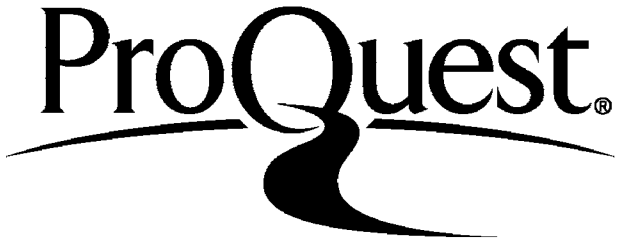
CARBOHYDRATES OF THE BROWN SEAWEED

Lessonia nigrescens

A thesis submitted by Mario Francisco Venegas Jara
in candidature for the degree of Doctor of Philosophy
of the University of London.

Royal Holloway College
Egham Hill
Egham
Surrey U.K.

February 1982.



This thesis is dedicated to my wife, my children,

and my parents.

ACKNOWLEDGEMENTS

The author would like to express his gratitude to his immediate Supervisor, Dr Elizabeth Percival, for the opportunity of working in her group, for her permanent and expert advice and encouragement, for her constructive criticisms and excellent guidance given day by day throughout the progress of this research. Her example has really had influence on the author and will always remain in his future life.

The author is also grateful to his official Supervisor, Dr Helmut Weigel, for his criticisms and suggestions.

Dr Paul Finch is thanked for his help and discussions on ^{1}H n.m.r. and ^{13}C n.m.r. studies.

Thanks are given to Professor Trevor G. Bonner, former Head of Chemistry Department, for providing all the facilities available in The Bourne Laboratory and his tremendous encouragement to the author when recently arrived in this country to start his postgraduate studies.

The author would like to thank the technical staff of Queen Elizabeth College, University of London for their co-operation in obtaining some mass spectra.

Dr G.E.Hawkes and the technical staffs of the ULIRS NMR services at Queen Mary College and King's College, London, University of London, are thanked for obtaining the ^{1}H n.m.r. and ^{13}C n.m.r. spectra.

The author is also indebted to everyone of the technical staff of The Bourne Laboratory who gave him assistance throughout these years. Special mention to Mr Robert Williams for the photographic work.

Special thanks are given to Dr Hans J Black, of Universidad del Norte, Coquimbo, Chile, for supplying samples

of Lessonia nigrescens and L. flavicans.

Alginate Industries Limited, U.K., is thanked for providing the seaweed for the present investigation.

The author wishes to thank Mrs Sylvia Partin for production of a neat typescript of this thesis.

Finally, grateful acknowledgements are given to Royal Holloway College for some financial support and in the same sense, special thanks are given to World University Service (W.U.S.) U.K., for a grant provided to the author during three years of this research.

ABSTRACT

The thesis describes the first extensive study of the carbohydrates of the brown seaweed Lessonia nigrescens, harvested in Chile.

It was hoped to discover a different metabolic pattern for the stems and for the fronds of this seaweed. In the initial studies sequential extraction with water, acid and alkali and a direct alkaline extraction were applied separately to stems and fronds. Each of the extracts was hydrolysed and the hydrolysates were analysed by paper and by gas liquid chromatography allied to mass spectrometry.

Crystalline mannitol was separated from both parts of the seaweed.

"Fucans" were present in all the extracts together with, in the alkaline extracts, alginic acid. Fractionation of the aqueous extract failed to reveal any laminaran, a typical (1 $\longrightarrow$ 3)-β-linked glucan of brown seaweeds.

Each of the "fucan" contained varying proportions of fucose (major), galactose, mannose, xylose, glucuronic acid and half ester sulphate. Varying proportions of protein were present in all the extracts. Molecular weights determinations revealed polydispersity with high proportion of the molecules equal to or larger than $\overline{M}_w$ of 6.7×10^5.

The only significant difference between stems and fronds was the higher proportion of glucuronic acid in the former.

Investigation of the alginic acids revealed that they consisted of mannuronic to guluronic acid in the ratio of 0.54. Viscosity and ^{13}C n.m.r. measurements were carried out.

Large scale partial acid hydrolysis of water soluble "fucan" from a mixture of stems and fronds led to the separation

of neutral and acidic oligosaccharides, an insoluble and a soluble polymer.

