0.5 M sodium sulphate. Fractions of 1 ml were collected and tested for carbohydrate and ketose (Forsythe, 1948). The main peak came off just after the void volume. The first 13 fractions, (the main peak (F_1)) did not appear to contain ketose. The second peak (F_2), fractions 19-40, gave a positive ketose test. The carbohydrate content of the two peaks was 5.3 mg and 2 mg respectively. After dialysis against distilled water, the two fractions F_1 and F_2 were hydrolysed. Paper chromatography in solvent A and D gave for F_1 the following sugars when developed with spray 1): Glucuronic acid, galactose, xylose and rhamnose and some oligosaccharides. Spray 2) revealed the presence of fructose in this fraction. F_2 gave mainly fructose with a trace of glucose.

As fructose was present in F_1, this method of fractionation of the polysaccharides was not satisfactory, but this experiment indicated a possible upper molecular weight of the fructan of 50,000, if one can apply the same rules to the fructans as to dextrans. A preliminary experiment using Sephadex G.50 showed that all of the polysaccharide applied to the column was excluded from it, indicating a lower molecular weight of the fructan of 10.000.

Separation by DEAE - cellulose column

The column was prepared as described in general methods. (p.29) After application of a solution of the polysaccharides to the top of the column, the neutral fraction was eluted with deionised water, and in this experiment, followed by elution of the acidic polysaccharide with 1 M potassium chloride. The neutral polysaccharide was isolated by precipitation with 80% ethanol after concentration of the eluates, and the acidic polysaccharide was freeze-dried after dialysis.

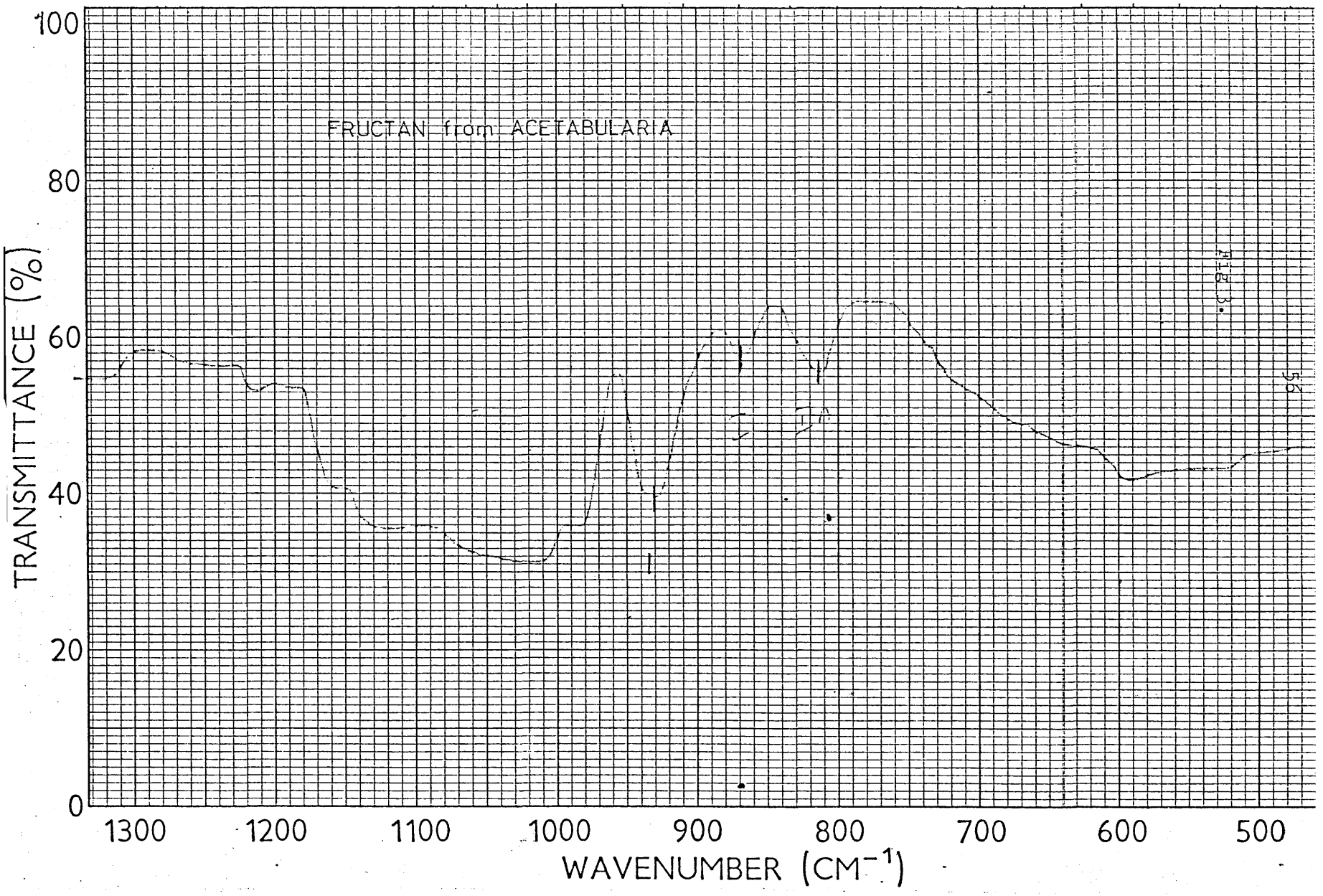
TABLE VI

Weight of polysaccharides obtained from batch I and II

	Stalk		Cap	
	Cold	Hot	Cold	Hot
Neutral polysaccharide	8 mg	44 mg	365 mg	3200 mg
Acidic polysaccharide	55 mg	89 mg	290 mg	500 mg

Examination of the neutral fraction

Hydrolysis, followed by paper chromatography of all four neutral fractions gave fructose as the main sugar, with traces of glucose. The amount of glucose relative to fructose appeared to be greater in the fractions from the stalk than in those from the cap. Iodine test for starch was negative. As there was too little material available from the stalks, no further studies were carried out on this fraction.


FRUCTAN from ACETABULARIA
TRANSMITTANCE (%)
100
80
60
40
20
0
1300
1200
1100
1000
900
800
700
600
500
WAVENUMBER (CM⁻¹)
FIG 3.
56

The fructan from the caps.

The fructan $[\alpha]_D$ - 36.3° (c, 0.5 in H_2O) was hydrolysed with oxalic acid and analysed by paper chromatography using solvents A and B. When developed with spray 2, one blue spot with the same mobility as fructose was visible. Spray 1 showed the presence of glucose. The latter was confirmed with glucose-oxidase spray. The fructose obtained after hydrolysis was purified by preparative paper chromatography and identified as its 2,3-4,5-di-O-iso-propylidene derivative, mp and mixed mp 93-95°C.

The average ratio between glucose and fructose determined by measuring the total carbohydrate content and the total fructose content, was found to be approximately 1:61.

Infrared spectrum of the fructan in a KBr disc in a Unicam SP1000 Infrared Spectrophotometer gave bands at 930, 870 and 815 (cm^{-1}) (see fig. 3) which is characteristic for a spectrum of Inulin (Verstraeten, 1964).

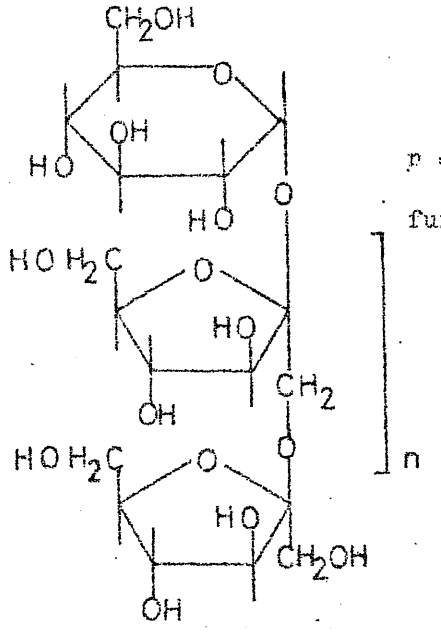
Methylation of the fructan was carried out as for the oligofructans described on p. 47 . Analysis of the methylated fructan gave identical methyl sugars to those obtained from the oligofructans. The 3,4,6-tri-O-methyl-fructose was present in a relatively greater amount.

Discussion

The neutral water-soluble polysaccharides, both from caps and stalks, seems to be a fructan. It has previously been reported that starch amyloplasts have been observed in the chloroplasts of Acetabularia mediterranea (Shephard 1970), but no positive starch test was obtained on the present samples. If any starch is present, it might be in such small quantities that it would be difficult to detect. It is proposed that the higher ratio of glucose to fructose in the fructan from the stalk, than in that from the caps, may indicate that there is some glucan in this fraction, or it may be that the fructan in the stalks has a relatively smaller molecular weight than that of the cap fructan.

The optical rotation of the fructan from the caps, $[\alpha]_D = -36.3^\circ$, is in quite good agreement with what has previously been reported for Inulin (-39°, du Merac, 1953). Methylation studies and the infrared spectrum of the polysaccharide, both confirmed that this fructan is of the Inulin type, i.e. linear (2 → 1) linked fructo-furanose units terminating in a molecule of sucrose. This agrees with du Merac's conclusions after comparison of X-ray powder photographs of her fructan with authentic Inulin. The average molecular size of the fructan eluted from the dialysis sac was found to consist of 33 units, while that remaining inside the dialysis sac had approximately 62 units per molecule. The results obtained by gelfiltration of the latter, indicate a

polydisperse polysaccharide with a lower molecular weight of 10,000 (i.e. 62 units per chain) and an upper limit of about 50,000 (i.e. 300 units per chain).



n = number of fructo-furanose units.

Formula of Inulin

ACIDIC POLYSACCHARIDES

Experimental

The following sulphated polysaccharides obtained earlier (p. 40) were now subjected to structural studies.

Cold water soluble polysaccharide -	stalk	(CS)	55 mg
Hot	"	"	"	(HS)	89 mg
Cold	"	"	caps	(CC)	290 mg
Hot	"	"	"	(HC)	500 mg

General properties of the different extracts

The optical rotations, the carbohydrate contents (calculated from a galactose-graph), the sulphate and uronic acid content of the four polysaccharides were measured (Table I) and the protein content of the HC fraction calculated from the nitrogen content (determined by Alfred Bernhard's laboratory), is given.

TABLE I

	$[\alpha]_D$	Carbohydrate content	Sulphate content	Uronic acid content		Protein
				cpc	carbazole	
CS	-14.8°	54%	24.3%	-	4.0%	-
HS	-13.6°	75%	17.6%	-	5.0%	-
CC	-4.5°	57%	27.8%	7.6%	-	-
HC	-13.8°	56.5%	21.3%	6.8%	-	7.7%

All results based on the freezedried weight of the polysaccharides.

Characterisation of the sugars present.

Hydrolysis (a)

Preliminary hydrolysis with formic acid and paperchromatography of aliquots of these polysaccharides (5 mg) indicated that the same sugars were present in each, but in different proportions.

(b)

The cold water extract of caps (100 mg CC) was hydrolysed with formic acid and the derived syrup was subjected to preparative electrophoresis in buffer (a) (3000 V, 25 mA, 2 hours) The compounds present were located by spraying guidestrips with spray (1). Glucuronic acid was used as a reference compound with glucose as endosmotic marker. Four bands were obtained. Three moved under the described conditions and had M_{glcA} = 1.06(1); 0.87(2); 0.69(3); respectively and one band (4) remained at the starting line.

Separation of acidic and neutral fractions

The four bands were eluted off the paper with deionised water and the eluate filtered through Millipore filter (pore size 25 μ) The following amounts were obtained: (1) - 5 mg; (2) 5 mg; (3) 10 mg; (4) 35 mg.

Each of the charged fractions was tested for sulphate (Ricketts et al, 1959). (1) was positive and (2) and (3) were negative.

Separation of the Acidic fractions.

Paper chromatography in solvent A, spray 1, revealed compounds with the following mobilities:

Fraction 1: Spots with R_{glc} 0; 0.13; 0.36; 0.89; 1.75

Fraction 2: Spots with R_{glc} 0.22; 0.92 (major) 1.61

Fraction 3: Spots with R_{glc} 0.33; 0.70; 0.92.

Fraction 1

Visual examination of the chromatogram indicated that the five constituents of this fraction were present in equal quantities, and that with R_{glc}=0, contained sulphate (toluidine blue spray).

Fraction 2

The major constituent (R_{glc} 0.92) which had the chromatographic mobility of glucuronic acid (solvents A and C) was separated by preparative paperchromatography (solvent A). Both it and standard glucuronic acid when subjected to electrophoresis in borate buffer gave 2 spots with $M_{glcU\ A}$ 1.00 and 0.76. It was converted into the methylester-methylglycoside, and reduced with borohydride. After hydrolysis, paper chromatography of the products showed glucose (major) and glucuronic acid (trace) (spray 2). A second paper sprayed with glucose oxidase gave a pink spot with identical mobility to that of glucose run as a control. G.l.c. of the derived TMS derivatives of the reduced material on column 3, gave a single peak with the same retention time (T, 2.54) as the first peak given by a standard glucose, but the second peak given by glucose (T, 4.55) was absent.

Fraction 3

These three components were separated and purified by preparative paper chromatography in solvent A. Hydrolysis of aliquots indicated that these compounds were oligo-uronic acids and will be dealt with later (p. 77).

Separation of the neutral fraction (fraction 4).

Paper chromatography (solvent A) revealed four compounds which were separated by preparative paper chromatography.

D-galactose. Paper chromatography showed the same mobility as authentic galactose in all solvents. It had $[\alpha]_D = +58^\circ$ (C = 0.3 (H_2O) determined by the phenol-sulphuric acid method) (Literature: D-galactose-$[\alpha]_D = +80^\circ$). The TMS derivatives of the sugar and the derived alcohol had identical retention times to those of standard galactose, T = 2.06 and 2.7, and galactitol, T = 2.84 (column 3) run as control. The 2,4-dinitrophenylhydrazone derivative gave mp and mixed mp 117-118°C (Dominguez, 1951).

Xylose A syrup which had the same paper chromatographic mobility as authentic xylose in all solvent systems was obtained. When subjected to g.l.c. (column 3) as its TMS derivative, it had T = 1.4 and 2.07 (Standard xylose T = 1.43 and 2.07). The TMS derivative of the reduced sugar gave T = 1.00, identical to that of standard xylitol trimethylsilylether.

4-O-methylgalactose. A sugar giving a brown colour when located by spray 1 had the following paperchromatographic mobilities:

Solvent A, R_{gal} - 1.75; Solvent B - R_{gal} - 1.39; Solvent D, R_{gal} - 1.42. (cf. 4-O-methylgalactose - R_{gal} 1.75; 1.39, and 1.42 in the respective solvents). The TMS derivative of the sugar had T - 1.66 and 1.87 (column 3) and that of the derived alcohol was 2.05. An aliquot on demethylation (Bonner et al 1960) gave only galactose.

Preparation of authentic 4-O-methylgalactose. α-Methyl 2,3,6-tri-O-benzoylgalactopyranoside (kindly given to us by Dr.Turvey, Bangor) was methylated after the method of Purdie (Hirst and Percival, 1963). The methylated product was dissolved in anhydrous methanol, ammonia was passed through the solution for 45 min, and then allowed to stand at 0°C overnight, followed by evaporation to dryness (Thompson and Wolfrom, 1963). This residue was hydrolysed to get 4-O-methylgalactose.

L-Rhamnose Paper chromatographic mobility as authentic L-rhamnose in all solvents. The TMS derivatives of the sugars and the derived alcohol had the same retention times as authentic samples (Rhamnose - T = 0.85 - 1.25, Rhamnitol - 1.45) on column 3. $[\alpha]_D$ = +7.9°(C = 0.33, H_2O; determined by the phenol sulphuric acid method) Literature +8.9°. The 2,4-dinitrophenylhydrazone derivative had mp and mixed mp 165°. (Dominguez, 1951).

The Relative Molar Proportions of the Sugars in the four Extracts.

An aliquot (ca. 5 mg) of each of the four polysaccharides was hydrolysed with formic acid, and after reduction, the TMS derivatives of the derived mixture of sugar alcohols were prepared, and analysed by g.l.c. on column 3. The amount present of each sugar was found by measuring the area under each peak and converted into the weight by

reading the corresponding area on a standard graph prepared under the same conditions (Table II)

TABLE II

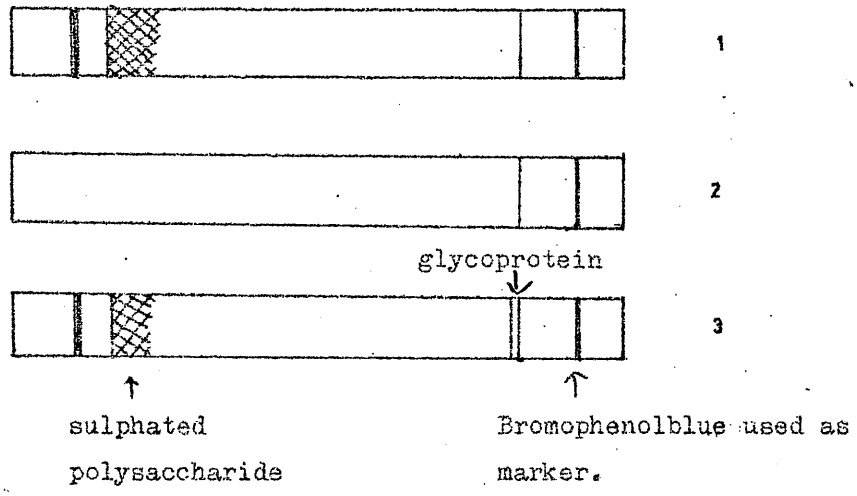
	CS	HS	CC	HC
Galactose	5.0	4.75	2.09	3.0
Xylose	2.0	1.0	0.59	0.55
4-O-methyl-galactose	0.2	0.55	0.59	55
Rhamnose	1	1	1	1
Glucuronic acid*	0.5	0.45	0.6	0.7

* calculated from the uronic acid content Table I.

Due to lack of material, no further studies were carried out on the polysaccharides from the stalks.

Gel Electrophoresis on the Hot Water Acidic Extract of the Caps (HC) (Steward et al, 1965)

a) Analytical gelelectrophoresis. The hot water soluble acidic polysaccharide (HC) was subjected to analytical gel-electrophoresis (Shandon analytical polyacrylamide electrophoresis apparatus, model SAE 2734) using tris-glycine buffer pH 8.3. One gel was stained with toluidine blue (1) for location of sulphated material (Ricketts et al, 1954), one with Amido black (2) for location of protein and a third with "Stains all" (Serva) (3), which will stain any charged material present. The following patterns were observed:



b) Preparative gelelectrophoresis was performed in a Shandon preparative polyacrylamide electrophoresis apparatus, model SAE 2782, using tris-glycine buffer, pH 8.3. The polysaccharide(HC) (15 mg) was applied to the top of the column, and fractions of two ml were collected. The absorption of every other tube was measured at 280 nm for location of protein. A protein fraction was obtained between tube 2 and 14 after elution of the bromophenol blue applied as marker. The tubes were also tested for the presence of carbohydrate. Even after 160 fractions had been collected no carbohydrate had been eluted. The elution was stopped and the gel was removed from the tube, and washed with water. The washings gave a positive carbohydrate test, but even after dialysis of this solution, the polysaccharide was contaminated with some polyacrylamide.

Attempted desulphation of the Cold Water Acidic Extract of the Caps (CC) (Rees, 1961).

25.2 mg polysaccharide CC (p.61) was dissolved in 5.0 ml water. 6 mg sodiumborohydride was added and this was allowed to stand at room temperature for 48 hr. 0.2 g sodium hydroxide and 30 mg sodiumborohydride was added. The flask was loosely stoppered and kept at 80°C for 4 hr. 30 mg sodiumborohydride was then added, and after 10 hours, the mixture was cooled, dialysed and freeze-dried. Weight of recovered material - 24.0 mg. Carbohydrate content 49.3%, sulphate content 24.0% uronic acid content 11.9% (cpc method). As seen the ratio between carbohydrate content and sulphate content is almost the same, both before and after alkali treatment (ca 2: 1).

Further preparation of Acidic Polysaccharides from the Caps.

Caps (11 g), after extraction with hot 80% ethanol, were extracted exhaustively with hot water on a steambath (5 times). The combined extracts were centrifuged, and the supernatant was dialysed, concentrated and freeze-dried (weight 1.8 g).

Fractionation of the Polysaccharide.

Portions of polysaccharide, ca.0.5 g, were applied to the top of a DEAE cellulose column in chloride from (ca. 2.5 x 30 cm) and eluted successively with water, 0.3 M KCl, 0.5 M KCl and 1.0 M KCl (500 ml of each). The following weights were obtained after dialysis and freeze-drying:

Water (fructan)	920 mg
0.3 M KCl	50 mg
0.5 M KCl	105 mg
1.0 M KCl	310 mg

Hydrolysis of the four fractions showed that the first two fractions contained mainly fructose and the latter two were devoid of fructose. No further studies were carried out on the former. The latter two fractions gave on hydrolysis the same sugars, but in different proportions (visually on paper chromatograms).