The neutral oligosaccharides which contained only galactose were separated into six entities and characterised by methylation and the anomeric linkage determined for two of them by enzymic and ^{1}H n.m.r. studies.

The eight acidic oligosaccharides proved to be very complex mixtures. However, two were characterised as 3-O-(D-glucopyranosyluronic acid)-L-fucose and 3-O-(D-glucopyranosyluronic acid)-D-glucuronic acid.

The insoluble residue, 10% of the starting material was mainly inorganic and protein.

The soluble polymeric material, 20% of the initial material (contained 80% carbohydrate) was shown by hydrolysis, molecular weight determination and ^{13}C n.m.r. to consist of approximately 44 alternating units of glucuronic acid and mannose.

CONTENTS

CARBOHYDRATES OF THE BROWN SEAWEED Lessonia nigrescens.

CHAPTER II General methods Page

LIST OF TABLES

List of tables (continued) Page

LIST OF FIGURES

List of figures (continued) Page

CHAPTER I

GENERAL INTRODUCTION

1.1. The term "algae" embraces an assemblage of organisms which exhibit great diversity of structure, reproduction and metabolism. Algae include all oxygen-evolving photosynthetic organisms except Bryophytes (mosses and liverworts) and vascular plants. Many colourless organisms are also referable to the algae on the basis of their similarity to photosynthetic forms with respect to structure, life history, and the nature of cell wall and storage products.[1]

As far as we know, life began in the oceans and the algae are certainly among the world's oldest known living organisms, fossil records dating back about 3×10^9 years[2]. The evolution of algae has been very slow compared to that of terrestial plants. Fossil evidence indicates that many of them have changed very little in hundreds of millions of years.[3-5] As new fossil evidence comes continually to light, ideas about the phylogenesis of algae are constantly being revised and there are inevitably disagreements between different authorities. There is unanimous agreement that the Blue-green algae (Cyanophyta) are by far the oldest and reasonable evidence that the Red algae(Rhodophyta appeared next, then the Green algae(Chlorophyta) and the Brown algae (Phaeophyta) last.[15]

Morphologically they are very primitive and the Blue-greens even lack a nucleus and other cell organelles.[6] Algae have a great diversity of form[7] ranging from unicellular organisms to the giant brown seaweed *Macrocystis pyrifera* which may attain up to sixty metres in length and *Lessonia flavicans* and *Lessonia nigrescens* which grow like trees to the size of four metres tall[8].

Although algae may occur in different habitats, in fresh

water, in the soil, in most places such as rocks, cliffs, walls and trees, they occur mainly in salt water. Being essentially photosynthetic organisms they grow only in parts of the sea where light is available. The free-floating types, most of them unicellular can be found in the upper layers of all the seas but most of the larger species are attached to rocks and are, therefore found only in the continental shelf areas, more particularly in those parts where the sea bottom is sufficiently stable to provide a firm anchorage.[7] Since intertidal algae are periodically exposed at low tide, they must be able to withstand desiccation and must possess a wide osmotic tolerance of dilution of the surrounding medium by rain and of increased salt concentrations resulting from evaporation. They must tolerate diurnal and seasonal changes of air temperature more extreme than those of the sublittoral habitat and considerably higher light intensities. In addition they must withstand the mechanical stresses by the force of the waves and in northern latitudes the denuding effects of ice in winter. Littoral zonation is affected by all of these factors, whereas as a rule in the sublittoral zone only light is of major importance as an ecological factor, apart from limitations imposed by various substrates.

In general it is found that sublittoral algae are less tolerant of extreme conditions than are the seaweeds of the shore and that tolerance limits of the various species are wider as one ascends through the littoral zones towards dry land.[9]

During low tide, algae of the tidal zone are exposed to full sunlight, while those of the sublittoral zone receive a light intensity that decreases with depth and turbidity of the water. With increasing depth the light alters not only in intensity but also in quality. The water acts as a light filter,

red light being completely absorbed in the upper layers, so that in the depth a blue-green twilight prevails. On physical grounds, therefore, red algae would be expected to be the most suited to deeper water since the prevalent light consists essentially of the wavelengths most strongly absorbed by the algae themselves, whereas Green algae, which utilize mainly the longer wavelengths of light for photosynthesis, must be best suited to the upper sublittoral and the littoral zones. A comparison of the light absorption and photosynthetic action spectra of seaweed thalli shows clearly that green algae effectively utilize light absorbed throughout virtually the entire red absorption band of chlorophyll whereas red algae exhibit their highest photosynthetic rates in the spectral regions absorbed by the phycobilins, phycoerythrin and phycocyanins.[10]

There is a tendency of the marine littoral flora to exhibit a descending sequence of predominantly green, brown and red algae indicating a correlation with the quality of the ambient light. Thus in deeper water where blue and violet light prevails the red pigments of the Rhodophyta appear adapted for its efficient absorption. Exceptions are not rare, however, many red algae grow in the tidal range while many green algae can be found surprisingly deep in the sub-littoral zone. On the other hand, it has been considered that the intensity of light is the chief controlling factor in the zonation of the algae at various depths. Many workers during the decades of the 30's and 40's confirmed that both wavelength and intensity of light are important factors on the life of the algae.[9]

Taxonomy is based on the degree of similarity or dissimilarity and relationships are proposed and tested in accordance with the principle of correlation of multiple factors or characters. While morphological characters

are usually thought to be of primary, and classical, importance in taxonomy, it should be noted that two biochemical characters, pigmentation and food reserves are fundamental in segregating this heterogeneous array of algae into major groups or divisions. The classification into divisions has been aided by the nature of the pigments present. All marine algae contain chlorophyll, but the colour of the chlorophyll in some divisions is masked by strongly coloured pigments.

Nearly all species of the Rhodophyta are coloured bright red by biliproteins, phycobilins, the Phaeophyta derive their brown colour from the xanthophyll, fucoxanthin the Cyanophyta's pigments are the phycocyanin and phycoerythrin, the Chrysophyta have a predominance of carotenes and xanthophylls which give them their characteristic golden colour; the Pyrrophyta also owe their colour, yellow brown, to a predominance of carotenes and xanthophylls; the Euglenophyta have some green pigments as chlorophylls unmasked by other colours.

1.2. The following four divisions:

Rhodophyta (Red algae); Chlorophyta (Green algae), Phaeophyta (Brown algae) and Cyanophyta (Blue-green algae) are well established and algae from these divisions are the most easily obtainable and most directly important economically and include the greater number of species which has been chemically studied.

1.3. Altogether algae represent about 25, 000 known species but the polysaccharides of only about one hundred of them have been studied structurally.

In the view of the very primitive morphology of algae it would be expected that the structure of the polysaccharides they elaborate could also be simple, however the reverse seems to be true. Many algal polysaccharides surpass even

the gum exudates of terrestial plants in apparent complexity, although recent investigation specially on alginates and carrageenans are making more clear that what is understood as "complexity" could be better described as "irregularity".

Many of the polysaccharides of the algae, although different in details, have their counterparts in land plants. This is the case of cellulose which is found in small proportion in many species of the major divisions mentioned above. Algae have also as constituents the various neutral sugars and sugar acids found in land plants, but a characteristic feature of seaweed polysaccharides is the presence of half ester sulphate groups attached to hydroxyl groups of the sugars. Terrestial plants are devoid of these groups, but they are common to animal polysaccharides.

Glucose, galactose and mannose, hexose sugars, are present in many of the seaweed polysaccharides and although they have an identical chemical formula their constituents atoms have different arrangements. This together with the way they are linked permits the elaboration of a large number of polysaccharides with different shapes and therefore with different properties. In addition the pentoses, xylose and arabinose and the deoxy hexoses rhamnose and fucose allow a further range of structures. Uronic acids glucuronic, mannuronic and guluronic and the half ester groups gives the possibility of various combinations with metal ions, as well as different conformations.[11]

Many different polysaccharides are found as constituents of the cell wall and some in the intercellular regions of algae. Some polysaccharides are almost certainly food reserve. Cell walls are the main components of plant tissues and they are responsible for their mechanical properties and must be suitable for withstanding the forces to which they are subjected. Even though macroscopic algae do not require

rigid structures which can withstand gravitational forces, they must be strong enough to resist damage caused by swiftly moving and turbulent water. The flexibility and tensile strength is given to the plant by the constituents of the cell walls and intercellular regions of the algae. Many of the polysaccharides of seaweed have functions which at present are not at all well understood. It is thought that the function of the half ester sulphates is probably to produce polysaccharides which are hygroscopic and mucilaginous in nature and therefore giving seaweeds the flexibility and ease of movement their environment requires. Another function is probably to prevent excessive dehydration when the plant is exposed to air at low tide. It appears that modifications in the structure of the macromolecule are brought about by the plant to enable the polysaccharide to fulfil a different function. Sulphate groups are thought also to perform functions associated with immobilised poly acids.