0.5 M - galactose > glucuronic acid > xylose=4-O-methylgalactose=rhamnose

1.0 M - galactose > rhamnose = glucuronicacid > xylose=4-O-methylgalactose.

Analysis of the two fractions 0.5 M and 1.0 M.

The molecular ratios of the sugars in the two fractions was measured by two methods.

a) An aliquot (10 mg) of each polysaccharide was hydrolysed with formic acid, reduced with sodiumborohydride, converted into the respective TMS derivatives and analysed by g.l.c. on column 3. The relative amounts of sugars were determined as described on p.65.

b) The hydrolysates were paper chromatographed in solvent B, the areas corresponding to each sugar cut out, eluted with water, the eluate filtered through Millipore filter and the amount of sugar present in each determined by the phenol sulphuric acid method.

The results of these measurements are presented in Table III.

TABLE III.

	0.5 M			1.0 M			
	a	b	average	a	b	average	1.OM-S
Galactose	3.50	3.40	3.5	1.25	1.05	1.15	1.3
Xylose	0.42	0.6	0.5	0.13	0.16	0.14	-
4-O-methyl-Galactose	0.40	0.65	0.5	0.1	0.1	0.1	-
Rhamnose	1	1	1.0	1	1	1	1.0
Glucuronic acid*			1.1			0.9	0.3

* calculated from the uronic acid content of the polysaccharides, Table IV.

The specific rotations, carbohydrate, protein, sulphate and uronic acid contents are given in Table IV.

TABLE IV.

	$[\alpha]_D$	Carbo-hydrate	Protein	Sulphate	Uronic acid Carbazole	Uronic acid cpc.
0.5 M	-21.6°	32.4%	20%	6.5%	5.7%	5.1%
1.0 M	-6.8°	53.5%	4.7%	27%	13.3%	10.2%

Investigation of the 0.5 M Fraction

Methylation and Identification of the Derived Methylated Sugars.

The polysaccharide 0.5 M (15 mg) was subjected to methylation after the modified Hakomori method. After this methylation one spot was obtained upon analysis of the methylated polysaccharide by TLC, solvent G, which indicated a fully methylated polysaccharide (Hammer, 1970). The methylated polysaccharide was hydrolysed with formic acid, and the derived hydrolysate subjected to paper chromato-

TABL V

Column 1	Column 2	Column 5	Corresponding to
4.10 > 3.61	(2.47) > 2.14	1.90	Galactose 2,4,6-tri-O-methyl (M)
6.20	3.18 – (2.47)	(2.96)	2,3,4-tri-O-methyl (M)
1.78-(1.65)	1.63 > 1.53	1.18	2,3,4,6-tetra-O-methyl
–	(4.8)	5.17	2,4-di-O-methyl
–	–	3.90	6-O-methyl (?)
–	–	6.55	2-O-methyl
–	–	8.85	free galactose
1.65 > 1.02	(0.63)	0.86	Rhamnose 3.4-di-O-methyl (M)
0.50	0.46	0.46	2,3,4-tri-O-methyl (M)
–	(1.01)	1.63	4-O-methyl
–	–	1.03	2,3-or 2,4-di-O-methyl
0.58-(0.50)	(0.46)	0.64	Xylose 2,3,4-tri-O-methyl
1.35-(1.65)	0.76 > 0.71	–	3,4-di-O-methyl
1.42-(1.65)-(1.75)	(0.63)-(0.76)	–	2,3-di-O-methyl (?)
1.35 (1.01)	–	–	2,O-methyl
2.37 – 3.02	(2.47)-(4.8)	–	Glucuronic acid 2,3,4-tri-O-methyl
–	(4.8)	(2.96)	2,3,-di- O-methyl
0.73-0.96-1.17-2.04-2.42 – 2.90	2.25	7.78	Unidentified peaks.

(M) – major peaks; figures in brackets indicate incompletely resolved peaks.

graphy in solvent B, and the compounds detected with spray 1. Free galactose was detected in addition to several compounds with R_G 0.25, 0.29, 0.43, 0.52, 0.59, 0.68, 0.80 (major) 0.93, 1.03. The R_G of Rhamnose is 0.29.

One half of the hydrolysate was converted into the corresponding methylglycosides and the other half converted into the alditol acetates. Both were analysed by g.l.c., columns 1 and 2 at 175°C and column 5 at 156°C. The results are presented in Table V.

Investigation of the 1.0 M Fraction

Desulphation by methanolic-hydrogenchloride (Kantor and Schubert, 1957)

a) Preliminary Experiment. The polysaccharide 1.0 M fraction (25 mg) (carbohydrate content 53.5; sulphate 27%) was shaken for 48 hours at room temperature with 0.08 M methanolic-hydrogenchloride (5 ml). Undissolved material was centrifuged off and washed with methanol and ether. The supernatant was hydrolysed, and paper chromatography revealed rhamnose as the major compound. The insoluble material (1.0 M - S) was dissolved in water and freezedried (Yield 15 mg. Found: carbohydrate content 73.5; sulphate 6.4; uronic acid 9.35%). The loss of carbohydrate by this treatment is calculated to be 2 mg (i.e. 15%).

b) Large scale desulphation. Freezedried polysaccharide (97.5 mg) was shaken with 0.08 M methanolic hydrogenchloride, 20 ml. The insoluble material (1.0 M-S) was recovered as previously described. (Yield 53.3 mg Found: carbohydrate content 88; sulphate 6.9; uronic acid 10.6). The ratio of glucuronic acid: rhamnose: galactose in this polysaccharide (1.0 M-S) is given in Table III (p. 70)

Infrared spectra before and after Desulphation of 1.0M Polysaccharide.

Infrared spectra were taken of the two polysaccharides separately in a KBr disc, using Unicam SP1000 Infrared Spectrophotometer. Polysaccharide 1.0M gave a broad band at 1240 cm^{-1}, another at 850 cm^{-1} and a weaker at 820 cm^{-1}. The spectrum of 1.0M-S gave a very small band at 1240 cm^{-1} and the one at 850 cm^{-1} had almost disappeared.

Methylation of the Desulphated Polysaccharide (1.0 M-S).

Identification of the derived Methylated sugars.

The methylation and analysis of the derived methylated sugars were carried out as described for the 0.5 M fraction p. 70 The methylated sugars were mainly the same, but evidence for the galactose and 2-O-methylxylose was not obtained. The proportions of the methylated sugars were different from those of the 0.5 M fraction.

Periodate Oxidation Studies on the 0.5 M, 1.0 M and 1.0M-S polysaccharides.

Each of the polysaccharides were dissolved separately in 0.2 M acetate buffer, pH 3.6. An excess of sodium meta-periodate (see Table VI) was added to all three solutions, and the reaction was carried out in the dark at room temperature. The reduction of periodate was measured at intervals. An aliquot (0.1 ml) of each solution was removed and diluted to 100 ml with deionised water before reading the optical density at 223 nm against a waterblank (Aspinall and Ferrier, 1957). The amount of periodate reduced was found from a calibration graph obtained by reading the optical densities of identical dilutions of equimolar solutions of sodium

meta-periodate and sodium iodate respectively. The respective amounts of sodium meta-periodate and sodium iodate for these graphs were identical to those used for the periodate oxidation of the polysaccharides.

The reactions were stopped after 24 hours by the addition of an excess of ethyleneglycol (1.0 ml). This was allowed to stand for two hours. After dialysis, the polyaldehydes were reduced to the respective polyalcohols by addition of potassium borohydride (20 mg). After 10 hours, excess borohydride was destroyed by adjusting the pH to 7.0 by addition of acetic acid, and the solutions were dialysed over-night. After concentration, the polyalcohols were freezedried and redissolved in 10.00 ml water, the carbohydrate contents of the polyalcohols were measured, and based on the loss of carbohydrate, the theoretic reduction of periodate per C_6-anhydro unit was calculated (i.e. if 1 mole IO_4^- was reduced per cleaved C_6 anhydro unit (Table VI). The polyalcohols were freezedried again.

TABLE VI

	0.5 M	1.0 M	1.0 M-S
Starting material) wt. carbohydrate)	7.4 mg (0.046 mM)	16.4 mg (0.1 mM)	16.7 mg (0.1 mM)
$NaIO_4$ added	21.4 mg (0.1 mM)	53.5 mg (0.25 mM)	53.5 mg (0.25 mM)
Buffer, volume	25 ml	25 ml	25 ml
Recovered carbohydrate	5.1 mg	12 mg	11 mg
Theoretic reduction of IO_4^-	0.30 IO_4^-/C_6	0.27 IO_4^-/C_6	0.34 IO_4^-/C_6
Experimentally found reduction	0.25 "	0.52 "	0.52 "

Analysis of the polyalcohols from the three polyalcohols.

a) Polyalcohol 0.5 M. This was hydrolysed and examined by thin layer chromatography (TLC) as for the other two polyalcohols (p.76) In this case glycerol was the major fragment followed by threitol and a little glycolaldehyde. No propyleneglycol could be detected. The ratio of uncleaved neutral sugars in the hydrolysate determined by g.l.c.(a) and the phenolsulphuric acid-method (b) (p. 69) are given below (Table VII).

TABLE VII

	Before oxidation			After oxidation		
	a	b	average	a	b	average
Galactose	3.50	3.40	3.50	6.0	5.65	5.8
Xylose	0.5	0.6	0.5	0.92	0.8	0.9
4-O-methylgalactose	0.6	0.5	0.5	0.95	0.8	0.9
Rhamnose	1.0	1.0	1.0	1.0	1.0	1.0

b) Polyalcohols 1.0 M and 1.0 M-S

Complete hydrolysis. An aliquot of each polyalcohol was subjected to formic acid hydrolysis, and the resultant hydrolysate was analysed by paper chromatography in solvents A,C and D. No glucuronic acid or xylose could be detected. The main sugar left in both polyalcohols was galactose, with smaller amounts of rhamnose. The molar ratio between galactose and rhamnose was determined by g.l.c. and by the

phenol sulphuric acid method (p. 69) (Table VIII).

TABLE VIII

	Rhamnose :	Galactose
Polyalcohol from 1.0 M	1 :	1.5
Polyalcohol from 1.0 M-S	1 :	2.0

G.l.c. analysis of the TMS derivatives of the reduced hydrolysates on column 3 at 164°C revealed small peaks with retention times relative to xylitol 1.0 of 0.71; 0.90; 0.97; 1.15; 1.98 in addition to the large peaks T = 1.45 and 2.85, given by rhamnitol and galactitol respectively. The peak 0.97 is probably due to xylitol and that of 1.98 to 4-O-methylgalactitol. Analysis at 155°C gave two additional peaks with T = 0.47 and 0.65. The former corresponded to that given by authentic threitol.

Thin layer chromatography of the hydrolysate, solvent F, spray 7, revealed the presence of a tetritol, glycerol and propylene-glycol as the major alcohols. Glycolaldehyde did not appear to be present in as large amounts as the alcohols, but some might have been lost on evaporation.

Mild acid hydrolysis Polyalcohol 1.0 M-S (5 mg) was dissolved in 1 ml 1 N H_2SO_4 and allowed to stand at room temperature for five hours. After neutralisation with barium carbonate, the filtrate was evaporated to a small volume. Upon addition of ethanol, no apparent precipitate was formed, indicating that the polysaccharide

had been extensively cleaved. Paper chromatography solvents A and B, showed the presence of threitol and glycerol in addition to small amounts of slower moving fragments. No free sugar could be detected.

Partial acid hydrolysis. The polysaccharide 1.0 M (200 mg) was hydrolysed as previously (p. 62) and fraction 3, containing the oligouronic acids was eluted from the electrophoretogram. Paper chromatography (solvent A) gave three spots (3a, 3b and 3c) which were separated and purified. The yields, optical rotations, chromatographic and electrophoretic mobilities and constituent sugars of each of these fractions are given in Table IX.

Degree of polymerisation (Timell, 1960).

Small measured aliquots of 3a, 3b, and 3c were dissolved in water and subjected to this procedure.

Fraction 3a. From its chromatographic mobilities it was thought to be a trisaccharide, consisting of either (a) GlcUA-Gal-Gal or (b) GlcUA-GlcUA-Gal. Standard phenol-sulphuric acid graphs were made of the following synthetic mixtures 1) GlcUA :Gal - 1:2, 2) GlcUA-GlcUA-Gal 2:1 3) GlcUA :Gal- 1:1.

If (a) represents the structure then the aliquot contained 41 mg of carbohydrate and it was calculated that the derived alcohol should correspond to 27.2 µg of glucuronic acid and galactose. It was found experimentally to be equal to 26 µg. If (b) represents

TABLE IX.

Fraction	Yield	$[\alpha]_D$	Paper chromatographic mobilities Solv.A		Electrophoretic mobilities Borate buffer		Sugar constituents
			R_{glc}	R_{glcUA}	M_{glc}	M_{glcUA}	
3a	6 mg	+2.3°(C=0.3)	0.38	0.77	0.89	0.75	Glucuronic acid Galactose
3b	8 mg	+6.0°(C=0.4)	0.75	1.13	0.95	0.76	Glucuronic acid Galactose
3c	8 mg	+ 27°(C=0.4)	0.89	1.39	-	0.67	Glucuronic acid Rhamnose

the structure the aliquot contained 56 µg, whose corresponding diuronic acid alcohol should contain 38.9 µg of uronic acid. Experimentally the value obtained was 39 µg. When calculated on the basis of aldotriuronic acid, the theoretical and experimental values were very different.

Fraction 3b. This oligosaccharide could be either on aldotriuronic acid as in 3a or an aldobiuronic acid. 3b cannot be triuronic acid (a) as the calculated and experimental found carbohydrate content for the derived aldotriuronic acid alcohol were very different. Considering trisaccharide (b) the aliquot is equivalent to 37 mg which theoretically contained 25.3 µg diuronic acid. The experimentally found value is 21 µg. If 3b is an aldobiuronic acid, the amount present in the sample corresponds to 34 µg and this contains theoretically 17.7 µg glucuronic acid. The experimentally found amount is 20 µg.

Fraction 3c. This oligosaccharide had the chromatographic mobility of an aldobiuronic acid. If this is so, then the amount present in the unreduced aliquot is equivalent to 79 mg. This should theoretically contain 43 mg of glucuronic acid. The experimentally found value is 46 mg.

Structural studies of the oligouronic acids.

Chromatographic analysis. The oligosaccharides were tested for the presence of (1 → 2) linkages by staining with the triphenyltetra-

zolium reagent (Feingold et al, 1956) No colour was developed for 3c, indicating a (1 → 2) linkage between the two sugar units. Both 3a and 3b gave pink colours with this reagent.

When development with diphenylamine-aniline reagent (Harris and MacWilliam., 1954) 3a produced a bluish spot which could indicate (1 → 4) linkage between the two sugars nearest the reducing end. The other two oligosaccharides gave brown and yellow colours respectively.

Reduction of the acidic moiety.

The three oligouronic acids (3a,3b and 3c) were converted into their respective methylesters - methylglycosides (Bollenback, 1963) followed by reduction of the acid to the corresponding sugar with potassium borohydride. An aliquot of each was hydrolysed and analysed by paper chromatography in solvent D and the sugars present located with spray 1. 3a and 3b gave glucose and galactose while 3c gave glucose and rhamnose.

Methylation. An aliquot of the derived neutral oligosaccharides was each subjected to three Purdie methylations. The oligosaccharides were dissolved in a small amount of methanol for the first methylation. After the third methylation, TLC was run of the derived product, solvent G. One spot occurred for each methylated oligosaccharide R_f-0.47; 0.75 and 0.65 respectively. After hydrolysis the mixtures were subjected to paper chromatography in solvents B and E (p. 27).

3a gave three spots, 3b and 3c gave two spots.

The methylglycosides of the hydrolysed, methylated oligosaccharides were prepared and analysed by g.l.c. on columns 1 and 2 (Table X).

TABLE X.

	Retention times of peaks obtained		Corresponding to
	Col.1	Col.2	
3a	1.00 - 1.36	1.00 - 1.34	methyl 2,3,4,6-tetra-O-methylglucosides
	1.65 - 1.82	1.52 - 1.61	methyl 2,3,4,6-tetra-O-methylgalactosides
	2.00-2.5-3.45-3.74-4.14	1.77-2.01-3.11*	Unidentified peaks
3b	1.00 - 1.36	1.00 - 1.33	methyl 2,3,4,6-tetra-O-methylglucosides
	1.65 - 1.82	1.51 - 1.61	methyl 2,3,4,6-tetra-O-methylgalactosides
		5.3	Unidentified peak
3c	1.00 - 1.36	1.00 - 1.34	methyl 2,3,4,6-tetra-O-methylglucosides
	1.76	0.75	methyl 3,4-di-O-methylrhamnosides
	3.36	2.02	Unidentified peaks

* The g.l.c. was unfortunately stopped soon after this peak came off.

The results obtained for 3a and 3b indicated that hydrolysis had occurred during reduction and methylation of these, as both tetra-O-methylglucose and tetra-O-methylgalactose were obtained. The product after reduction of 3a was checked by paper chromatography and this revealed two spots, which indicate that a hydrolysis takes

place during this reaction.

Methylation after the method of Bishop and Perila (1961). Ca.1 mg 3a was suspended in 0.2 ml redistilled dimethylformamide, cooled to 0°C before addition of 0.2 ml methyl iodide and 0.2 g silver oxide. This was shaken at 0°C for 2-3 hr followed by 18-20 hr at room temperature. After filtration and washing with chloroform, the combined filtrations and washings were evaporated under reduced pressure, hydrolysed and converted into the methylglycosides. Ca. 1 mg 3b was treated the same way. The results obtained by g.l.c. analysis, col.1 and 2 are given in Table XI.

TABLE XI.

	Retention times of peaks obtained		Corresponding to
	Col.1	Col.2	
3a	1.75 - 1.90	1.50 - 1.62	methyl 2,3,4,6-tetra-O-methylgalactosides methyl ester
	2.32 - 3.00	1.75 - 2.20	methyl 2,3,4,-tri-O-glucuronosides
	1.4,6-3.86-4.15-4.40	0.74-2.10-2.44	Unidentified peaks
3b	1.67 - 1.82	1.49 - 1.60	methyl 2,3,4,6-tetra-O-methylgalactosides methyl ester
	2.6 - 2.86	1.75 - 2.20	methyl 2,3,4-tri-O-glucuronosides.
	3.96 - 4.17	2.40 - 4.7	Unidentified peaks

Neutral oligosaccharides

The neutral fraction obtained (p.62) by electrophoresis showed the presence of oligosaccharides when paper chromatographed in solvent A (R_{gal} - 0.29(A) and 0.68 (B)). After preparative separation of these two areas, and analysis in solvents B and D, (A) gave one spot (R_{gal} 0.23 and 0.17 respectively), and (B) gave rise to three compounds: B_1 (R_{gal} - 0.18 and 0.12), B_2 (R_{gal}-0.39 and 0.43)* and B_3 (major) (R_{gal}- 0.57 and 0.61)**

They each gave a red colour on a paper chromatogram when sprayed with triphenyltetrazolium hydroxide (Feingold et al, 1956) indicating the absence of (1 → 2) linkages. When treated with diphenylamine-aniline-reagent (Harris and MacWilliam , 1954) B_3 was the only one which gave blue colour, indicating that this might contain (1 → 4) linkage. No further evidence was obtained.

The four neutral oligosaccharides were separated and purified by preparative paper chromatography in solvent B. The degree of polymerisation was measured by the method of Timell (1960), and gave the following results: A - 2.70, B_1- 1.8, B_2 - 2.0, B_3 - 2.04.

Formic acid hydrolysis of aliquots of the four oligosaccharides showed on paper chromatography the following constituents:

A - Galactose, traces of xylose

B_1- Galactose, traces of xylose and rhamnose

B_2- Galactose, traces of xylose

B_3- Galactose, traces of xylose and 4-O-methylgalactose.

* (cf.1,6-linked galactobiose, R_{gal}-0.39 and 0.43)

** (cf. 1,3-linked galactobiose, R_{gal} - 0.57 and 0.61)

Discussion

Preparation of the acidic polysaccharide.

As mentioned on p 37 , Zetsche (1967) found different proportions of sugars in hydrolysates of stalks and caps, and due to this finding, the water soluble polysaccharides of Acetabularia were extracted from the caps and stalks separately. The extractions were carried out with cold and hot water, and the sulphated polysaccharides were separated from the neutral fructan on a DEAE-cellulose column (p. 55).

The four polysaccharides thus obtained, had a somewhat different negative specific rotation, and they contained different proportions of sulphate and uronic acid. (Table I, p.61). Acidic polysaccharide was also obtained by initial extractions of the alcohol extracted caps with hot water (p. 40) and this was separated into two fractions by consecutive elution of the DEAE cellulose column with 0.5 M KCl and 1.0 M KCl. The two fractions again had different negative specific rotations, sulphate and uronic acid contents (Table IV, p. 70) from the above four polysaccharides.