There is growing evidence that many algal polysaccharides exist in the native state, at least partly as proteoglycans; this means that various constituents form a complex structure in which different polysaccharides may be held together by covalent links to protein[12]. Procedures for isolation usually entail some degradation of these complex macromolecules. In other words, extraction methods used to obtain "pure" compounds must therefore break down much of their macro-structure, but preparations of sulphated polysaccharides of the Chlorophyta and "fucans" from the Phaeophyta commonly contain some protein. The nature of the carbohydrate protein linkages, apart from those of Porphyridium cruentum awaits further study.

Although much of the work on the primary stages of carbon fixation in photosynthesis was carried out on the

green alga, Chlorella, very little is known about the steps involved in the biosynthesis of algal polysaccharides. A few nucleosides have been extracted from algae and as with higher plants, it is probable that they are the precursors of polysaccharides[13], but extraction of the enzymes which bring about the synthesis has proved extremely difficult.

The majority of the chemical studies on the polysaccharides of algae have been carried out on extracts prepared from whole plants, and in many cases a sequential method of extraction has been used with a series of reagents or physical conditions bringing out the more easily extracted materials in the early stages[7]. Thus the whole seaweed including cell contents, cell wall and intercellular regions are extracted in a series of solutions, and an insoluble residue remains, in which carbohydrates are still present.

Each stage of sequential extraction of an alga contains a mixture of polysaccharides. They can be separated to some extent by various fractionation techniques[7], but after even a series of such separation it cannot be expected that the product will consist of molecules of identical size, and composition and analysis will give results which are only averages with little information on the degree of polydispersivity and homogeneity.

The instinct of seaweed workers has been to extract the polysaccharides under the mildest conditions, but it must be recognised that this effort has not always been productive and, when it has been successful the product might have been so complex that it had been intentionally degraded into simpler fragments before it has been studied.

With several species variations have been found in the fine structure of polysaccharides between different batches of the same algal material. Well established causes of

such variation are differences in the average age of the algal population, the time of year when it was harvested and conditions of drying and storage.

A brief description of carbohydrates of the Cyanophyta (blue-green algae), Chlorophyta (green algae) and Rhodophyta (red algae) will be given in this chapter. Carbohydrates of the Phaeophyta will be described in Chapter 3.

Little in the way of chemical investigation, apart from a determination of the constituents[14], has been carried out on the cell wall polysaccharides of members of the Pyrrophyta, Euglenophyta, Chrysophyta, Xanthophyta and Charophyta.

1.4. The carbohydrates present in algae can be roughly divided into the following three groups.

1. Low molecular weight carbohydrates (soluble in 80% ethanol).
2. Water soluble polysaccharides
   - 2.1. Food reserve material
   - 2.2. Other soluble polysaccharides
3. Cell wall material or structural polysaccharides

1.5. Carbohydrates of the Chlorophyta (green seaweeds).

1.5.1. Low molecular weight carbohydrates

Sucrose seems to be the main low molecular weight carbohydrate present in green seaweeds[16]. In addition, glucose and fructose have been found in all the species investigated. Small quantities of other sugars, some alditols and myoinositol have been found in a few species.[17, 18]

1.5.2. Water soluble carbohydrates

1.5.2.1. Food reserve material

Most of the species so far investigated were found to metabolize a small proportion of starch-type glucans which closely resembles those of terrestial plant starches comprising

both amylose and amylopectin[19]. Among the Prasinophyceae, Tetraselmis carteriiformis[20] yielded only a typical amylopectin, but a Platymonas species yielded a starch with 23% of amylose[21]. A representative of the Charophyceae, Nitella translucens contains a starch with about 12% of amylose[22]. One species, Urospora penicilliformis, is reported to contain pure amylose[23].