Nevertheless all six fractions contain the same sugars namely: D-glucuronic acid, D-galactose, D-xylose, 4-O-methylgalactose and L-rhamnose. The glucuronic acid was identified by having the same chromatographic and electrophoretic mobilities as an authentic sample. When reduced to the corresponding sugar, glucose was obtained, which was confirmed by D-glucose oxidase spray.

The galactose present is believed to be D-galactose as the optical rotation was +58° (literature +80°). The amount of galactose present in the sample was measured by the phenol-sulphuric acid method, and one must allow for some experimental error in the measurements. If $[\alpha]_D$ +58° were the exact value for the galactose, this could be a mixture of D- and L-galactose. (Literature L-galactose-$[\alpha]_D$= -78°). It was calculated that the D-galactose in a sample of this specific rotation would account for 86% of the mixture. Due to the high proportion calculated, and the fact that the 2,4-dinitrophenylhydrazone was easily formed, it is considered that the galactose present is only of the D-configuration, and the lower specific rotation obtained is due to experimental error.

This is the first time 4-O-methylgalactose has been reported as a constituent of a green algal polysaccharide. Its identity was confirmed by demethylation which gave galactose, and its paper chromatographic mobilities and retention times by g.l.c. which were identical to those of a prepared standard sample of 4-O-methylgalactose.

The xylose was identified by its mobility on paper and g.l.c. chromatography and also by reduction to xylitol and analysis on g.l.c.

L-Rhamnose was characterised in the same way and also by the formation of the crystalline 2,4-dinitrophenylhydrazone.

Analytical gel electrophoresis of the unfractionated hot water extract from the caps (p. 66) separated a major slow moving band of sulphated polysaccharide and a small band of fast moving material which appeared to comprise sulphated polysaccharide and protein. It is possible that this is a sulphated glycoprotein but the quantity is certainly very small. An attempt to fractionate the two materials by preparative gel electrophoresis was unsuccessful. The sulphated material appeared to react with the polyacrylamide gel and did not move in the electrical field applied to the column. It was only after extrusion of the gel and extraction with water that any carbohydrate could be recovered and even after dialysis this was contaminated with polyacrylamide.

Analysis for nitrogen on the hot water extract (7.7% protein), 0.5 M (20% protein) and 1.0 M (protein 4.8%) showed that most of the protein was concentrated in the 0.5 M fraction, confirming the presence of protein by the gel electrophoresis results, and also explains the low carbohydrate content of the 0.5 M fraction (Table IV p. 70).

Relative molecular proportion of the sugars in the various extracts and fractions. These were determined by g.l.c. as the TMS derivatives of the derived alcohols by measuring the peak areas and conversion into weights by reference to standard

graphs (see Table II, p. 66 ; and Table III p 70). In order to test the accuracy of this technique the two fractions 0.5 M and 1.0 M, were also analysed by eluting from paper chromatograms the areas corresponding to the sugars present in the respective hydrolysates and determining the amount present with phenol sulphuric acid. The two sets of results are given in Table III and it can be seen that they are in reasonable agreement.

The proportions of glucuronic acid were obtained by calculation from the percentage of uronic acid found by the modified carbozole method or cpc method, and the total carbohydrate content of the polysaccharides.

It can be seen (Tables II and III) that there are large variations in the molar proportions of the sugars present in the different extracts, particularly in the proportions of galactose to rhamnose which varies from 5:1 in the cold extract of stalks to just over 1:1 in the 1.0 M fraction from the hot water extract of the caps. Apart from the 0.5 M fraction the ratio of glucuronic acid to rhamnose is in the region of 1:2. The xylose, although on the whole a minor constituent, is present in greater proportion than the glucuronic acid in the hot extract of stalks. In the 1.0 M fraction of the caps, the material obtained in largest amount and therefore investigated most fully, the xylose is only

present to the extent of 1.4 partsin 24 parts of total sugars. The 4-O-methyl galactose is also a minor constituent and appears to be present in largest proportions in the hot water extract from the caps.

Desulphation Since there was no reduction in sulphate relative to carbohydrate on treatment of the cold water extract (CC) with alkali, it can be deduced that the sulphate groups were not located on any carbon atom in the sugar molecule where there is trans free hydroxylgroups or on either C-3 or C-6 of galactose units where the C-6 or C-3 of the respective units carried free hydroxyl group, i.e. where 3,6-anhydride formation could occur on removal of sulphate.

Preliminary experiments with 0.08 M methanolic hydrogen chloride (p. 72) effected reduction of sulphate from 27% to 6.4% with a loss of only 15% of carbohydrate. Large scale desulphation of this material gave a reduction of sulphate to 6.9% with a 10% loss of carbohydrate. Analysis of the molar proportion of the sugars in the desulphated materials (Table III) showed a small reduction in the proportion of rhamnose. Only trace quantities of xylose and 4-O-methylgalactose could be detected.

Infra red examination of the 1.0 M fraction before and after desulphation confirmed these findings. A large band at 1240 cm^{-1} characteristic for S=O stretching vibration and a moderate sized band at 850 cm^{-1} characteristic of axial sulphate was

present in the initial material, the former had been very much reduced, and the latter had virtually disappeared in the partially desulphated material. A small band at 820 cm^{-1} indicative of a sulphated primary hydroxyl group (Lloyd et al, 1961, Lloyd and Dodgson,1961) apparently remained unchanged after desulphation.

From these results it may be deduced that a large proportion of the sulphate groups substitute axial hydroxyl groups, i.e. C-4 of galactose, and/or C-2 of L-rhamnose (in IC conformation,) and that a smaller proportion resistant to hydrolysis with methanolic hydrogen chloride, are located on C-6 of galactose units. It should be pointed out that the sulphate content (27%) of the 1.0 M fraction is too high to be accommodated as monosulphated galactose. It is therefore possible that a number of (1 → 3) linked galactose (see methylation results p. 94) are disulphated at positions C-4 and C-6. The possibility of a small proportion of (1 → 3) linked rhamnose sulphated on C-2 (cf. Percival and Wold, 1964) is also indicated.

Periodate oxidation (p. 73) The three polysaccharides 0.5 M, 1.0 M and 1.0M-S were each subjected to periodate oxidation.

The reduction of periodate for the 0.5 M fraction was equivalent to 0.25 mol per C_6- anhydro unit (Table VI, p. 74). The calculated value, from loss of carbohydrate, (2.3 mg) by this reaction was 0.30 mol IO_4^- per C_6-anhydro unit. This was based on the reduction of 1 mol IO_4^- per sugar unit degraded, and as the

calculated and experimentally found values are in fairly good agreement, it is likely that most of the sugar units cleaved, are sulphated end groups or interchain residues which only reduce one mole periodate per unit of sugar.

The reduction of periodate for the 1.0 M and 1.0 M-S materials (Table VI) was each equivalent to 0.52 mol per C_6-anhydro unit. The calculated values, based on loss of carbohydrate by the oxidation, if 1 mol IO_4^- was reduced per cleaved sugar unit was found to be 0.27 and 0.34 mol IO_4^- per C_6-anhydro unit respectively. These values do not agree with those experimentally found, which then indicate that a considerable proportion of the sugar units oxidised reduced 2 mol IO_4^- each, thus indicating that many of the sugar units cleaved have been end units, or 1,6-linked galactose residues.

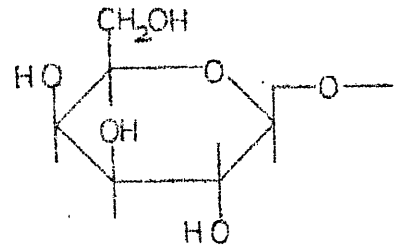
It was surprising that the same amount of periodate was reduced by these two materials. This can only be explained if the hydroxyl groups which carry a sulphate ester in the initial polysaccharide, labile to methanolic hydrogen chloride, are not adjacent to a free hydroxyl group. The loss of rhamnose, probably as end groups, during the desulphation, has produced a new end group vulnerable to periodate oxidation to the same extent as the initial rhamnose.

Analysis of the two polyalcohols show that the ratio of

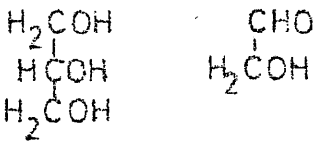
rhamnose to galactose (p. 76) is smaller in both polyalcohols than in the starting materials, indicating that rhamnose has been oxidised preferentially to galactose in both samples. Xylose and glucuronic acid both appeared to have been completely cleaved by the oxidation.

Paper, thinlayer and gas liquid chromatography analysis of the fragments obtained on hydrolysis of the polyalcohols indicated the presence of threitol, glycerol, propyleneglycol and glycolaldehyde in all three polyalcohols except that propyleneglycol could not be detected in the 0.5 M fraction. Threitol could be derived from either 1,4 linked galactose or end group galactose-4-sulphated (Fig. 4b.). Glycerol is derived from end group galactose and/or 1,4-linked xylose (Fig.4a,d). Propyleneglycol can be derived from 1,2-linked rhamnose (Fig.4c) and glycolaldehyde is derived from the reducing end of the cleaved units, except from 1,2-linked rhamnose which gives glycerose (Fig. 4c). This unfortunately has the same chromatographic mobility as glycerol and would therefore be masked.

a)

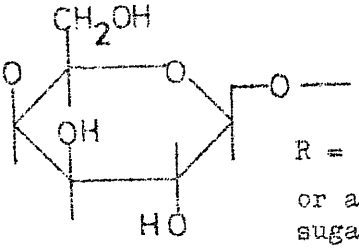


(1) IO_4^-
(2) red
(3) H^+



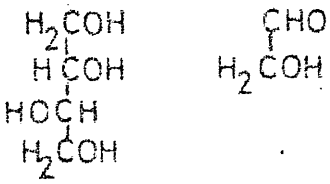
Glycerol Glycolaldehyde

b)



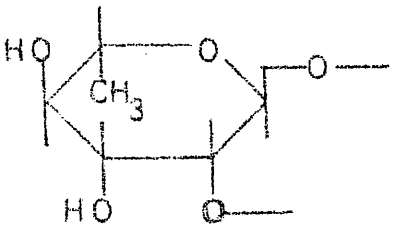
$R = SO_3^-$ or another sugar unit

(1) IO_4^-
(2) red
(3) H^+

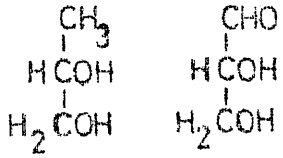


Threitol Glycolaldehyde

c)

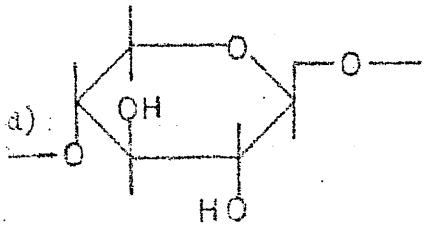


(1) IO_4^-
(2) red
(3) H^+

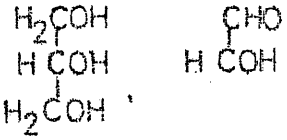


Propyleneglycol Glycerose

d)



(1) IO_4^-
(2) red
(3) H^+



Glycerol Glycolaldehyde

Fig 4

From these studies it can be deduced that the galactose is mainly 1,3-linked and sulphated at C-4 and that some of the units may be end groups partially sulphated at C-4. That a proportion of rhamnose is either 1,4-or/1,2-linked and that the rest is probably triply linked. Had it been 1,4-linked with sulphate on C-2, then these units would have been vulnerable in the desulphated material. It should be emphasized however, that these experiments were carried out on small quantities of material and the presence of a small percentage of this structural unit could well have escaped detection. Since the glucuronic acid and xylose were completely cleaved in the two 1.0 M materials, they must be present as end groups or 1,4-linked units. The fact that the 0.5 M fraction contains a higher proportion of glucuronic acid and xylose can possibly explain why all the xylose is not cleaved on periodate oxidation. Studies by Painter and Larsen (1970) have shown that polysaccharides containing these sugars are very liable to form acetal linkages between cleaved and non-cleaved units (Fig. 5) and complete oxidation of potentially vulnerable units is hindered.

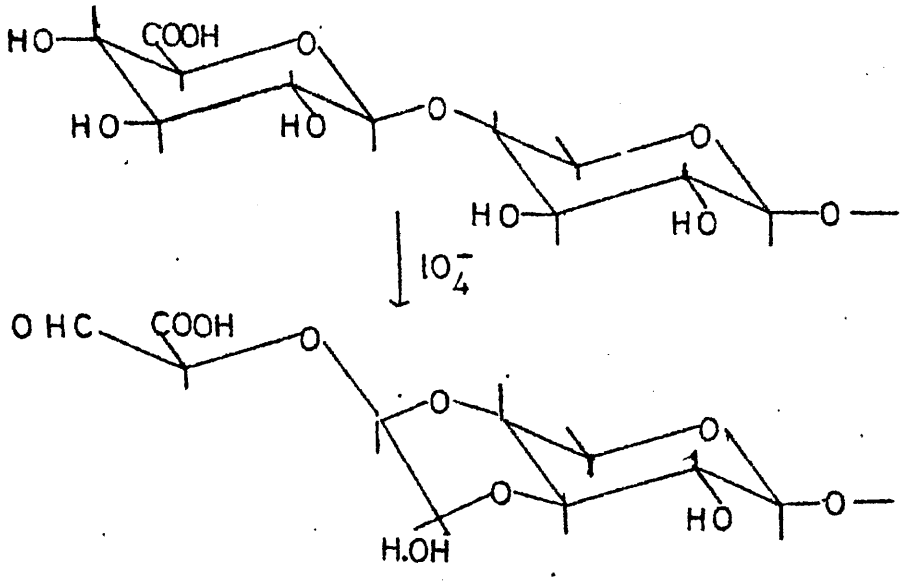


Fig 5

It is interesting that the 1.0 M and the 0.5 M fractions give ~~very~~ similar results on periodate oxidation and it can only be assumed that they have a very similar macro-molecular structure.

These interpretations of the results of periodate oxidation must be viewed with a certain amount of caution since the quantities involved did not permit complete characterisation of all the fragments, particularly the slower moving, non-reducing fragments obtained from the mild acid hydrolysis of the polyalcohols.

Methylation studies (p. 73) Methylation of the 1.0 M fraction was attempted, but due to the high sulphate content complete methylation proved impossible. However, after partial desulphation, permethylation was achieved. The methylated product

after hydrolysis was analysed on g.l.c. as the methylglycosides and as the methylated alditol acetates. Methyl 2,3,4,6-tetra-O-, -2,4,6 tri-O-(major), 2,3,4, tri-O- and 2,4-di-O-methyl-galactosides were indicated. Confirmation of these derivatives was obtained as the alditol acetates in addition to 2- and 6-mono-O-methylgalactose alditol acetates. From this it can be concluded that galactose is mainly 1,3-linked with a smaller proportion of end-group and 1,6-linked galactose. The 2,4-di-O-methylgalactose is probably derived from 1,3-linked 6-sulphated units and the mono-methyl derivatives from sulphated galactose occurring at branch points. No hexaacetyl galactitol could be detected proving the absence of free galactose in the methylated polysaccharide.

The main methylated rhamnose derivative was 3,4-di-O-methylrhamnose, but a considerable amount of tri-O-methylrhamnose was also present. Evidence for a mono-methylrhamnose, most probably 4-O-methylrhamnose was also obtained. Rhamnose is therefore present mainly as end groups and 1,2-linked or 2 sulphated units. Di-O-methyl rhamnose derivatives are difficult to characterise and the presence of 2,4-di-O-methylrhamnose (i.e. 1,3-linked units) is not ruled out from these results.

End group glucuronic acid and xylose were found to be present, and evidence for either 2,3 or 3,4 di-O-methylxylose (i.e. 1,4- or 1,2-linked xylose units) was obtained.

The most surprising fact that emerges from these methylation studies is the high proportion of end group units, indicating a large number of single unit branches.

Methylation of polysaccharide 0.5 M gave the same overall picture as for the methylated desulphated polysaccharide, 1.0 M-S. The main difference is that this methylated polysaccharide contained free galactose and a relatively greater amount of mono- and di-O-methylgalactose, indicating sulphate on these units. The amount of di-O-methylxylose seemed also to be greater.

There is a general agreement between the results obtained by periodate and methylation studies, but no evidence for 2,3,6-tri-O-methylgalactose (arisen from end group galactose-4-sulphate) was obtained, but the presence of small amounts of this methylated sugar would be masked by other compounds present.

Acidic oligosaccharides Partial acid hydrolysis of the cold water extract of the caps, polysaccharide CC, gave rise to three acidic oligosaccharides (p. 77). One of them, 3c, was identified as 2-O-β-D-glucuronosyl-L-rhamnose. The uronic acid was identified as glucururonic acid by its chromatographic and ionophoretic mobilities, and as glucose after reduction. When a paperchromatogram of the aldobiuronic acid was sprayed with the triphenyltetrazolium reagent, no colour developed, indicating a (1 → 2) linked disaccharide.

Methylation and hydrolysis of the reduced oligosaccharide gave rise to 2,3,4,6 tetra-O-methylglucose and 3,4-di-O-methylrhamnose. These results together with a negative rotation, and a DP of 2, indicated the above mentioned structure for this aldobiuronic acid (Fig.5).

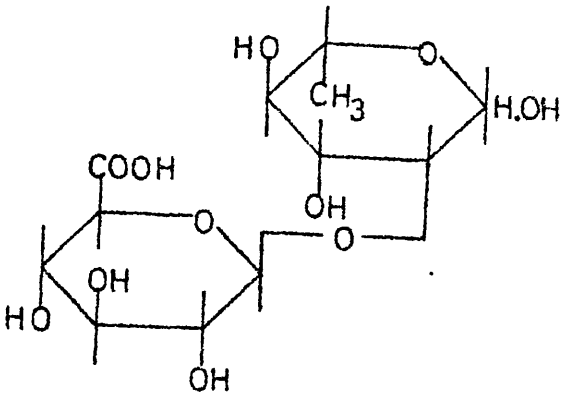


Fig 5:

Oligosaccharides 3a and 3b both have low specific rotation and give glucuronic acid and galactose on hydrolysis. From its chromatographic mobilities and DP determination, 3a appears to be a trisaccharide and its most probable structure is glucuronosylgalactosylgalactose. It was methylated both before and after reduction (p.80,82) and gave on hydrolysis the two methylated products 2,3,4-tri-O-methylglucuronic acid and 2,3,4,6-tetra-O-methylglucose respectively, indicating that the glucuronic acid residue occupies the non-reducing end. However in each case tetra-O-methylgalactose was also obtained

Four neutral oligosaccharides were believed from their chromatographic mobilities to be three disaccharides and one trisaccharide. Unfortunately on hydrolysis each proved to be a mixture consisting mainly of galactose with traces of xylose, rhamnose and 4-O-methylgalactose. All indicated the absence of (1 → 2) linkage and one the presence of (1 → 4) linkage (p. 83). Disaccharides B_2 and B_3 are tentatively identified as mainly galactosyl (1 → 6) galactose and galactosyl (1 → 3) galactose respectively, due to the same chromatographic mobilities as authentic sample. This shows the presence of mutually linked galactose units in the macromolecule.

Summary

The present studies on the green alga Acetabularia mediterranea show that it metabolises a whole family of sulphated uronic acid containing polysaccharides. Depending on the method of extraction of the alga and the fractionation procedure, polysaccharides with different content of sulphate, glucuronic acid, galactose, xylose, 4-O-methylgalactose and rhamnose are obtained. Methylation and periodate oxidation show however that they are all built up on the same general plan and can be regarded as a family of related polysaccharides. This type of polysaccharide has not been found before in Nature, and the most suitable name would be sulphated glucuronoxylorhamnogalactans.

The main features of these polysaccharides are (1 → 3) linked galactose units sulphated mainly on C-4 and to a lesser extent on C-6. Rhamnose is mainly (1 → 2) linked or possibly sulphated, and xylose, glucuronic acid, galactose and rhamnose are all present as end groups. Due to the evidence for a relatively high proportion of end groups found by methylation studies, the polysaccharide is thought to be highly branched with very short branches.

Glucuronic acid is linked both to galactose and rhamnose units, and evidence for mutually linked galactose units was obtained from partial hydrolysis studies.

THE MANNAN

Introduction.

Pure mannans are not found widely distributed in nature. The only ones among higher plants which have been studied, come from ivory nuts (Phytelphas macrocarpa) (Aspinall et al 1953 ; 1958) and from coffee beans (Wolfrom et al, 1961). They are all highly insoluble and consist mainly of β-(1 → 4) linked D-mannopyranose residues.

Among the algae, mannan has been shown to be present in the cellwall of certain Rhodophyceae and Chlorophyceae.

The mannan from Porphyra umbilicalis is the only one which has been chemically studied of those from the red algae (Jones 1950). This mannan was subjected to methylation and periodate oxidation studies. The results together with a negative rotation ($[\alpha]_D = -41^\circ$ in formic acid), indicated a β-(1 → 4) linked mannan with an average chainlength of 12-13 D-mannose residues. This mannan had the same insolubility as was found for the ivory nut mannan. Miwa and Iriki (1960) did a survey of the nature of the cellwall of various green algae, and found that the cellwalls of Codium, Acetabularia and Halicoryne consisted of mannan. On the basis of periodate oxidation and negative rotation, they tentatively concluded that the cellwall of these algae consisted of β-(1 → 4) linked mannose units.

The cellwall of Codium fragile has been extensively studied by Love and Percival (1969). The polysaccharide was

extracted with 20% sodium hydroxide and purified by precipitation as its copper complex. After decomposition of this complex, the polysaccharide, $[\alpha]_D = -41^\circ$, contained 95% mannose and 5% glucose. Enzymic hydrolysis of this polysaccharide yielded an homologous series of $\beta(1 \rightarrow 4)$ linked mannose oligosaccharides and a trace quantity of a disaccharide which on hydrolysis gave mannose and glucose. This was tentatively identified as mannose $(1 \rightarrow 4)$ glucose. Periodate uptake was 0.87 mol/C_6-unit, and the polyalcohol still showed traces of mannose on paper chromatography after hydrolysis of an aliquot. Methylation studies showed the presence of 2,3,6-tri-O-methyl mannose, 2,3,4,6 tetra-O-methyl mannose and a mixture of two di-O-methyl sugars. Since no chemical studies of the mannan from Acetabularia have been reported other than those mentioned above by Miwa and Iriki (1960), it was decided to study this mannan in more detail.

Isolation of the Mannan. The residual weed (originally 31.4 g caps and 11.6 g stalks) after exhaustive hot water extraction and 4% sodium hydroxide extraction (see p. 40) was extracted with 100 ml and 50 ml 20% sodium hydroxide respectively at 70°C for 9 hours under nitrogen atmosphere with continuous mechanical stirring. The extraction mixtures were centrifuged, and after cooling, the supernatants were filtered through glasswool. Fehling's solution was then added to precipitate the mannans as their copper complex. After complete precipitation

had occurred, the precipitated polysaccharides were filtered off and washed thoroughly with water. The precipitates had a blue colour. After washing, 100 ml 1% hydrochloric acid in ethanol was added on to each filter to break the complexes and the precipitates changed colour to white. These white residual precipitates were washed on the filters with ethanol, acetone and ether and left to dry.

	Stalk	Cap.
Weight of mannan	194 mg	341 mg
$[\alpha]_D$	-37° (c=0.19 in 90% H.COOH)	-38.1° (c=0.25 in 90% H.COOH)

Identification of the sugars present. An aliquot of each of the two mannans was hydrolysed separately with formic acid. The derived hydrolysates were analysed by paper chromatography and by g.l.c. Paper chromatography in solvents A and D showed mainly mannose with a faint trace of glucose. The latter was confirmed by glucoseoxidase. G.l.c. analysis of the trimethylsilylated hydrolysates gave on column 3, both for the caps and the stalks, two peaks only at T 1.70 and 2.90 identical with the retention times of the peaks of authentic mannose. An aliquot of the hydrolysates was reduced, and then converted to the trimethylsilylethers before analysis by g.l.c. Both caps and stalks then gave one peak only, T = 2.45 identical to the retention time of the peak from authentic mannitol.

A hydrolysate of mannan, 10 mg, was subjected to preparative paper chromatography in solvent A. The mannose fraction was eluted off the paper with water filtered through Millipore-filter and evaporated to dryness. The mannose had $[\alpha]_D$ = +17.5°(C = 0.04 in water). The derived phenylhydrazone had mp. and mixed mp.199-200°C (Bourquelot and Herissey, 1899). The mannan from the caps and from the stalks 41.9 mg and 39.6 mg respectively, were each dissolved in 10.00 ml 90% formic acid. An aliquot of each solution was withdrawn for estimation of the carbohydrate content, which was found to be 93.1% and 102% respectively.

Methylation of the mannan. It proved difficult to methylate the mannan completely by the method of ~~Grandal~~ Bjerndal and Lindberg,(1969), but the procedure devised by Unrau and Choy (1970) of Haworth methylation,followed by Hakomori's,proved successful.

Haworth methylation. 100 mg polysaccharide was dissolved in 20 ml 30% sodium hydroxide by stirring and slight heating in an atmosphere of nitrogen. The mixture was cooled to 0°C in an icebath and 80 ml 30% sodium hydroxide and 30 ml dimethylsulphate was added dropwise over a period of 6 hours. The reaction was carried out at 0°C and with continuous stirring. This was followed by stirring at room temperature overnight. The pH of the reaction mixture was adjusted to 8 by dropwise addition

of 8 N-H_2SO_4. The partially methylated polysaccharide was then extracted with chloroform and the solution taken to dryness. The partially methylated polysaccharide was then dissolved in 5 ml dimethylsulphoxide in an atmosphere of nitrogen with continuous stirring. 1.0ml of the carbanion (Sandford and Conrad, 1966) was added. The solution turned turbid, but after 15 minutes stirring it became clear, and stirring was continued for another 4 hours. Methyliodide (0.3 ml) was then added dropwise, keeping the reaction-mixture at 20°C and continuous stirring until a clear solution occurred. After dialysis overnight, the methylated polysaccharide was extracted with chloroform. TLC of the methylated polysaccharide in solvent 6 gave one spot.

Hydrolysis and examination of the methylated sugars. The methylated mannan was hydrolysed with formic acid and chromatographed in solvent A, three brownish-spots appeared after spraying the paper chromatogram with spray 1. R_f - 0.82 - 0.74 - 0.60; the compound with R_f 0.74 was the major sugar, and the other two were present in almost equal amounts (visual examination on paper or TLC chromatograms).

Thin layer chromatography was performed on silica gel using solvent 6. Three compounds with R_f 0.33 - 0.16 - 0.05, were revealed with spray 1. The compound with R_f 0.16 was the major compound and the other two were present in almost equal amounts.

After methylglycosidation of the hydrolysate, the derived methylglycosides of the methylated sugars were analysed by g.l.c. on columns 1,2 and 6.

Results obtained by g.l.c. of the methylated mannan.

Column 1

Peaks obtained T = 0.97, 1.34, 1.79, 1.93*, 3.32, 4.22(M), 6.25
Methyl-2,3,4,6-tetra-O-methylmannoside - 1.30
Methyl-2,3,6-tri-O-methylmannoside - 1.29 - 2.27(M) 3.24
Methyl-2,3,4,6-tetra-O-methylglucoside 1.00,1.38

Column 2

Peaks obtained T = 1.04, 1.28, 2.29(M) 3.24 4.48
Methyl-2,3,4,6-tetra-O-methylmannoside - 1.28
Methyl-2,3,6-tri-O-methylmannoside - 1.29, 2.27(M) 3.24
Methyl-2,3,4,6-tetra-O-methylglucoside 1.00, 1.38

Column 6

Peaks obtained T = 0.99, 1.40, 1.82, 2.00*, 3.39, 4.42(M) 6.50
Methyl-2,3,4,6-tetra-O-methylmannosides 1.33
Methyl-2,3,6-tri-O-methylmannosides 1.82, 3.34, 4.42(M)
Methyl-2,3,4,6-tetra-O-methylglucosides 1.00, 1.40

* unidentified peak
(M) major peak

As can be seen, the following methylated sugars were present: methyl 2,3,4,6-tetra-O-methylmannoside, methyl-2,3,6-tri-O-methylmannoside and evidence for a very small peak of methyl 2,3,4,6-tetra-O-methyl β-glucoside was obtained but any α-glucoside would be masked by the mannosides. On columns 2 and 6 an unidentified peak occurred, T - 4.48 and 6.50 respectively. This is in the region of di-O-methylmannosides. All standards, except 3,6-di-O-methylmannose

were available, but none of those gave any peaks corresponding to the unknown.. It is therefore tentatively concluded that the di-O-methylmannose is 3,6-di-O-methyl-mannose.

By measuring the areas under the peaks, the ratio of tri-O-methyl to tetra-O-methyl sugar was found to be for Col.1, 13:1 and col.2, 17:1.

Periodate-oxidation. (Aspinall and Ferrier, 1957)

Periodate-oxidation in acetate-buffer. Periodate-oxidation was carried out in parallel on the mannan from Acetabularia caps and the ivory nut mannan (a linear β (1 → 4) linked mannan)and 0.2 mM (32 mg) of each were treated with 0.5 mM-sodium periodate in acetate buffer (pH 3.6-10 ml) in the dark at 2°C. The amount of periodate consumed, was measured by removing an aliquot (0.1 ml), diluting to 100 ml with deionised water and reading the optical density at 223 nm against a water blank in a Unicam SP500 Spectrophotometer. A calibration curve was obtained by measuring the optical densities of 0.5 mM-$NaIO_4$ and 0.5 mM-$NaIO_3$ under the same conditions. The molar consumption of periodate per C_6 - anhydrounit at different time intervals could be found from this calibration graph. The reaction was followed at 2°C for 48 hours and then kept at room temperature for another 20 hours. The reaction at 2°C was complete

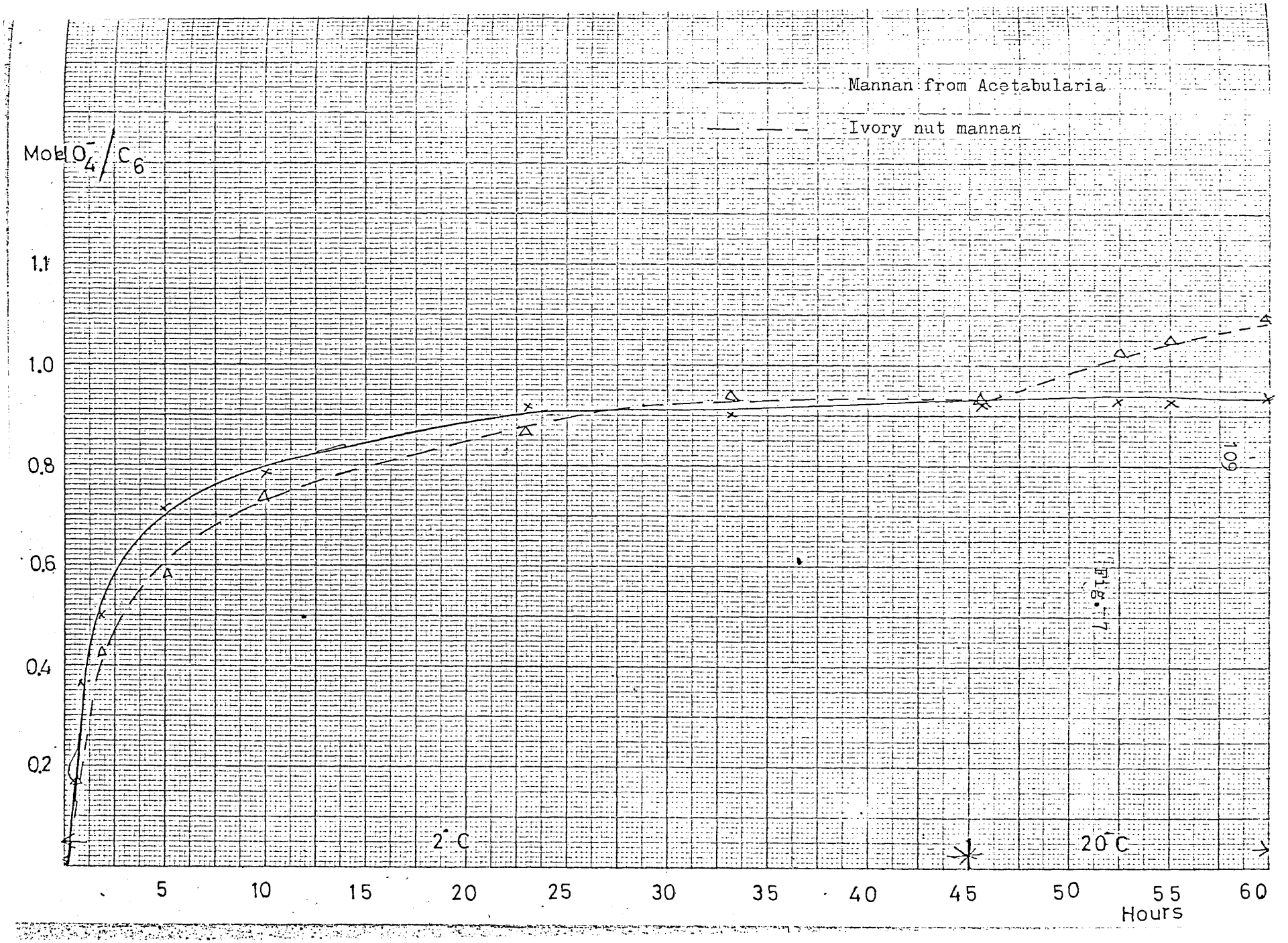

Mannan from Acetabularia
Ivory nut mannan
Mol SO_4^-/C_6
1.1
1.0
0.8
0.6
0.4
0.2
2° C
20° C
5
10
15
20
25
30
35
40
45
50
55
60
Hours

Fig. 17

after 20 hours in both cases (0.94 mol IO_4/C_6 unit). That of the cap-mannan remained unchanged after transferring to room temperature, whereas that of ivory nut mannan increased to 1.05 mol IO_4/C_6 unit. (Fig. 7). The reactions were stopped by adding ethyleneglycol. After dialysis overnight, the derived polyaldehyde was reduced with sodiumborohydride to its corresponding polyalcohol.

Analysis of the polyalcohol. A phenol-sulphuric acid test on the two polyalcohols showed that carbohydrate was still present, in that derived from the cap-mannan, while that from the ivory nut mannan was negative.

A few mg of the polyalcohol from the cap-mannan was hydrolysed with formic acid. Thin-layer-chormatography in solvent F and spray reagents 3 and 7 /p.27 showed the presence of glycolaldehyde, glycerol and erythritol. When paper chromatographed in solvent A a faint spot corresponding to mannose was visible after development of the paper chromatogram with spray 1.

The hydrolysate was analysed by g.l.c. as the trimethylsilyl ethers on column 3, at 156°C. The presence of erythritol and glycerol was confirmed, and the ratio between the two alcohols was found. The peak areas were measured and the amount present found from appropriate standard graphs. The ratio between erythritol and glycerol was ca. 14:1.

Periodate-oxidation carried out in water at 2°C.

Periodate oxidation of 0.2 mM-mannan (32 mg) was carried out as previously described, but water was used instead of buffer. The periodate oxidation was the same. After a reaction period of 24 hours, an aliquot (2 ml) was withdrawn, and the release of formic acid was determined by titration with 0.005 M-sodiumhydroxide (carbonate-free). The excess periodate was destroyed with ethyleneglycol before titration. The formic acid released corresponded to 0.072 mole per C_6-anhydro unit. As the reaction was carried out at 2°C the formic acid was most probably only derived from the non-reducing end of the mannan, thus indicating an average chain-length of 14. The polyalcohol from this periodate oxidation was obtained as before (weight 24 mg.) (polyalcohol 2).

Polyalcohol 2, 5 mg, was subjected to a second periodate oxidation in 0.01 M-$NaIO_4$ at room temperature. The reaction mixture was allowed to stand for 15 hours, and no reduction of periodate had taken place. The polyalcohol was recovered as before.

Mild acid hydrolysis of polyalcohol 2.

Polyalcohol 2, 11 mg, was hydrolysed with N-sulphuric acid, 1 ml, for 5 hours at room temperature. After neutralisation with barium carbonate and filtration, the derived syrup was analysed by TLC (solvent F, and sprays 3 and 7). Compounds with the following mobilities were detected: R_f 0.45-0.24-0.15-0.00. The three first-mentioned compounds had the same R_f values as glycol-aldehyde,

glycerol and erythritol respectively.

When subjected to paper chromatography in solvent D, the following spots were obtained: $R_{mannose}$ = 2.39-1.42-1.25-0.72-0.32 (trace). The three first mentioned had the same $R_{mannose}$-values as glycolaldehyde, glycerol and erythritol respectively.

Erythritol was the major compound. The compound with $R_{mannose}$ = 0.32 was not studied further due to the small amount present.

The compound with $R_{mannose}$ = 0.72 was purified by preparative paper chromatography and hydrolysed with formic acid. Paper chromatography revealed mannose and erythritol.

An attempt to determine the molecular weight.

Stalk-mannan, 10 mg, was dissolved in 5% sodiumhydroxide and dialysed. The mannan remained in solution. After concentration it was applied on top of a column of Sephadex G-100 (2.54 x 30 cm.) Fractions of 2 ml were collected, using an automatic fraction collector. Every second fraction was tested for carbohydrate content. Carbohydrate started to come off the column when Blue Dextrandid, i.e. after the 25th fraction, and continued to appear for about 88 ml. Three "fractions", tubes 25-29, 31-49 and 51-69 were combined. A part of all three were hydrolysed and all gave mannose with a trace of glucose. Fraction 31-49 was reapplied to the column and the elution pattern was similar to that obtained previously for this fraction. Fraction 51-69 gave the same result. As some of the mannan is excluded from the column and some retarded, it appears that the mannan applied to

the column has a wide molecular weight range, the upper limit being somewhat larger than 100,000 by comparison with Dextran (i.e. 550 units per chain)

Discussion

The residual weed from both caps and stalks after exhaustive hot water extractions, was extracted with 4% sodiumhydroxide. This treatment extracted some mannan in addition to a small amount of the water-soluble polysaccharide. Extraction of the residual material (p. 102) with 20% sodium hydroxide at 80°C and isolation of the polysaccharide via its coppercomplex, yielded a pure mannan from both stalks and caps. Analysis of a hydrolysate of the mannans by paper chromatography and g.l.c. gave mainly mannose, but a faint trace of glucose was present. The polysaccharides had $[\alpha]_D = -38.1^\circ$ (cap mannan) and $[\alpha]_D = -37^\circ$ (stalk mannan). The mannose had the same chromatographic mobility on paper as authentic mannose, and the TMS derivatives of the sugar and the derived alcohol had the same retention values as authentic mannose and mannitol on g.l.c. Mannose, purified by preparative paper chromatography, had $[\alpha]_D = +17.5^\circ$ (C = 0.04 in water) and the phenylhydrazone had mp and mixed mp 199-200°C. The glucose present was confirmed with glucoseoxidase spray.

Due to the high insolubility of the mannan, methylation by the ~~Grandal~~ Bjørndal and Lindberg method was unsuccessful, but when the mannan

was first given one Haworth methylation which was followed by one methylation by the Hakomori method, an apparently fully methylated polysaccharide gave on hydrolysis 3 compounds, which could be tentatively identified(by paperchromatography and TLC) as the tetramethylsugar, trimethylsugar and dimethylsugar. The trimethyl sugar was present in largest amount, and the tetra- and di-in almost equal quantities. Examination of the methylglycosides by g.l.c. showed that the mixture consisted of 2,3,4,6 tetra-O-methyl-mannose, 3,4,6-tri-O-methylmannose, probably 2,3,4,6 tetra-O-methyl glucose, and one di- O -methylmannose, which has been tentatively identified as 3,6-di-O-methylmannose. Whether this di-O-methyl-mannose has arisen due to undermethylation or comes from a true branchpoint can not be said for certain, but there is a strong evidence for its being a branch point. The average chainlength found by measuring the peak areas under the tetra-methylmannose peak including any possible tetramethylglucose and the tri-methylmannose peaks was 14 to 18 mannose units.

When the mannan was oxidised with sodium periodate, 0.94 mole of periodate was reduced per C_6-anhydrounit. Periodate oxidation was also carried out on the ivory nut mannan at the same time in order to compare the two mannans. The periodate oxidation pattern of the two mannans resembled each other when the reaction was carried out at 2°C, but when they were transferred to room

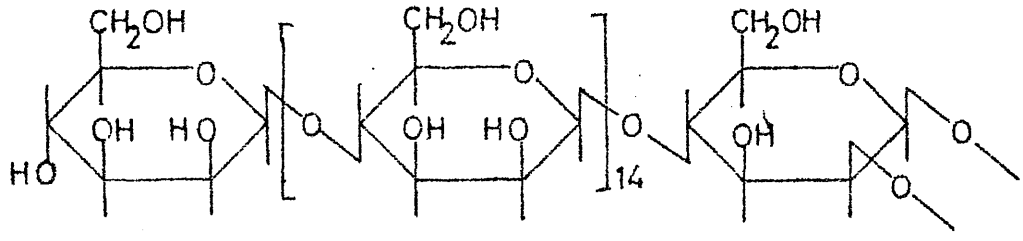
temperature, no increase in the uptake of periodate occurred for the mannan from Acetabularia, while that of ivory nut mannan continued to a reduction of 1.05 moles of periodate per C_6-anhydro unit. A test for carbohydrate content of the two derived polyalcohols gave a positive test for the cap mannan, while that of the ivory nut mannan was negative. These results show that the mannan from the cellwall of Acetabularia is not identical to the ivory nut mannan, although they have fairly similar properties, both physically and chemically.