Green algal starches, like some others, can be very hard to separate from protein, but there is no evidence for a covalent linkage.[24]

From a sample of fresh Acetabularia mediterranea (Order Dasycladales) crystalline fructan was isolated, which gave an X-ray diffraction diagram identical with that of inulin[25]. Hot water extraction of the caps of Acetabularia crenulata subsequently yielded 10.3% (dry basis) of a fructan containing residues of D fructose and D glucose in a molar ratio 33:1[26]. A closely related species Batophora oerstedi yielded 6.5 - 12.5% of inulin.[27]

Inulin has been also reported in six species of the Order Cladophorales, but other members of the Order contained only (2 → 1)-linked, fructose-containing oligosaccharides, built on to sucrose as in inulin.[17]

Small amounts of a (1 → 3)-linked glucan, tentatively assumed to be laminaran, have been reported in the marine species Caulerpa filiformis[28] and Cladophora rupestris[29], but it is not absolutely clear whether it functions as a food reserve. Codiolum pusillum contains β-(1 → 3)-linked, D-glucose-containing oligosaccharides[30] and Caulerpa simpliciuscula contains a low molecular weight β-D-glucan that resembles laminaran in containing a small proportion of (1 → 6) in addition to (1 → 3) linkages.[31]

1.5.2.2. Other soluble polysaccharides

According to Preston[32] these polysaccharides are

mainly constituents of the continuous amorphus phase of the cell walls and comprise 30-70% of the dry weight in different algae[33].

The major polysaccharides in all the species examined are polydisperse heteropolysaccharides in which at least some of the hydroxyl groups of the sugar residues are substituted by half ester sulphate groups.

The different genera of green seaweeds fall into several main groups according to the type of polysaccharide produced.

The first group contains mainly galactose, arabinose and xylose units and has been obtained from species of Caulerpa[35], Cladophora[29], Chaetomorpha[34], Codium[36], and Rhizoclonium[17].

The second group contains mainly rhamnose, xylose and glucuronic acid and has been obtained from Enteromorpha compressa[37], Ulva lactuca[38], Acrosiphonia arcta[39], Urospora wormskioldii[30], Urospora pencilliformis[23] and Codiolum pussilum[30].

The polysaccharide from Acetabularia crenulata[26] is of an intermediate structure as it comprises galactose, xylose, rhamnose and glucuronic acid as the main components.

The polysaccharides of the first group, sulphated xyloarabinogalactans have a positive specific rotation, whereas in the second group, sulphated glucuronoxylorhamrans and the intermediate one, sulphated glucuronoxylorhamnogalactan have a negative specific rotation.

Structural studies in all of these and similar polysaccharides have established that the proportion of the sugars vary from genus to genus and even among species of the same genus. The modes of linkage and the points of attachment of the sulphate groups have been established for the majority of these polysaccharides[40]. They are all extremely complex, highly branched molecules which do not

appear to have long chains of a single sugar since evidence of the mutual linkage of the different sugars have been obtained for the different groups. In the first group of polysaccharides, galactose and arabinose are the main sugars in most species, but appreciable amounts of xylose and traces of rhamnose have been found. All attempts to fractionate the polysaccharides into homopolysaccharides have been unsuccessful.

The water soluble polysaccharides obtained from the species mentioned in the second group were found to be heteropolymers with L-rhamnose and D-xylose as the major sugars and smaller quantities of D-glucose. The uronic acid content was found to be around 20% while the sulphate content varied, about 17% in Ulva[38] and Enteromorpha[37] and 8% in Acrosiphonia[39].

Partial acid hydrolysis of all three polysaccharides gave a relatively high proportion of an aldobiouronic acid, 3-O-β-D-glucopyranunosyl-L-rhamnose.

The polysaccharide from Ulva lactuca has been most extensively studied. Methylation studies of the desulphated and carboxyl reduced polymer have indicated the linkages for rhamnose and xylose and tentative evidence for the glucose linkages was also obtained.

It has been deduced from alkaline desulphation and periodate oxidation experiments that the position of sulphate ester is mainly at C-2 of rhamnose and to a lesser extent at C-2 of xylose[41].

An acidic oligosaccharide was isolated in high yield after Smith degradation and from this it was deduced a structure which represents a repeating unit in the polysaccharide.[42]

The unicellular Acetabularia crenulata[26] metabolises a polysaccharide which comprises fructose as the main sugar and a second polysaccharide containing rhamnose, glucose, galactose

and lesser amounts of xylose and 4-O-methyl galactose. Due to the evidence for a relative high proportion of end groups the polysaccharide is thought to be highly branched with very short branches.