Both measurement of the release of formic acid and the ratio of erythritol to glycerol formed during periodate oxidation, indicated an average chainlength of 14 mannose units, which is in good agreement with that found from measuring the peak areas of the methyl sugars derived after methylation and hydrolysis of the mannan.

The products after periodate oxidation, reduction and hydrolysis of the polyalcohols, erythritol and glycerol, were identified by paper chromatography, TLC and by g.l.c. Glycolaldehyde which was also present, had the some mobility both by paper chromatography and TLC as an authentic sample but g.l.c. proved impossible as it is too volatile. The amounts obtained were insufficient to separate and prepare crystalline derivatives. Mannose was shown to be present after formic acid hydrolysis of the polyalcohol. When subjected to mild acid hydolysis, a non-reducing compound was separated which gave erythritol and

mannose on hydrolysis.

In an attempt to determine the molecular weight of the mannan by Sephadex-gelfiltration the elution pattern indicated a considerable polydispersity with an upper limit of molecular weight about 100,000 if comparable to dextran. This polydispersity might have arisen during extraction of the polysaccharide as alkali is known to degrade polysaccharides. Although no definite conclusions can be drawn regarding the molecular weight of the mannan, it is probably at least 100,000, which is equivalent to ca.550 sugar units per molecule.



Proposed structure for the mannan.

This mannan from the cellwall of Acetabularia seems to be quite similar to the mannan of the cellwall of Codium (Love and Percival, 1964) although the periodate oxidation studies by Miwa and Iriki (1960) agree with the present findings, other results indicate that the cellwall of Acetabularia consists of a branched mannan with an average chainlength of ca.15, rather than linear molecules consisting of 16 sugar units as the above authors propose.

The studies on the low molecular weight carbohydrates, the fructan and the mannan from Acetabularia have been accepted for publication in Carbohydrate Research.

PART II

Photosynthetic studies on Carbohydrates in algae.

1. ACETABULARIA MEDITERRANEA

Although Acetabularia sp. have been extensively studied from biological and physiological viewpoints, relatively few studies have been carried out regarding the metabolic and photosynthetic role of the carbohydrates in this alga.

Shephard et al (1968) allowed both whole cells of Acetabularia mediterranea and chloroplast preparations to photosynthesise in the presence of $^{14}CO_2$ for 10 minutes. The samples were then extracted with 80% ethanol, and this extract subjected to two-way paper chromatography. The extent of labelling was measured for all compounds detected, and it was found that the carbohydrates acquired the main part of the radioactivity in the 80% ethanol fractions. In all experiments except one, sucrose was highest labelled, followed by glucose. No other free sugars were reported, but their presence was masked by other compounds present under the described paper chromatographic conditions.

The extent of labelling was almost equal in the 80% ethanol soluble and insoluble fractions. When chloroplasts were allowed to photosynthesise in the presence of $^{14}CO_2$ for 70 minutes, the major part of the radioactivity was found in the alcohol soluble fraction (of this 49% was present in free sugars). A weak acid hydrolysate of the residue showed that of the radioactivity detected in soluble compounds 56% was found in glucose

and 44% in galactose.

Bidwell et al (1970) allowed chloroplasts from A.mediterranea to grow in the presence of $^{14}CO_2$ in the light, followed by a dark and then a light period. Samples were taken frequently throughout the experiment and applied to paper chromatograms which were developed two-dimentionally. The change of radioactivity in the various compounds was measured. The results showed that sucrose attained the highest radioactivity,it was mainly formed during the light periods, and did in these experiments not show any sign of saturation. They conclude that sucrose is the major end product of photosynthesis in chloroplast of A.mediterranea. No mention of other sugars was made, which is strange as their previous paper mentioned the presence of labelled glucose in chloroplasts from this alga.

To the author's knowledge no further photosynthetic studies have been carried out on the 80% ethanol soluble carbohydrates of this alga.

General Methods.Experimental.

The Acetabularia cells were allowed to photosynthesise in radioactive synthetic seawater. This was de-aerated prior to use to reduce the carbon dioxide content before addition of $NaH^{14}CO_3$ (ca.300 μC). After growth in radioactive medium, the alga was removed and rinsed thoroughly, followed by growth in inactive

medium for a certain period. Samples were removed at time intervals. Each sample (10 cells) was transferred to 80% ethanol at 70°C and ground up and extracted. After centrifugation, the radioactivity present in the ethanolic extracts was measured.

After deionising with Biodeminrolit resin (carbonate form), and concentration of the derived neutral extract, an aliquot was subjected to paper chromatography in solvent A. The compounds present were located by radioautography and the radioactivity present in each compound was measured by counting on both sides of the corresponding part of the paper chromatogram. The counting was performed by an ultra thin window gas flow Geiger-Müller counter, and the average of the two counts per sample used for calculations.

Experiment I

Cells before cap initiation (A), caps only (B) and cells with caps (C), were each allowed to photosynthesise in radioactive medium. After 10 min. this was followed by photosynthesis in inactive medium for 2-3 hours. Samples were taken at time intervals and treated as described previously. The incorporation and change of radioactivity in the various compounds can be seen from graphs A, B and C.

Experiment II

Cells with caps were grown for 2 min. in the presence of $^{14}CO_2$, followed by growth in $^{12}CO_2$ containing medium for 2 hours. Samples were treated as previously described (Graph D).

Experiment III

Cells with caps were grown for 2 hours in the presence of $^{14}CO_2$ in the light, followed by 2 hour growth in the dark in the absence of $^{14}CO_2$. In this case a sample of 15 cells were taken after the light period and one sample after the dark period. The samples were treated as described (Graph E).

Discussion

Studies on the relative molecular ratio of the sugars present in the polysaccharide fractions of caps and stalks separately (Zetsche, 1967), indicated that these two parts of the alga synthesised different types of polysaccharides (see p 37).

Due to this report, it was decided to carry out biosynthetic studies on cells before cap formation, caps only and cells with caps (Exp.I), to see if there was any difference in the biosynthesis of the low molecular weight carbohydrates in these three experiments. As seen from graphs A,B and C, the main picture is the same for all three experiments.

Experiment II was performed to see if a shorter period of growth in the presence of $^{14}CO_2$ would give rise to a different pattern, but as observed from graph D, the main pattern is the

same as for the longer feeding period. The only difference is a smaller amount of radioactivity present in glucose, relative to the radioactivity of sucrose, compared with the other experiment.

In each experiment, the radioactivity of sucrose increased rapidly during the feeding period. This increase continued for a short while thereafter, followed by a rapid decrease of radioactivity, which then gradually flattened off. Glucose followed to a lesser extent the same pattern, but the uptake of radioactivity during the feeding period was small compared with that of sucrose. In contrast to these two sugars, fructose acquired hardly any radioactivity during the feeding period, but the radioactivity present in this sugar increased throughout the experiment. The radioactivity of the trisaccharide, F_3 (graphs A,B and C) (see p.126-128), followed more or less the same pattern as sucrose in all experiments. The maximum extent of labelling for both occurred after ca.15 mins.

Experiment III was carried out to find out if a dark period would give a different pattern of radioactivity from those already described. Graph E, (p. 130) shows that the pattern for sucrose, glucose and fructose are similar to those obtained previously. From this experiment it was observed that the radioactivity of F_3, dropped considerably during the last two hours of the experiment, that of F_4 dropped less, while that of

F_5 remained almost constant. It was also observed that the total radioactivity of oligofructans, higher than F_5, increased during this period.

The amount of radioactivity incorporated into myo-inositol was very small, and only in experiment I, A and III could any be detected. The amount of radioactivity present decreased throughout the experiment, indicating that it is being transformed into other products.

Sucrose is clearly the first free sugar formed in Acetabularia mediterranea. This is in agreement with what has been found previously for other green algae (see p. 4). Sucrose is not a storage product in this alga, as the radioactivity decreases rapidly after transfer to inactive medium. It is an active metabolite and is converted in to other products via various routes. A.mediterranea contains a homologous series of fructans.

Edelman and Jefford (1968) have demonstrated that the same homologous series of fructans present in Helianthus tuberosum is built up from sucrose, by transfer of fructofuranose from one sucrose unit to the fructose moiety of another sucrose unit. This reaction continues and thus forms the homologous series in the plant. It was shown that one enzyme system (SST) is responsible for formation of the trisaccharide and another (FFT)

for the further formation of higher oliogmers. The glucose released from the one sucrose residue was believed to be transformed into sucrose again. When studying the patterns of changes in activities of the various carbohydrates present in the present 80% ethanolic extract of A.mediterranea, one can see that there is a possibility for the same reactions in this alga.

As seen from graphs A,B,C and E, the pattern of change in radioactivity of sucrose and F_3, follow each other or can be said to be in equilibrium with each other. From graph E, it can be deduced that F_3 is transformed into F_4 which again is converted to F_5 and so on, which is the same type of reaction as was found for the oligofructans from Helianthus tuberosum. Part of the sucrose is probably hydrolysed by invertase to form glucose and fructose. From both the synthesis of the oligofructans and from hydrolysis of sucrose, glucose is set free. The change in radioactivity of glucose follows almost the same pattern as sucrose, implying that the glucose is phosphorylated and transformed into sucrose again. If this was so, the amount of glucose present would be small which was found to be the case in this alga (p. 43) As mentioned in contrast to glucose the radioactivity of fructose increases throughout all experiments indicating that the fructose is probably not metabolised further, or only very slowly.

The proposed pathways for the metabolism of the low molecular weight carbohydrates are summarised in Fig.I.

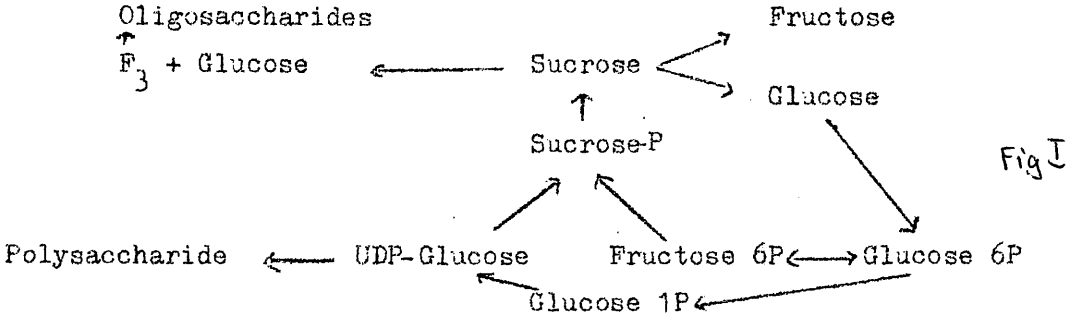


Fig I

[H.Mahler and Cordes, 1969; Hassid, 1967; Bassham and Calvin, 1957)

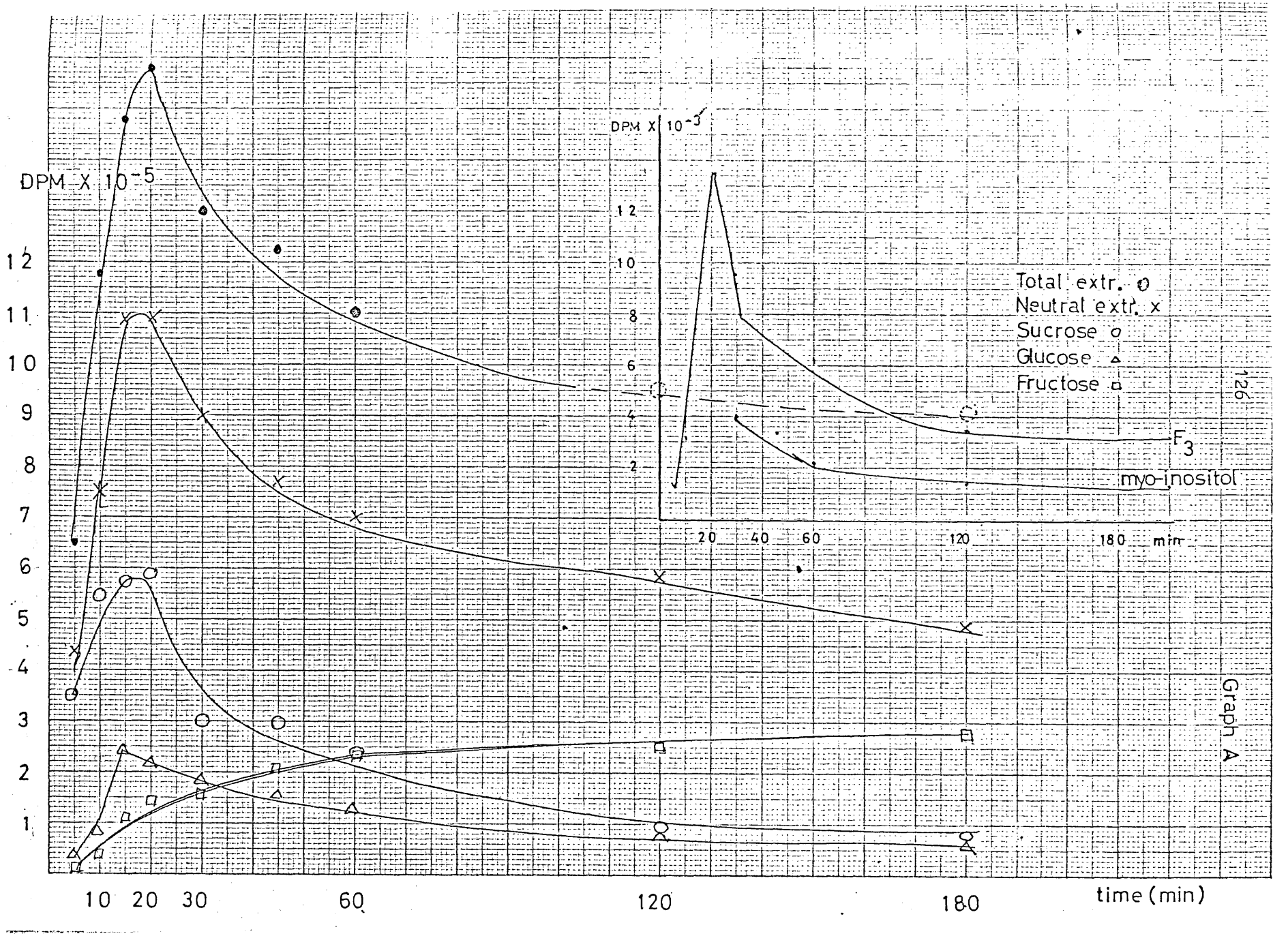
In other words:

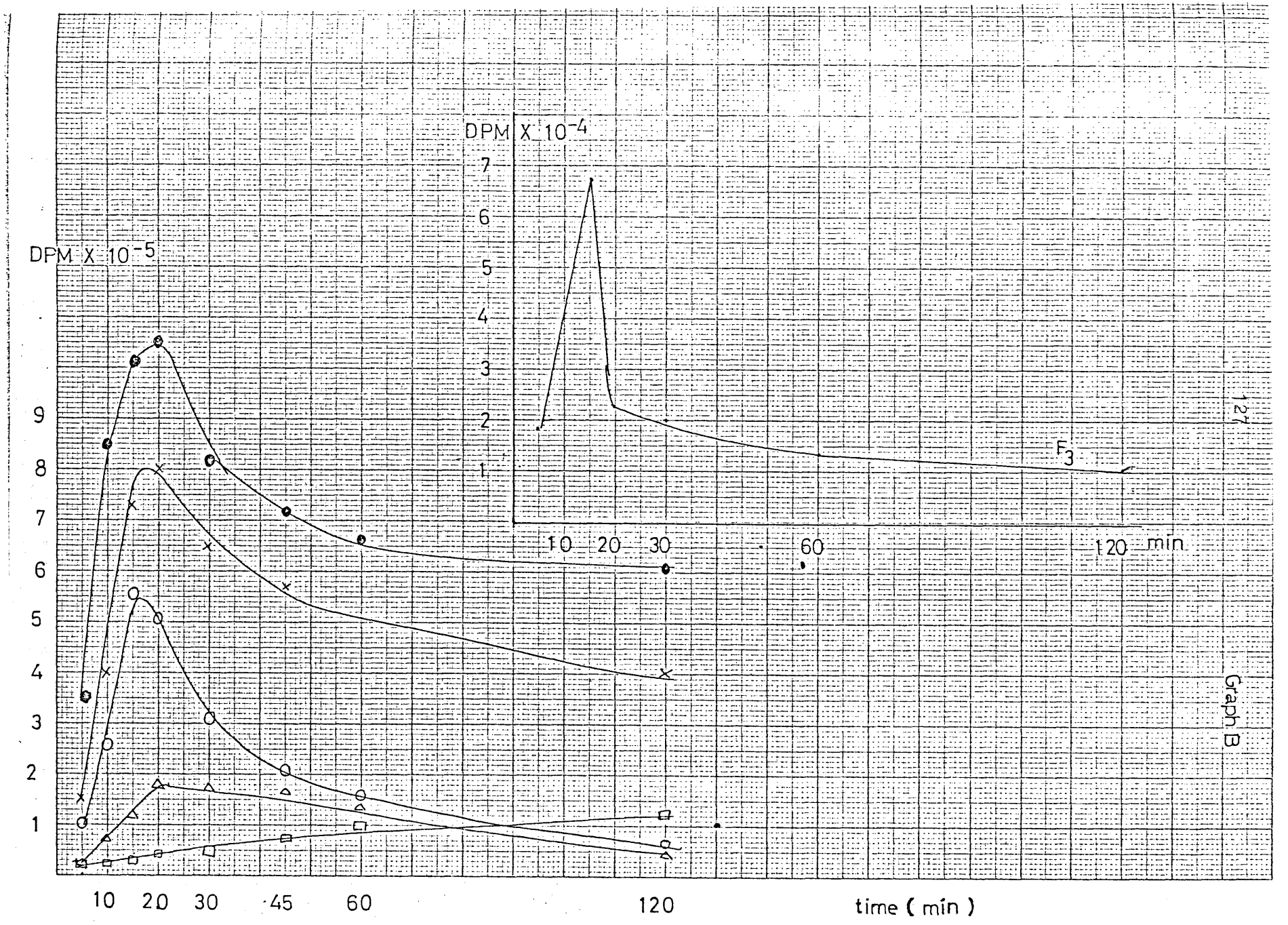
I Sucrose → Glucose and Fructose

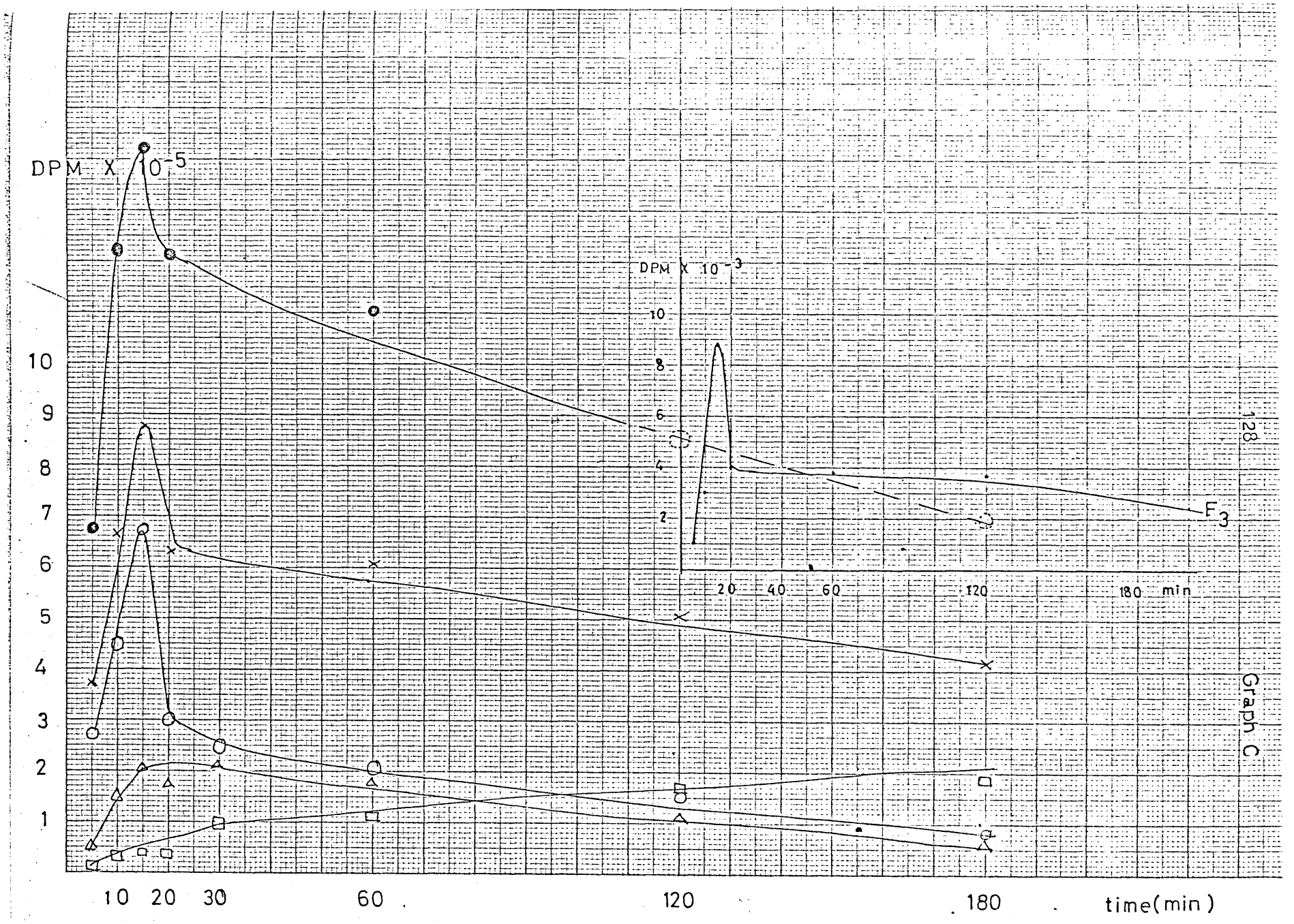
Sucrose → F_3 → higher homologues + Glucose