1.5.3. Structural polysaccharides

1.5.3.1. Cellulose:

The most highly organised algal cellulose is found in Valonia and species of Cladophora and Chaetomorpha.

A beautifully crystalline form of cellulose is present in the vesicles of Valonia species which are tropical marine members of the Order Siphonocladales. It exists in the cell wall as long, regular microfibrils arranged in a criss-cross manner. They give essentially only glucose on hydrolysis and very sharp X-ray diffraction patterns characteristic of Cellulose-I[43, 44]. Other marine species such as Cladophora rupestris and Chaetomorpha melagonium also give clear cellulose I patterns in the native state, others give them only after extraction of encrusting hemicelluloses and still others do not give them at all.[45, 46]

Due to difficulties in isolating the pure cellulose no structural studies have been carried out.

1.5.3.2. D-Xylans

In marine species that lack cellulose, most notably those of the Order Siphonales, its function is taken over by β-(1 → 3)-linked D-xylans.[28, 47] The fibres are more soluble in concentrated alkali than are those of cellulose or mannan, but they are initially isolated by extractions with dilute acids and alkalis.

The xylans of Caulerpa filiformis[28], C. brachypus, C. racemosa, C. anceps, Halimeda cuneata, Udotea orientalis, Chlorodesmis formosana and Pseudodicho tomosiphon constricta[48, 49] consist of chains of ca. 50 β-(1 → 3)-linked xylose

units. From X-ray studies it is deduced that the 1, 3-β-xylans consist of hexagonally packed double stranded helices present as microfibrils[50, 51]. Reconsideration of these results[52] supplemented by polarised infrared observations have shown that these polysaccharides exist in right handed helices with three xylans chains forming a triple helix thus giving a strong fibrous structure.

Xylose and glucose are the dominant monosaccharides in the polysaccharide of the gametophytic macrothallus of Bryopsis maxima; Bryopsis plumosa; Bryosidella neglecta and Derbesia tenuissima.[53]

1.5.3.3. D-Mannans

Some of the genera that do not contain cellulose have been shown to contain fibrous mannans as the principal skeletal polysaccharides

Codium fragile[36] and Acetabularia crenulata[26, 54] synthesise a(1 → 4)-β-Mannan. Electron micrographs of the walls of these two algae[55] show fibrils with a heavily encrusted appearance.

Codiolum pussilum, the erect stage in the life history of Urospora wormskioldii synthesises a(1 → 4)-β-Mannan, branched at C-6 on some of the units.[30] Bryopsis plumosa and Derbesia tenuissima (tropical, marine species) contain cellulose (and xylan in small proportion) in their gametophytes, but mannan in their sporophytes.[56]

An unsulphated α-(1 → 3)-linked mannan has been isolated from a hot water extract of Urospora penicilliformis[23].

1.6. Carbohydrates of the Rhodophyta (red seaweeds).

1.6.1 Low molecular weight carbohydrates.

A galactoside called floridoside when first isolated[57] was later shown to be a 2-O-α-D-galactopyranoside.[58] It is present in many species of red algae[59, 60] and it seems

to be a final product of photosynthesis and a reserve material in the red algae[61]. In some species, as in Polysiphonia[62], 2-O-α-D- mannopyranosyl-D-glyceric acid seems to be the main sugar derivative.

In addition, O-α-D-mannopyranosyl-(1 → 3)-O-α-D-galactopyranosyl-(1 → 2)-glycerol has been isolated from Furcellaria fastigiata[63] and 1-O-α-D-galactopyranosyl-glycerol, iso-floridoside from Porphyra umbilicalis[64] and other red seaweeds.[60]

In some species of red algae there have also been found various alditols and inositols.

1.6.2 Water soluble polysaccharides

1.6.2.1. Food reserve material

The Rhodophyta metabolize a starch, known as floridean starch, as their food reserve material.[65] It differs from the Chlorophyta starch in that it is devoid of amylose[66]. It was first isolated from Furcellaria fastigiata by Kylin in 1913. It is attacked by all the enzymes which hydrolyze starch. It gives a red violet colour with iodine, similar to that given by glycogen. Floridean starch differs from land plant amylopectins in having average chain lengths of about 9-19, with most values falling in the range of 10-14, compared with 25 for land plant starches[67,69]. It is essentially an α-(1 → 4)-linked glucan with (1 → 6)-branch points[66,68], though a small proportion of α-(1 → 3)-linkages has been reported in some species.