II Glucose → phosphorylated → sucrose
→ polysaccharides

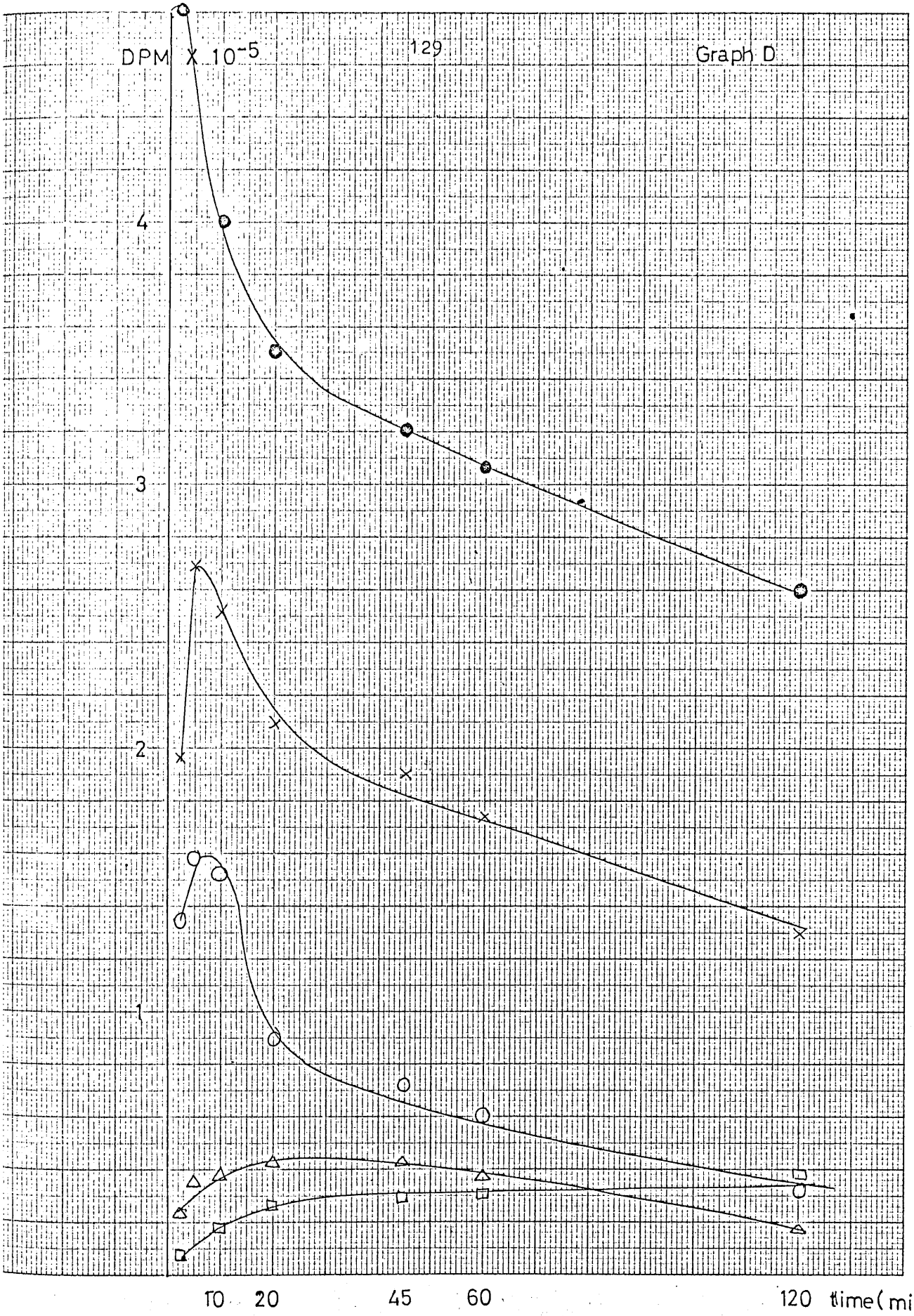
III Fructose — is probably an end product and not metabolised further.

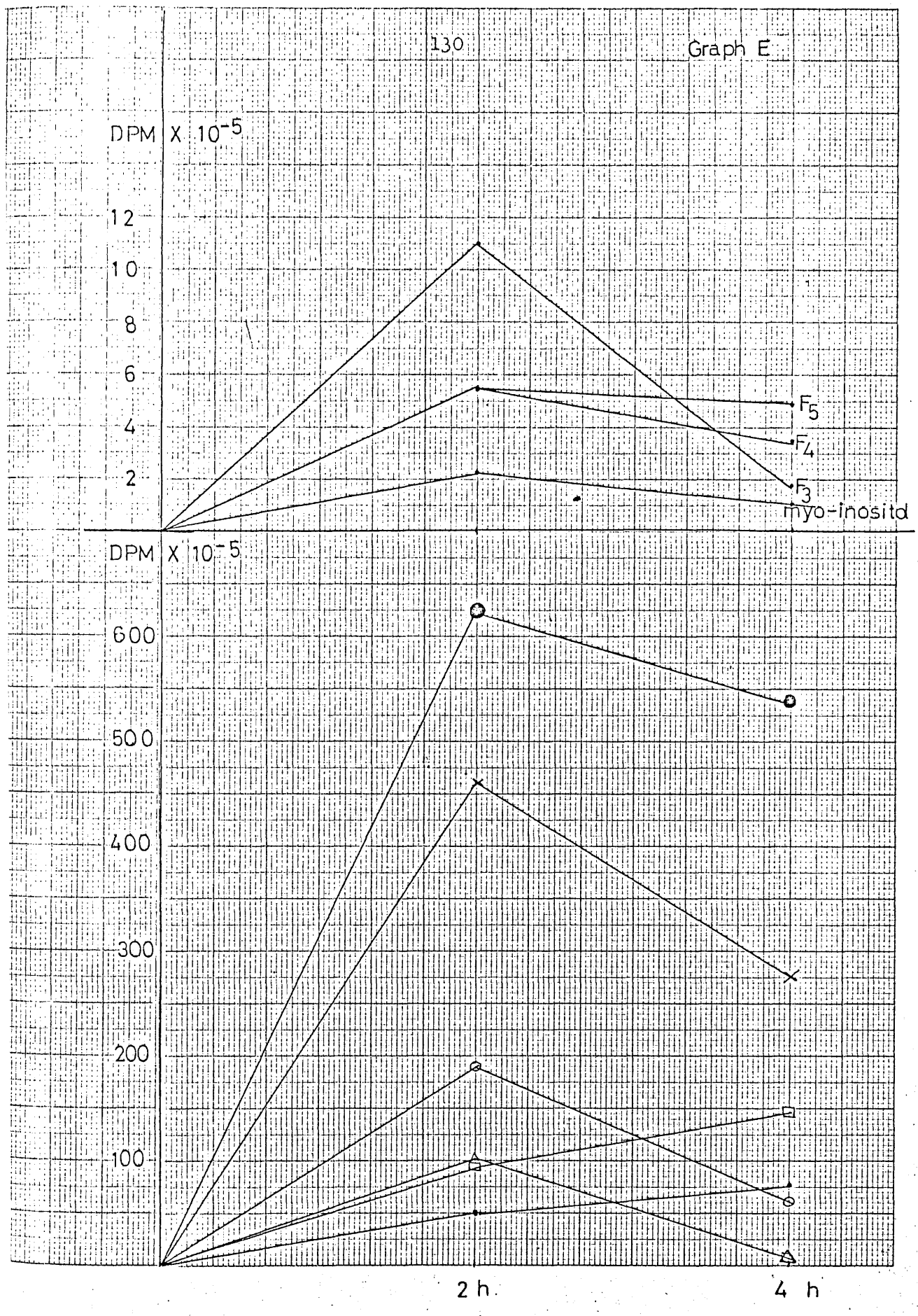

DPM X 10⁻⁵
12
11
10
9
8
7
6
5
4
3
2
1
10 20 30 60 120 180
time (min)
DPM X 10⁻³
12
10
8
6
4
2
20 40 60 120 180 min
Total extr. o
Neutral extr. x
Sucrose o
Glucose △
Fructose □
F3
myo-inositol
126
Graph A


DPM X 10⁻⁵
DPM X 10⁻⁴
F3
10 20 30 60 120 min
10 20 30 45 60 120 time (min)
Graph B
127


DPM X 10⁻⁵
10
9
8
7
6
5
4
3
2
1
DPM X 10⁻³
10
8
6
4
2
20
40
60
120
180 min
F3
10
20
30
60
120
180
time(min)
128
Graph C

Graph D




Graph E
DPM X 10^{-5}
12
10
8
6
4
2
F_5
F_4
F_3
myo-inositd
DPM X 10^{-5}
600
500
400
300
200
100
2 h
4 h

2. FUCUS VESICULOSUS

Brown algae are known to synthesize D-Mannitol (Black, 1950), 1-O-D-Mannitol-β-D-glucopyranoside, 1,6-O-D-Mannitol-di(β-D-glucopyranoside (Bouveng and Lindberg, 1955), laminaran, a β(1 → 3) linked glucan (Quillet, 1958), alginic acid (Black, 1950), fucoidan (Kylin, 1913, 1915) and several polysaccharides termed "glucuronoxylofucan" (Haug & Larsen, 1963; Larsen et al, 1966; Percival, 1968; Bourne et al, 1969; Mian, 1971). Several workers have shown interest in the biosynthesis, metabolism and respiratory role of these carbohydrates.

Kylin (1913) claimed that laminaran was a reserve substance. MacPherson and Young (1952) suggested on the basis of seasonal variations that mannitol and alginic acid are direct products of photosynthesis, and Black (1954) on the same grounds postulated fucoidan as a main storage carbohydrate of the Fucaceae.

The application of radioactive techniques in photosynthetic studies yielded more information about the function of these carbohydrates in brown algae. Fucus sp. have been most extensively studied from this aspect, but some studies have also been carried out on Eisenia bicyclis. Bidwell et al (1958) showed that when Fucus vesiculosus was allowed to photosynthesize in seawater containing $^{14}CO_2$, mannitol quickly became labelled. In contrast, when fronds of F. vesiculosus were supplied with Mannitol-U-^{14}C

in seawater little of this was metabolised, and it was concluded therefore that mannitol was a storage product, rather than an active metabolite (Bidwell and Gosh, 1962). Later work, however, indicated that plants probably respire endogenous cellular mannitol in preference to exogenously supplied mannitol (Bidwell et al, 1964). The large pool of mannitol existing in F.vesiculosus would therefore make it more difficult for mannitol-U-^{14}C supplied in the medium to serve as a substrate for respiration.

Yamagushi et al (1966) found that when the brown alga Eisenia bicyclis was fed with $^{14}CO_2$ in the light and was allowed to continue growth in the dark in inactive medium, the mannitol acquired radioactivity quickly during the first period, followed by a decrease in the activity in the second period. The total weight of mannitol increased in the light and decreased in the dark. The decrease in radioactivity and amount of mannitol during the dark period, suggested that mannitol is an active metabolite and is converted into other substances through unknown pathways. On the other hand, the amount present did not change as much as the radioactivity during both the light and dark period, which indicates that of the mannitol present, the recently formed molecules appear to be

metabolized preferentially. This implies that part of the mannitol is a storage product.

In a similar experiment with F.vesiculosus the same changes in the radioactivity of mannitol was observed by Bidwell (1967) as was found in E.bicyclis.

The conclusions to be drawn about the role of mannitol in brown algae are therefore as follows: Mannitol is a major product of photosynthesis, part of it probably serving as a substrate for respiration and the rest serving as a storage product.

In the experiments carried out on E.bicyclis (Yamagushi et al, 1966) it was found that laminaran also quickly became highly labelled in the light in presence of $^{14}CO_2$, and that the activity decreased in the dark in the absence of $^{14}CO_2$. The authors concluded that mannitol and laminaran are interconvertible in the same way as are sucrose and starch in higher plants, but the metabolic pathway is not known. This is in agreement with Nisizawa's (1938, 1940 a and b) observations of diurnal changes as well as changes in seasonal growth in mannitol and laminaran in E.bicyclis. The content of laminaran was highest in the summer and that of mannitol highest in the winter.

When F.vesiculosus was subjected to pulse labelling experiments (Bidwell, 1967) it was observed that the "fuccidan" (i.e. acid soluble polysaccharide) acquired radioactivity rapidly

during the supply of $^{14}CO_2$, and slowly thereafter, tending to increase more rapidly in the dark than in the light. This "fucoidan" consists of laminaran, and several sulphated fucose-containing polysaccharides. As Bidwell did not fractionate the polysaccharides his conclusion that there is no relation between mannitol and the polysaccharides in brown algae similar to that found between sucrose and starch in higher plants is inapplicable.

Goldenberg and Marechal (1963) have demonstrated the synthesis of a $\beta(1 \rightarrow 3)$ linked glucan from UDP-glucose, using a transferase from the freshwater flagellate, Euglena. Lin and Hassid (1966) have shown that UDP-glucose is present in brown algae, and suggest this as a precursor for laminaran, and that parallel seasonal variations of laminaran and mannitol may indicate that mannitol has a biological relationship to the ~~poly~~glucan.

As already mentioned, sulphated polysaccharides containing fucose, xylose, glucuronic acid and galactose have been found both in the acid and alkali-extracts of brown algae; the acid soluble sulphated polysaccharide can be fractionated into several polysaccharides, see p. 10. Bidwell et al (1958) found in their early experiments that the polysaccharide that acquired radioactivity most rapidly was the "alginic acid"

(i.e. alkali soluble polysaccharides), and claimed therefore that "alginic acid" was in a state of active metabolism, undergoing breakdown and resynthesis at rates corresponding to starch in plants. When fronds of F.vesiculosus were supplied with radioactive pyruvate and acetate (Bidwell and Gosh, 1963a) most of the activity in the polysaccharide fraction was recovered in the "insoluble alginic acid" fraction, and hardly any was detected in laminaran and fucoidan. This indicated different precursors for the latter polysaccharides from those of "alginic acid". Other experiments showed that the radioactivity of "alginic acid" was increasing both in light and in dark (Bidwell, 1967), (this"alginic acid" contains all the alkali-soluble polysaccharides) which implies that "alginic acid" is either a long term storage material or is permanently laid down in the weed. This interpretation of these results are however questionable, as no separation was carried out of the polysaccharides extracted with alkali.

When F.gardneri Silva discs were infiltrated with mannose-U-^{14}C (Lin and Hassid, 1966) they both gave rise to labelled respiratory CO_2, fucoidan, "alginic acid" and "residual" fractions. Activity was also found in sugarphosphates, sugarnucleotides and glyconic acids. All enzymes required for the transformation of D-mannose to GDP-D-mannuronic acid and GDP-L-guluronic acid, which probably would be the precursors for

the synthesis of alginic acid were shown to be present. Both GDP-D-mannuronic acid and GDP-L-guluronic acid have been found in F.gardneri Silva (Lin and Hassid, 1966 b).

Yamagushi et al (1966) found an increase in the radioactivity in the fucose of the "crude laminaran fraction" (i.e. acid soluble polysaccharides) after E.bicyclis had been transferred to inactive medium. From this it was considered that fucoidan or fucose-containing polysaccharides are storage carbohydrates.

GDP-fucose has been found in F.gardneri Silva (Lin and Hassid, 1966 b) and this may be a precursor for the fucose containing polysaccharides although this biosynthesis has not yet been demonstrated in algae. Ginsberg (1960) has shown evidence for conversion of GDP-D-mannose to GDP-L-fucose using an enzyme system from Aerobacter aerogenes.

It has also been demonstrated that most of the constituents of brown seaweed polysaccharides are derived by various reactions from the common precursor GDP-D-mannose, and it has been proposed that this has the same role in brown algae as UDP-D-glucose has in higher plants (Lin and Hassid, 1966 b).

The fucose containing polysaccharides are highly sulphated, and Bidwell and Gosh (1963) found that the molar ratio of incorporation of sulphate ^{34}S to ^{14}C in "fucoidan" was 600:1 implying that there is a considerable turnover of sulphate in

"fucoidan", presumably without breakdown and resynthesis of the carbohydrate chain. It is also probable that sulphation follows polysaccharide formation in the synthesis of fucoidan.

Recent studies on carbohydrates in Fucus vesiculosus (Bourne et al, 1969) showed that the "fucoidan" and "alginic acid" fraction studied by Bidwell (1967) each consisted of more than one polysaccharide. Black and Dewar (1949) had shown that Fucus sp. synthesize up to 7% of laminaran which together with sulphated fucose-containing polysaccharides, is extracted with dilute acid. As mentioned on p.10 , this sulphated polysaccharide can be further fractionated into several polysaccharides.

The alkali-soluble polysaccharides consist of alginic acid and a sulphated polysaccharide called glucuronoxylofucan. These could be separated by precipitation of the alginic acid as its calcium salt. (Bourne et al, 1969).

With this knowledge and also the possibilities of measuring the proportions of the sugar residues and their respective radioactivities in the individual polysaccharides it was decided to carry out further metabolic studies on the carbohydrates of Fucus vesiculosus.

Materials:

Experiment I.

Sterile fronds, without vesicles (2-5 cm long) of Fucus vesiculosus were used. The alga was collected at Point Pleasant, Halifax, Nova Scotia, Canada, in August 1969. The fronds were cleaned and the surface wiped to remove epiphytes etc.

Experiment II.

F.vesiculosus fronds, as for experiment I, were used. They were 1-2 cm long, and were collected in August 1970. After cleaning, the fronds were stored in sterile seawater of 15°C over-night and used for growth experiments the following morning.

Experimental and results:

Exp.I.(1) The fronds (50 g. wet, blotted weight) were placed in 10 l seawater at 20°C, illuminated for 10 min. Then 1.0 mCi $Na_2{}^{14}CO_3$ was added, and the sample was illuminated for a further 10 min. The fronds were removed from the seawater, rinsed in inactive seawater and pulverized in a mortar containing liquid nitrogen. The resulting powder was extracted as described below.

Exp.I.(2) was similar to exp.I (1), except that the fronds (50 g.) were allowed to photosynthesise in 5.0 l seawater containing 2.0 m Ci $Na_2{}^{14}CO_3$ for 3 hours.

Exp.II. The fronds (35 g. wet, blotted weight) were allowed to photosynthesise in sterile seawater, 1005 ml, that had been freed from carbon dioxide by acidifying to pH3, purging with nitrogen for 20 min. and readjusting the pH to 8 with 1 N NaOH(CO_2^{--} free) The pH of the seawater was brought to 8,3 by the addition of $NaH^{14}CO_3$ [equivalent to 0.18 m Mole CO_2 and 0.91 m Ci.]. After growth in the presence of $^{14}CO_2$ for 10 min, the fronds were removed from the medium and rinsed thoroughly with seawater, 1.5 l, to remove activity adhering to the surface. One sample II_3 (8.8 g. blotted weight) was removed, and the rest was allowed to continue growth under the same conditions as before in inactive seawater, 1.8 l. After 30 min, another sample II_4 (blotted weight 11.2 g.) was removed, and to the remaining sample II_5 (14.8 g.) 2 m Mole of $NaHCO_3$ was added. Growth was stopped after a further 1.5 hours.

The temperature was maintained at ($20.5 \pm 1.5^{\circ}C$) by circulation of cold filtered seawater.

To facilitate comparison, all results given are calculated on 10 g. blotted weight.

Extraction procedure.

1. 80% ethanol at $70^{\circ}C$, carried out 5 times for 2-3 hours each.
2. The residue from 1, was extracted 5 times with HCl, pH2.0, at $70^{\circ}C$, for periods of 6-10 hours, with continuous stirring.

3. The residue from 2. was extracted with 3% Na_2CO_3 at 50°C for 6-12 hour periods until negligible radioactivity was found in the extract

Extract 1. 80% ethanol extract.

The combined extracts were filtered through glasswool, concentrated to 120 ml, and the total radioactivity was measured (Table I). Charged materials, such as amino acids, organic acids, sugar phosphates etc., were then removed by treating the extract with Biodeminrolite-resin in carbonate form. The neutral filtrate and washings, containing the low molecular weight carbohydrates, were concentrated to a known volume and the radioactivity measured (Table I). The solutions were then concentrated to a minimum volume, ethanol was added and the mixture set aside overnight in the refrigerator. Mannitol crystallised out. This was filtered off, washed with cold ethanol and ether and dried. It was weighed and its radioactivity measured (Table I).

The alcoholic supernatant and washings separated from Experiment II, were concentrated and subjected to paperchromatography in solvent A. Radioautography revealed 3 radioactive compounds with mobilities corresponding to mannitol, β-D-glucosyl-1-mannitol and β-D-diglucosyl-(1 - 6-mannitol) (Bouveng and Lindberg 1955). The respective radioactivities of these compounds were measured by liquid scintillation counting. (Table I).

TABLE I

Radioactivity of the low molecular weight carbohydrates in F.vesiculosus. (DPM x 10^{-4} / 10 g. blotted weight of weed.

	Exp.I		Exp.II		
Fraction	1	2	3	4	5
Ethanol extract	3300	16000	7800	9800	9800
Neutral part	1500	14000	5200	7200	7500
Mannitol (615 mg/10g.)	1200	11000	5100	7000	7300
Glucosyl-1-mannitol	- *	-	72	23	17
Diglucosyl-1-6-mannitol	-	-	7	8	8

* not measured.

Extract 2.Acid soluble material.

The combined extracts were filtered through glasswool and the total radioactivity of the filtrate was determined (Table II). It was poured into ethanol to give a final concentration of 80% ethanol. This was allowed to stand at 4°C over-night and the precipitated polysaccharide was filtered off. After drying, the precipitated polysaccharides were separated on a DEAE-cellulose column (see p. 29).

The alcoholic supernatant was concentrated and its activity (Table II) was determined.

Examination of the acid soluble, alcohol soluble fraction from Exp.II.

Electrophoresis in neutral buffer followed by scanning for location of radioactive compounds showed that less than 2% of the total radioactivity of the extract was present in charged compounds, most of the radioactivity in all three samples retained in neutral material on the starting line of the electrophoretogram. After elution, this neutral material was subjected to paper chromatography in solvent A and B. This showed the presence of 4 oligosaccharides together with fucose and a fast moving component. The radioactivity of each of the components was measured (Table II). The oligosaccharides were separated by preparative paperchromatography in solvent B and hydrolysed. Paper chromatography of the hydrolysates in solvents A and B showed the presence of fucose and galactose in all four compounds. The hydrolysate of the oligosaccharide with R_{fucose} 0.39 also gave a spot with the mobility of mannose; the fastest moving oligosaccharide, R_{fucose} = 0.57, contained an unknown with R_{fucose} 1.4. The immobile oligosaccharide contained all the above mentioned sugars, together with xylose and a trace of glucuronic acid.

Acid-soluble, alcohol-soluble polysaccharide

Previous studies by Black and Dewar (1949) have shown that this extract contains laminaran and fucoidan. In the present experiments separation was achieved on a Whatman DE52 microgranular cellulose column by successive elution with water, 0.5 M potassium chloride and 1.0 M potassium chloride. The three fractions were

TABLE II

Radioactivity and chromatographic mobility (solvent A) of components of the acid-soluble, alcohol-soluble supernatant. (Activity in DPM x 10^{-4})

		1	2	3	4	5
Total activity of acid extracts		450	1600	580	250	383
Total activity of acid supernatant		420	1200	440	170	83
	R_{fucose}					
Oligosaccharide 4		0.00		73	55	29
"	3	0.25		110	24	14
"	2	0.39		-	-	-
"	1	0.57		84	10	6
Fucose		1.00		85	32	18
"Fast spot"		1.4		79	43	12

separately dialysed, freezedried and weighed. Their activities were measured (Table III) and constituents determined.

Fraction 1. Laminaran

Hydrolysis of an aliquot gave only glucose, which was confirmed with glucose-oxidase spray.

Fraction 2. Xyloglucuronogalactofucan (B).

This fraction was slightly contaminated with alginic acid.

The latter was removed by precipitation with 2% calcium chloride. The weight, radioactivity and carbohydrate content of the recovered, purified, B, [Sulphate 18.9%] were measured. After hydrolysis, the proportions and radioactivities of the individual sugars were determined (Table V). p.148.

Fraction 3. Fucoidan (C). [Sulphate-content 23.3%]. Hydrolysis was carried out and the proportions and radioactivities of the individual sugars were determined as for fraction 2. This fraction, in addition to fucose, also contained glucuronic acid, galactose and xylose.

TABLE III

Radioactivity of acid soluble polysaccharides in *F. vesiculosus* fronds at intervals during (Exp.I) and following (Exp.II) the supply of $^{14}CO_2$ (DPM x 10^{-4})

Fraction	Weight of carbohydrate. mg.	Exp. I		Exp.II		
		1	2	3	4	5
1.Laminaran	1.5	18	27	9.4	1.6	5.4
2.Xyloglucurono-galactofucan(B)	128	2.9	298	19	97	98
3.Fucoidan	53	0.39	91	2.7	11	20

Extract 3. Alkali-soluble material.

The extracts were combined and the radioactivity measured both before and after dialysis. The dialysed extracts were poured into ethanol to give a final concentration of 80% ethanol. This

was left at 7°C overnight and the precipitated polysaccharide filtered off, washed with ethanol and air-dried. It has previously been found that this supernatant comprises alkaline degradation products of the polysaccharides (Bourne et al, 1969). The radioactivity in this supernatant was measured (Table IV) but further investigations were not carried out.

The precipitated polysaccharide was dissolved in 1% ammonia, and the alginic acid precipitated as the calcium salt after addition of 2% calcium chloride. This was filtered off, washed with 2% calcium chloride solution, ethanol and ether and air-dried. This was then dissolved in 5% sodium carbonate, dialysed and freeze-dried. The carbohydrate content and radioactivity was measured (Table IV).

Preliminary studies suggested that the alkali soluble, calcium soluble polysaccharide was contaminated with a β-(1 → 3) linked glucan. This was therefore incubated with a β-(1 → 3)-glucanase. The reaction was carried out in deionised water, in a dialysis sac at room temperature. After several changes of dialysis water, and 24 hr incubation time, no more carbohydrate was released from the dialysis bag. The enzyme was inactivated by boiling the solution and removed by centrifugation. The resultant, pure polysaccharide was freeze-dried, weighed and the carbohydrate content and radioactivity was measured (Table IV). /p.148 The polysaccharide (A) was hydrolysed and the proportions and radioactivities of the individual sugars were determined. (Table V). This polysaccharide

has previously been named glucuronoxylofucan, but these studies indicate that a more correct name is xylogalactofucoglucuronan, and this name is used hereafter.

TABLE IV.

Radioactivity in alkali soluble fractions and of the polysaccharides present in this extract.

($DPM \times 10^{-4}$)

	mg.carb.	1	2	3	4	5
Alkali extract	-	240	2100	890	490	580
Alkali ext.sup.after dialysis	-	20	430	2.1	2.1	4.0
Alginic acid	220	17	290	120	290	400
Xylogalactofuco-glucuronan	37	8.1	320	38	64	60

Residual material

The residue after the alkali extractions was hydrolysed with 72% H_2SO_4 in an icebath for 30 min (Blake and Richards, 1970). After addition with cooling of 50 times the volume of water, the mixture was heated at 100°C for 3 hours, then neutralised with $Ba(OH)_2$ and $BaCO_3$ and filtered. The radioactivity and carbohydrate contents of the residual materials in this hydrolysate were then measured (Table VIII). The hydrolysates from experiment I were then treated with Biodeminrolit (in the carbonate form) to remove any amino acids and glucuronic acid. The radio activity in the resultant "neutral"

TABLE V

Galactoglucuronoxylofucan Rel.Prop.

Activities in 1 g Polysaccharide

Polysaccaride		DPM x 10^{-4}					
			I			II	
	Rel.Prop.	1	2	3	4	5	
	Total wt.						
Xylogalactofuco-glucuronan (A)							
Fucose 259 mg/g Poly.	9.6mg.	1.1	97	3400	310	610	660
Galactose		1.0	21	890	160	270	260
Xylose		0.95	20	850	130	230	270
Glucuronic acid		1.2	59	2700	260	440	330
Oligosaccharide		-			160	170	110
Xyloglucurono-galactofucan (B)							
Fucose 773 mg/g Poly.	98 mg.	7.8	6.6	824	49	340	430
Galactose		1.0	3.3	192	31	62	85
Xylose		0.5	4.3	230	22	81	54
Glucuronic acid		0.8	7.1	958	10	120	67
Oligosaccharide		-			32	79	130
Fucoidan (C)							
Fucose 900 mg/g Poly.	50 mg.	20		1570	34	160	270
Galactose		1.0		28	5.4	19	36
Xylose		0.9		35	2.6	6.6	17
Glucuronic acid		0.3		63	4.2	5.5	15
Oligosaccharide		-			4.5	14	42

TABLE VI

Specific radioactivities in polysaccharides of F.vesiculosus fronds at intervals during (Exp.I) and following (Exp.II) the supply of $^{14}CO_2$.
(DPM x 10^{-3}/ mg.carbohydrate)

	1	2	3	4	5
Laminaran	55	140	58	19	40
Xylogalactofucoglucuronan (A)	2.2	85	10.3	17.2	16.3
Xyloglucuronogalactofucan (B)	0.23	23	1.5	6.8	7.6
Fucoidan (C)	0.09	17	0.5	2.0	3.8
Alginic acid	0.62	13	1.6	2.4	3.9

TABLE VII

Radioactivity and weight of the fucose-containing polysaccharide present in 10 g. blotted (DPM x 10^{-4})

Polysaccharide	Wt. of Carb.	1.	2	3	4	5
Xylogalactofucoglucuronan (A)	37 mg.	8.1	320	38	64	60
Xyloglucuronogalactofucan (B)	128 mg.	2.9	298	19	97	98
Fucoidan (C)	53 mg.	0.39	91	2.7	11	20

hydrolysate was measured. Paper chromatography in solvents A and B showed that the neutral part of the residue consists of glucose (major) together with galactose, xylose, fucose and an unknown compound with R_{fucose} (solvent A) 1.40. The activities in the various sugars were measured.

TABLE VIII

Radioactivity of residual material after extractions of 10 g. blotted weed.
(DPM x 10^{-4})

	1	2	3	4	5
Total residue	33.7	425	141	211	251
Neutral material	6.5	51	- *	-	-
Galactose	2.0	11.3	-	-	-
Glucose	3.0	25.8	-	-	-
Fucose	0.7	7.5	-	-	-
Unknown	0.7	6.7	-	-	-

* not measured.

Periodate oxidation of Xylogalactofucoglucuronan (A), Xyloglucurono-galactofucan (B) and Fucoidan (C).

Experimental

A sample of each of the polysaccharides was oxidised with 0.015 M IO_4^- for 7.5 hours. The reaction was stopped by adding excess ethyleneglycol. The oxidised polysaccharides were then

Results

TABLE IX

	Starting material sample	Before periodate oxidation: Weight carbohydrate	Activity DPM	Periodate uptake mol/C_6 unit	After periodate oxidation: Wt.mg. carbohydrate	DPM polyalcohol	DPM dialysate	Cleaved carbohydrate	Calculated uptake mol 104/C_6
A	I_2	4.85 mg	369,000	0.8	1.40	133,000	14.287	3.45 mg.	0.69
B	II_4	6.3 mg	47,200	0.44	3.25	31,200	3.835	3.05 mg.	0.48
C	II_5	3.96 mg	16,680	0	2.45	11,730	768	1.51 mg.	0.38

TABLE X

Radioactivity present in the individual sugars of the initial polysaccharides

Sugar unit	A	B	C
Glucuronic acid	101.200	9.440	740
Galactose	41.830	4.840	1.770
Xylose	40.580	6.290	885
Fucose	161.100	26.630	13.260

TABLE XI

Radioactivity present in the individual sugars after periodate oxidation

Sugar unit	A	B	C
Glucuronic acid	6575	1120	325
Galactose	4055	1010	405
Xylose	6290	575	220
Fucose	16,080	7775	6540

reduced to their respective polyalcohols by adding 2% KBH_4 in 0.05 M boric acid, and allowed to stand over-night. Excess borohydride was destroyed with acetic acid and the solutions dialysed. The polyalcohols were freeze-dried, hydrolysed, and the hydrolysate subjected to paper chromatography in solvents B and C, and to electrophoresis in neutral buffer.

The radioactivities and carbohydrate contents of all the samples were measured before and after each operation (Table IX). The radioactivities of the individual sugars present in each polysaccharide were calculated from results in Table V, and the radioactivity in the uncleaved sugars in the polyalcohols were measured as outlined in general methods (Table X). The periodate uptake was measured after the method of Aspinall and Ferrier (1957).

The radioactivity in uncleaved sugars after periodate oxidation is obtained from paper chromatography of the neutral sugars and for glucuronic acid by paper chromatography in solvent C and electrophoresis. (Table XI).

Discussion

When Fucus vesiculosus fronds were grown for 10 minutes in the presence of $^{14}CO_2$ (Exp.I p.139) mannitol became highly labelled, and its radioactivity was ten times higher after growth for 3 hours in the presence of $^{14}CO_2$(Table I, exp.I). In experiment II, again the radioactivity of mannitol was very high, but in the absence of $^{14}CO_2$ in the medium, this remained almost constant, after a small increase during the first 30 minutes. The quickly acquired activity of mannitol is in full agreement with the findings of Bidwell (1967) and Yamagushi et al (1966) and the pattern of change in radioactivity, or rather lack of change, in experiment II, supports the theory that mannitol serves both as a storage product and as a substrate for respiration.

The change in radioactivities of the glucosyl-mannitol and diglucosyl-mannitol suggests that the glucosyl-mannitol is formed first, probably by transfer of a glucose unit to mannitol, and then another glucose unit is added to form the diglucosyl-mannitol. The extent of labelling in the glucosyl-mannitol is relatively high after the first 10 min., when one takes into consideration the small amounts present. This activity decreases during the 2 hour period in the absence of $^{14}CO_2$, while that of

diglucoside-mannitol (also present in small amounts) appear to be unchanged. The two oligosaccharides are probably built up sequentially by the transfer of glucose to C-1 and C-6 of mannitol.

The amount of laminaran metabolised by Fucus vesiculosus is very small compared with that of mannitol, but it was relatively highly labelled (Table III)/ p.145 which again agrees with the results of Yamagushi et al (1966). These results indicate that mannitol is probably partly converted into laminaran via glucosyl-mannitol and diglucosylmannitol; which supports the theory of the Japanese workers that mannitol and laminaran probably play the same role in brown algae as sucrose and starch do in green algae and higher plants.

It was calculated from the uptake of $^{14}CO_2$ and the radioactivity of carbondioxide supplied during the 10 minutes metabolism in experiment II, that the alga would take about 1000 hr.to double its weight. For this reason any increase in weight during the experiments would have negligible effect on the weights of the different polysaccharides, and therefore for simplicity and to permit comparison, the average carbohydrate weights of the different polysaccharides in 10 g. of blotted weed is given (Tables III and IV). p.145 and 147.

The different samples of weed were extracted under identical conditions by acid and by alkali and the two extracts

were fractionated into their individual polysaccharides. Hydrolysis of the precipitated polysaccharides from the acid extract gave on paper chromatography fucose as the major sugar, together with glucose, glucuronic acid, galactose, xylose and trace quantities of a sugar with the mobility R_{fucose} 1,4 (Solvent A), slightly faster than 3-O-methyl-fucose (Bernardi and Springer, 1962). This indicated the presence of a glucan as well as a heterofucan. The polysaccharides were successfully separated on a DEAE-cellulose-column into (1) laminaran, (2) a sulphated xyloglucurono-galactofucan (B), with fucose content ca.78% of the total carbohydrate content, and (3) fucoidan (C) in which the fucose content was over 90% of the carbohydrate content (Table V). A third sulphated polysaccharide (A), also fucose-containing, was obtained from the alkali extract of the alga. This has previously been named by Bourne et al (1969) "glucuronoxylofucan", but analysis of the constituent sugars of the present polysaccharide indicate that a more correct name is xylogalactofuco-glucuronan (A). Galactose was not reported to be present by the above authors, although its presence was suspected (Brush, 1970). It should be pointed out that separation by paper chromatography of galactose and glucuronic acid is difficult when the latter is in excess. A hydrolysate of (A) also contained a trace of the material with R_{fucose} 1,4, but the amount present was too small to measure.

Fucose-containing polysaccharides comprising different proportions of fucose, xylose, galactose and glucuronic acid, have

already been reported (Percival and McDowell, 1967) and it seems that the brown algae synthesise a wide variety of this type of polysaccharides.

The three above mentioned fucose-containing polysaccharides are not therefore considered to be three distinct polysaccharides metabolised by the alga, but the result of a somewhat arbitrary fractionation of a family of polymers based mainly on fucose. In this framework, polysaccharide (A) represents one end and polysaccharide (C) the other end of the family spectrum.

After precipitation of the polysaccharides present in the acid extracts, considerable radioactivity remained in the supernatant. It was first thought that this supernatant contained mainly degradation products of the polysaccharides, but if that were the case, one would expect the radioactivity in the supernatant and in the polysaccharide to increase with the same rate. However, the radioactivity in the supernatant decreased as the culture experiment proceeded whereas that of the polysaccharides increased (Table II)p.144. It is probable that some of the radioactivity in the supernatant is derived from degraded polysaccharides, but only a small fraction can be due to this. Paper chromatographic examination of this supernatant revealed the presence of fucose, the compound with R_{fucose} 1,4, and four oligosaccharides (Table II). If these substances had been present as such in the alga, they would have been soluble in 80% ethanol. As they were not present

in the 80% ethanol extract they must therefore have arisen from highly labelled, acid-labile higher oligosaccharides. Because the radioactivity of these oligosaccharides decreased after the alga had been transferred to inactive medium, it is postulated that they are part of the metabolic pool for synthesis of the polysaccharides. The radioactivity in such a pool would be expected to decrease after transfer to inactive medium because there would be no new $^{14}CO_2$ available. This concept of a metabolic pool would explain the rapid increase of radioactivity in polysaccharides (B) and (C) in the first half hour in the absence of $^{14}CO_2$ and the continued increase in radioactivity in polysaccharide (C) in the following 1.5 hours. (Table III, p.145 sample 5).

The extent of labelling of the three fucose-containing polysaccharides (Table VII)/, p.149 their high specific activity (Table VI), p.149 and the change of activity in all three during the period of the experiment indicate that they are all being actively metabolised. The activity in (A) was the highest of the three after growing for 10 minutes in radioactive medium, it almost doubled during the next 30 minutes in inactive medium, and then decreased slightly during the following 1.5 hours. In contrast, the other two polysaccharides, particularly fucoidan (C), acquired relatively little radioactivity, considering the respective weights of A, B

p.148
and C,/during the 10 min. growth in $^{14}CO_2$. During the following 30 minutes, however, the radioactivity of polysaccharide (B) increased five-fold and that of fucoidan (C) four-fold. The radioactivity of polysaccharide (B) remained almost constant during the following 1.5 hours, while that of fucoidan (C) almost doubled. (Table VII). It seems that some of the radioactive fucose-containing metabolic precursors remaining in the metabolic pool after $^{14}CO_2$ was removed from the growing medium, were used to synthesise polysaccharides (B) and (C).

The decrease in radioactivity of (A) (Table VII) and the increase in the radioactivities of the other fucose-containing fractions during the last 1.5 hours, with concomitant decrease of radioactivity in the "precursor pool" (acid supernatant) suggest the following tentative pathway for the biosynthesis of fucoidan (C):

xylogalactofucoglucuronan (A) → xyloglucuronogalactofucan (B) → fucoidan (C)

As would be expected from the high proportion of unlabelled fucose in the polysaccharides at the beginning of the experiments, the fucose in polysaccharides (B) and (C) is relatively less labelled than that of (A) after 10 minutes, but radioactivity of fucose in these two polysaccharides increased considerably throughout the experiment. If there had been no relationship between the biosynthesis of these three polysaccharides, one would expect the

same changes in radioactivity for each of them throughout the experiment, but this is clearly not so.

In both experiment I and II, there is a marked increase in radioactivity in the alginic acid throughout the experiment. The increase continues even after transfer to inactive medium, which indicates that there must be a ppol of radioactive precursors present in the alga. This may well be in the acid supernatant, as only 78 x 10^4 DPM (of the 270 x 10^4 DPM which was utilized from the supernatant) was incorporated into fucose-containing polysaccharides during the first 30 min. in inactive medium (Table II)/p.144, and the difference is even more pronounced in the following 1.5 hours.

Yamagushi et al (1966) found that the radioactivity in the "alginic acid" fraction increased during photosynthesis and remained unchanged during growth in the dark - indicating that "alginic acid" is probably a direct product of photosynthesis. However, this "alginic acid" fraction probably also contained polysaccharide (A).

The hydrolysate of the residual material after acid and alkali extraction was found to be highly radioactive, but a a high proportion of this was removed by ion-exchange treatment (Table VIII)/p.150 and was therefore probably due to aminoacids, peptides and uronic acids. Chromatographic analysis of the neutral material showed mainly glucose with less galactose, a

little fucose and an unidentified compound (R_{fucose}-0.60). Glucose was found to be highly labelled, but all the others contained a certain degree of labelling.

The glucose is probably derived from cellulosic cellwall material. Yamagushi et al (1966) called this the "cellulose" fraction and showed that it was completely hydrolysed to glucose by a cellulose preparation from Trichoderma viride. These authors found that this fraction acquired activity throughout growth in presence of light but the change in activity during growth in the dark was complicated. Bidwell (1967) found in the "insoluble residue" an increase in activity in the dark and a decrease in the light, indicating active metabolism with probably more than one compound involved.

The periodate oxidation of the polysaccharides A,B and C was carried out in order to see which if any of the constituent sugars resisted oxidation. If no radioactivity was found in the area on the paperchromatogram corresponding to a particular sugar, this would indicate that this sugar was oxidised by periodate and a) was present as endgroups, or b) that it was present in the inner part of the molecule linked to two other sugar units in such a way that it was vulnerable to periodate oxidation, or it could be present as a combination of a) and b). If one considered a)

to be the case for the glucuronic acid and xylose units, the theory put forward concerning the biosynthetic relationships among the polysaccharides A, B and C would be incorrect, but if b) or a combination of a) and b) was the case, the theory would still be valid.

If after the periodate oxidation there still remained radioactivity in the areas corresponding to all the constituent sugars this would be further evidence for the proposed theory, assuming that no acetal formation took place which hindered complete periodate oxidation. As is evident from Table XI,p.152, all the sugars remained after periodate oxidation.

Their presence on paperchromatograms was checked in several solvents and also by ionophoresis. In each case their respective radioactivity was measured. This was to ensure that the radioactivity was actually present in these sugars and not in other breakdown products of oxidation. As the same counts were obtained in at least two different systems for each of the sugars, one can consider that they are correct.

Other investigations in this laboratory have provided evidence that no acetal formation occurs with fucoidan (C), but that in a glucuronic acid rich material acetal formation does occur. From these results one can assume that no acetal formation has taken place during the present oxidation experiments of fucoidan (C)

and the results obtained therefore support the proposed theory for the biosynthetic connection among polysaccharides A,B and C.

Summary

This work supports the earlier findings in Fucus vesiculosus and Eisenia bicyclis, insofar that mannitol is a major product of photosynthesis of brown algae, that it is a storage product, and is partly converted into laminaran.

There also seems to be evidence for a relationship in the biosynthesis of the fucose-containing polysaccharides, and the theory that xylogalacto-fucoglucuronan (A) is synthesised first, and eventually transformed into fucoidan (C) via xyloglucuronogalactofucan (B) is put forward. It is not proposed that these three polysaccharides exist as distinct individuals, but that they are all members of a family of fucose-containing polysaccharides synthesised by the alga, with xylogalactofucoglucuronan (A) as one end of the family spectrum and fucoidan (C) as the other end.

This theory is supported by periodate oxidation studies of the three fractions.

It is suggested that the low molecular weight carbohydrates found in the supernatant of the acid extract are derived from precursors for the biosynthesis of these polysaccharides.

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3. ULVA LACTUCA

Introduction

The metabolism of carbohydrates in green marine algae has received little attention, only a few papers concerned with this problem can be found in the literature.

Bidwell (1958) allowed the green seaweeds Cladophora and Ulva lactuca to photosynthesise in the presence of $^{14}CO_2$ for 5.5 hours and 17 hours respectively. After extraction of the algae with 80% ethanol, 73% and 59% of the radioactivity in this fraction from Cladophora and Ulva respectively was found in sucrose. The carbohydrate fraction of the insoluble part accounted for 24% of the radioactivity from Cladophora and 14% in that from Ulva.

Craigie et al (1966) carried out studies on the green algae Chaetomorpha, Cladophora, Enteromorpha, Monostroma spp., and Ulva lactuca. They were allowed to photosynthesise in the presence of $^{14}CO_2$ for 2 hours. The algae were extracted with 80% ethanol, and these extracts and the insoluble residues were investigated separately. Sucrose acquired the main proportion of the radioactivity in the 80% ethanol extracts from all species except Cladophora, where most of the radioactivity was found in unidentified, neutral compounds. Fructose and glucose also became labelled, fructose higher than glucose in all samples except in U.lactuca. Cladophora also sythesised labelled mannitol.

The main part of the radioactivity in the "insoluble fraction" was present in the carbohydrate material. In all samples, after hydrolysis, glucose had the highest radioactivity. The residue from Chaetomorpha had traces of radioactivity in arabinose and xylose, Cladophora in arabinose, xylose and galactose. Enteromorpha, Monostroma and Ulva all gained considerable radioactivity in rhamnose, and the two first mentioned acquired smaller amounts in mannose and xylose. These authors concluded that the photosynthetic products of these green algae resemble those of Chlorella and higher plants in storing sucrose, glucose and fructose. The labelled glucose derived from the polysaccharides was thought to have arisen from starch.

The photosynthetic products from Ulva lactuca have recently been studied in more detail by Patil and Joshi (1970, 1971). These authors allowed the alga to photosynthesise for 6 hours in radioactive seawater ($^{14}CO_2$). Samples were taken at 10 sec, 30 sec, 5 min, 30 min, 1 h. and 6 h. The extent of labelling of most compounds present in the 80% ethanol extract were measured. The radioactivity of the various components of the residue was measured after total hydrolysis of this. No radioactivity was found in any carbohydrates until after 5 min.

Sucrose was then most highly labelled, followed by glucose and fructose. As the experiment proceeded, it was observed that after 1 hour, glucose had the highest labelling and after 6 hours, fructose was the highest labelled sugar present in the 80% ethanol extract.

After hydrolysis of the residues of the last four samples, it was found that 34%, 32%, 38% and 50% of the radioactivity respectively was in the carbohydrate fraction. The radioactivity of the individual sugars in each sample was measured. After 5 min, most of the activity was present in glucose, followed by xylose and rhamnose. Little was found in glucuronic acid. As the experiment continued for 30 min, activity increased in glucose, while that of rhamnose and xylose decreased. After 1 hour there was still increase in the activity of glucose, and also in that of xylose and glucuronic acid. After 6 h, 80% of the radioactivity in this carbohydrate fraction was found in glucose, followed by xylose, glucuronic acid and rhamnose. The radioactivity of glucuronic acid increased throughout the experiment.

When studying their results, it is observed that the actual radioactivity in the 80% ethanol extract of U.lactuca does not increase throughout the experiment. An increase would be expected during an experiment of this kind

as the weed has access to radioactivity in the medium during the whole period. Patil and Joshi (1970) report a lower radioactivity in this extract of the samples after 30 min. and 1 hr., than after 5 min.

For the "insoluble residue" (i.e. insoluble in 80% ethanol) it is observed that the extent of labelling of rhamnose and xylose from the hydrolysate is smaller in the samples from 30 min. and 1 hr., than in that after 5 min. No attempt to fractionate the polysaccharides has been made. It should be pointed out that glucose, for instance, probably is present in three different types of polysaccharides, and without any separation of these, conclusions about their role in the carbohydrate metabolism in U.lactuca can not be drawn from radioactive experiments.

Due to these discrepancies, interpretation of their results is very difficult. Their conclusions, however, from these experiments are that the synthesis of sucrose dominates up to 30 min. from the start of the experiment, and this is followed by domination of glucose-synthesis after 1 hour. The glucose is thought to be the main constituent of the "insoluble fraction", and also that it serves as a metabolic pool for synthesis of the complex polysaccharides as well as for other fractions.

The present experiments were carried out at the same time as those of Patil and Joshi, and as will be shown, the respective results obtained are not in complete agreement.

Material

The alga, Ulva lactuca, was collected at Point Pleasant, Halifax, Nova Scotia, Canada, in August 1969. It was cleaned for adhering epiphytes etc., and stored overnight at 15°C in filtered seawater.

Experimental and results.

A. Ulva lactuca (50 g. blotted weight) was placed in 1500 ml filtered, sterile seawater at 18°C and illuminated for 15 minutes. 1.0 mCi $NaH^{14}CO_3$ was added and the sample illuminated for a further 10 minutes. It was then taken out of the medium, rinsed thoroughly in fresh seawater and pulverised with liquid nitrogen by grinding in a mortar.

B. The weed (50 g. as for A) was treated similarly, except that the amount of seawater was 6.0 l containing 2.0 mCi $NaH^{14}CO_3$. The illumination period was for 3 hours.

Extraction of the Alga

The finely ground powder was extracted with 80% ethanol, by refluxing for 6 hours, repeated 3 times), followed by exhaustive extractions with hot water on a boiling waterbath in an atmosphere of carbondioxide. The residue after the aqueous extractions was hydrolysed completely with 72% sulphuric acid (Blake and Richards, 1970). The radioactivities of these fractions were measured (Table 1).

TABLE I

Radioactivity of various fractions of Ulva lactuca (DPM x 10^{-5})

	80% Ethanol extract	Hot water extract	Residue
Sample A	783	242	602
Sample B	3135	1367	4307

80% Ethanol soluble fraction

The combined extracts were deionised with Biodeminrol-it-resin in carbonate form, to remove amino-acids, sugar phosphates and other charged compounds and then examined by paper chromatography in solvents A and D. The components present were identified by paper chromatography and g.l.c. and the radioactivity in the various sugars and alcohols was measured (Table II).p.171.

Hot water extract

The total extracts were dialysed. During dialysis a precipitate formed inside the dialysis sac, which was removed by centrifugation, before freeze-drying the polysaccharide. Previous studies had shown that this fraction was a mixture of starch and sulphated glucuronoxylorhamnans (Brading et al, 1954, Percival and Wold, 1963). The starch was removed by treatment

TABLE II

Radioactivity in the components of the 80% ethanol soluble, neutral fraction (DPM x 10^{-5})

	Difference between total and neutral extracts	Total de-ionised extract	Oligo-saccharide	Myo-Inositol	Sucrose	Glucose	Fructose	Xylose	Ribose	Unknown.
A	460	315.9	36.65	2.53	143.7	72.02	42.96	4.74	7.27	5.32
B	950	2169.0	997.9	49.89	1054.0	366.6	314.6	52.06	93.28	13.2

of the polysaccharide with α-amylase inside a dialysing sac in distilled water. The dialysing water was changed frequently, and the reaction was stopped after 24 hours by heating the mixture to boiling point. After cooling the precipitated enzyme was removed by centrifugation. The solution containing sulphated polysaccharide, after concentration, was freeze-dried. The carbohydrate content was measured of the polysaccharide mixture and of the precipitate formed in the dialysis sac. The radioactivity of the various fractions was measured (Table III).

TABLE III

Radioactivity present in various parts of the hot water fraction (DPM x 10^{-5})

	Total extract	Dialysate	Precipitate in sac y	Polysaccaride fraction x	Starch	Glucuronoxylorhamnans
A	242	127	32.6	77.9	35.4	24.9
B	1367	257.5	230.7	894.8	174.5	559.3

x weight 805 mg, carbohydrate content 50% for both A and B.

y carbohydrate content, A = 3.44 mg, B = 16.7 mg.

Investigation of the sulphated polysaccharide

As the glucuronic acid is highly degraded by normal acid hydrolysis (Haq and Percival, 1966) the polysaccharide was ~~just~~ given a mild hydrolysis with 2.25% oxalic acid at 100°C for 4 hours. After neutralisation, the derived neutral and acidic oligosaccharides were separated on Amberlite 1R-45 ($HCOO^-$) resin and eluted successively with water and 1 M formic acid. The acidic fraction was converted into its methylester-methyl glycosides followed by reduction of the acid to the neutral sugar with sodium borohydride. After another hydrolysis of the neutral and the reduced acidic fraction with 90% formic acid, an aliquot of the derived syrup was subjected to paper chromatography in solvent A and the radioactivity present in each sugar was measured. Another aliquot was reduced and the ratio of the alditols present was determined by g.l.c. The results obtained from the two fractions were combined to find the molecular ratio of the sugars present in the polysaccharide and the radioactivity present in each sugar (Table IV).* The relative specific radioactivity per sugar unit was calculated (Table V).

Residual material

The residue was hydrolysed with 72% sulphuric acid (Blake and Richards, 1970), deionised with Biodeminrolit resin in carbonate form, and an aliquot of the derived neutral syrup was subjected to paper chromatography in solvents A and D.

*No glucose was detected in a complete acid hydrolysis of the acidic fraction.

TABLE IV

Sugar -		Glucose	Xylose	Rhamnose	Glucuronic acid
Rel.prop.		1	4.55	5.7	2.28
DPM	A	49.38	38.65	123.7	28.93
$X10^{-4}$	B	313.2	1376	2417	1102

TABLE V

Relative specific radioactivity

	Glucose	Xylose	Rhamnose	Glucuronic acid
A	49.38	8.5	21.7	12.7
B	313.2	302	422	483

TABLE VI

Radioactivity in the residual sugars (DPM x 10^{-5})

	Total radio-activity	Neutral fraction	Glucose	Mannose	Xylose	Rhamnose
A	602.4	378	251	87.7	22.0	17.4
B	4307	2950	2235	389.4	135.7	191.7

Another aliquot was analysed by g.l.c. after conversion of the sugars to their corresponding trimethylsilyl-derivative. The presence of glucose, mannose, xylose and rhamnose was confirmed, glucose being the main sugar of this material. The radioactivity of the respective sugars was measured as described previously (Table VI).

Discussion

When Ulva lactuca was allowed to photosynthesise in the presence of $NaH^{14}CO_3$ for 10 min. (A) and 3 hours (B) respectively, it was found that the low molecular weight fraction incorporated a higher percentage of the radioactivity after 10 min. (A) than after 3 hr. (B), whereas the highest amount of radioactivity was found in the residue after 3 hr.(B), which agrees with the fact that the cellwall is built up later than the soluble products.

In the neutral fraction of the 80% ethanol soluble material, sucrose was the highest labelled carbohydrate in both experiments. The radioactivity acquired by glucose and fructose was also high, while that of the other compounds were relatively low. The high content of radioactivity in sucrose after 10 min. agrees with the finding that sucrose is the first free sugar to be formed by photosynthesis (Bassham and Calvin, 1957). The increase in the radioactivity in sucrose and fructose between 10 min and 3 h. was proportionally almost identical, while that of glucose was not so pronounced. Patil and Joshi (1970) found sucrose to be the highest labelled sugar after short periods of photosynthesis of U.lactuca. After 1 hour, glucose had the highest labelling and after 6 h. fructose was the highest labelled sugar present in their 80% ethanol extract. Our results agree with these observations for the short period of photosynthesis, but in our case after 3 hours, sucrose was still the highest labelled carbohydrate and the increase

in radioactivity was proportionately somewhat greater for fructose than for glucose during this period, but the total radioactivity of glucose or fructose does not exceed that of sucrose.

The radioactivity present in the charged components of the 80% ethanol soluble compounds was greater in (A) than in (B) (Table II)/ p.171 indicating that charged compounds are formed before neutral sugars; the same was observed by Patil and Joshi (1970).

The hot water extracts contained 14.9% (A) and 15.5% (B) respectively, of the total radioactivity present in the alga. Loss of radioactivity during dialysis of these extracts was greater for (A) than for (B), indicating that smaller molecules (precursors?) are formed first and then are probably converted into higher molecular weight compounds. One would therefore expect the dialysable material to have relatively less radioactivity after a longer time of growth in radioactive medium and hence a greater fraction of radioactive non-dialysable polysaccharide.

The non-dialysable fraction consists mainly of two polysaccharides, starch and a sulphated glucuronoxylorhamnan (Brading et al, 1959; Percival and Wold, 1963). Starch is a storage product, but possibly also an active metabolite. It has the higher radioactivity of the two in (A), but the lower in (B) indicating that it is a more direct product of photosynthesis than the sulphated polysaccharides, the latter are probably synthesised via different precursors and are eventually laid down in the alga either as long term storage material or as part of the cellwall.

The radioactivity present in various sugars of the sulphated polysaccharide, show that the glucose acquires radio-activity quickest, followed by rhamnose, glucuronic acid and then xylose (see Table IV). After 3 h. growth (B), one can see that the relative specific radioactivity per sugar unit (Table V) is highest for the glucuronic acid and lowest for glucose, but the difference is relatively small compared with the differences in exp.A. The sugars are almost equally labelled after 3 h. Glucose has the smallest increase in radioactivity which indicates that this part of the polysaccharide probably has a high "turn-over-rate" relative to the parts containing the other sugars. It could be that glucose is transformed into some of the other sugars at the polysaccharide level or that comparatively little glucose is incorporated after the initial stage. It appears that rhamnose is laid down in the polysaccharide before the other two sugars.

Glucose contains the highest amount of the radioactivity laid down in the insoluble residue and this is followed after 10 min., in decreasing order of activity by mannose, xylose and rhamnose (Table VI). An even higher proportion of radioactivity in glucose is found after 3 hours. The radioactivity in rhamnose seems to have increased proportionately to the same extent as glucose, while mannose and xylose did not show such large increases. The neutral fraction (carbohydrates) of the residue contains a higher proportion of the radioactivity of the total fraction in (B) than in (A), which indicates that the carbohydrates are a less direct product of photosynthesis

than the other constituents of this insoluble residue.

Patil and Joshi (1971) investigated the total 80% ethanol insoluble fraction as such and they found a decrease in the radioactivity of the xylose and rhamnose after a certain period of their experiment. This cannot be so, as the alga has access to $^{14}CO_2$ all the time. The present results agree with their observations concerning the increase of radioactivity in glucuronic acid, but any further comparison is difficult as the alga has been examined differently in their and the present studies. The discrepancy in their results also make comparisons difficult.

It should be pointed out that one ought to separate the polysaccharides before trying to analyse them, in order to get as true a picture as possible of the extent of labelling in the various sugars. Glucose, for instance, is probably present in three different polysaccharides of Ulva lactuca; starch, sulphated glucuronoxylorhamnan and in the cellwall. The metabolism of these three polysaccharides would be expected to be different, and one would therefore expect a different extent of labelling of glucose in these three polysaccharides. In the present experiment, it is clearly observed a different extent of labelling of the glucose in these polysaccharides, and, also a different order of increase in radioactivity ~~in the~~ between exp.A and exp.B. (Tables II,IV and VI)p.171,174.

The conclusions drawn from these experiments are tentative, and further studies, preferentially pulse labelling experiments will

be required to obtain more information about the role of these carbohydrates during photosynthesis in Ulva lactuca.

APPENDIX

Culture of Acetabularia

The method for culturing the alga is basically the same as described by Shephard (1970).

The cysts of Acetabularia mediterranea (kindly given to us by Professor Bidwell, Queen's University, Kingston, Ontario) were put in synthetic seawater, in light (ca.250 ft c, cycled 12 hourlight/12 hours dark and temperature of 23°C). After a few days, phototactic gametes were released from the spores. They were cleaned by allowing them to swim through a 25 cm long sterile U-tube towards light, and transferred from this tube to the culture-vial. They were allowed to grow and after a few weeks, it was observed that growth had commenced. When the culture-vials became too crowded, the cells were transferred to a number of vials, and left to grow until they had developed caps of normal size. It normally took 4-5 months before the cells were fully grown. The medium was changed periodically.

Culture Medium

A Synthetic Medium for Acetabularia

	Amount/liter	Instructions
Major salts		
NaCl	24 gm	The phosphate is added last; then
$MgSO_4 \cdot 7H_2O$	12 gm	the pH is adjusted to 7.8 with 1N
$CaCl_2 \cdot 2H_2O$	1 gm	HCl (about 5 ml/liter);
Tris	1 gm	finally the solution is autoclaved
KCl	0.75 gm	
$NaNO_3$	40 mg	
K_2HPO_4	1 gm	
Micronutrient salts		
Na_2EDTA	12 mg	This solution is made up separately
$ZnSO_4 \cdot 7\ H_2O$	2 mg	and added to the major salt
$Na_2MoO_4 \cdot 2\ H_2O$	1 mg	solution before autoclaving
$FeCl_3 \cdot 6\ H_2O$	0.5 mg	
$MnCl_2 \cdot 4\ H_2O$	0.2 mg	
$CoCl_2 \cdot 6\ H_2O$	2 µg	
$CuSO_4 \cdot 5\ H_2O$	2 µg	
Bicarbonate		
$NaHCO_3$	100 mg	These solutions are made up
Vitamins		separately and added to the salt
Thiamine HCl	300 µg	solution through a sterilizing
p-Aminobenzoate	20 µg	membrane filter after the auto-
Ca-Pantothenate	10 µg	claving
Vitamin B_{12}	4 µg	

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