1.6.2.2. Other soluble polysaccharides.

The water soluble polysaccharides from the red seaweed are mainly galactans. They are essentially linear polymers of alternating (1 → 3)- and (1 → 4)-linked galactose units. This simple pattern is masked sometimes by other features, depending on the alga from which the polysaccharide has

been isolated. Differences are due to the presence of both D- and L-galactose, 3,6-anhydro-D and L-galactose, 6-O-methyl-D-galactose and occasionally galactose substituted at O-4 and O-6 with pyruvic acid and with various degrees of sulphation[70]. These units offer a broad spectrum of polysaccharides comprising chains of alternate (1 → 3)- and (1 → 4)-linked galactose or modified galactose units.[71] The individual polymers differ in their finer details of structure possibly due to a particular environment. These polysaccharides occur in nature as hydrated viscoelastic gels probably serving both structural functions and also as antidesiccants[72].

Galactans can be divided into three groups, agar, porphyran and carrageenan type, although polysaccharides related to more than one of these types have been found.[70, 7]

Agar

Agar is synthesised by a wide variety of species of the red seaweeds of which the most important are from the genera Gelidium and Gracilaria, species generally called agarophytes. It is the common name for a mixture of polysaccharides which originally were thought to comprise two polysaccharides only, neutral agarose and the sulphated agaropectin[73], but recent studies have indicated may exist as a complex series of related polysaccharides ranging from an almost neutral to a highly charged galactan. It has been possible to separate the extremes of the structure which can be defined as follows.

A) Neutral agarose:

A non-sulphated fraction separated from agar[74], which consists of chains of alternating (1 → 3)-linked β-D-galactose and (1 → 4)-linked 3,6-anhydro-α-L-galactose.

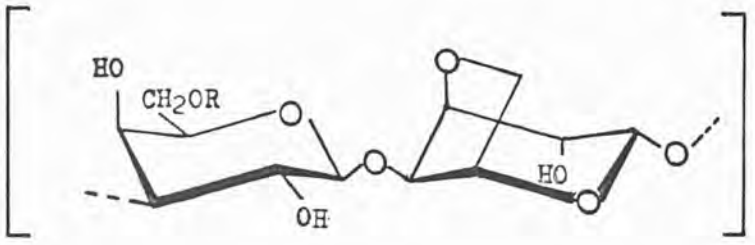


Fig. 1.1. Agarose. R = H or OMe

B) Pyruvylated agarose with a little sulphate:

The molecules with substitution of pyruvic acid consist of the same alternating pattern with about one in twenty D-galactose residues being pyruvylated as 4,6-O-(1-carboxy-ethylidene)-D-galactose units[75] and a few of the (1 → 4)-linked residues present as L-galactose-6-sulphate (3-10% sulphate).

C) A highly sulphated galactan:

These molecules contain little or no 3,6 anhydro galactose or pyruvic acid but consist of alternating (1 → 3) and (1 → 4)-linked galactose residues mono- and di-sulphated.

Some seaweeds which are not at present used commercially yield extracts which are similar to agar in containing the L-isomer of 3,6-anhydro-galactose or galactose-6-sulphate. Examples of these are galactans from the Porphyra species, but here the repeating pattern is masked not only by 6-O-methyl on the D-galactose units but as much as 50% of the anhydro-L-units are replaced by L-galactose-6-sulphate[7]. Laurencia[76], Ceramium rubrum[77] and a number of genera belonging to the Grateloupiaceae[78] have C-2 of the D-galactose units substituted by methoxyl. 3-, 4-, and 6-O-methyl galactose residues have also been found as constituents of the galactans.

Agarase, an enzyme separated from the bacterium Pseudomonas atlantica has been used to characterise agar in marine algae.[79] ^{13}C n.m.r. analysis[80] established the structure of the enzymic hydrolysis products of agarose and characteristic ^{13}C n.m.r. spectra have been obtained for agars from different genera and for a variety of carrageenans as well.[81]

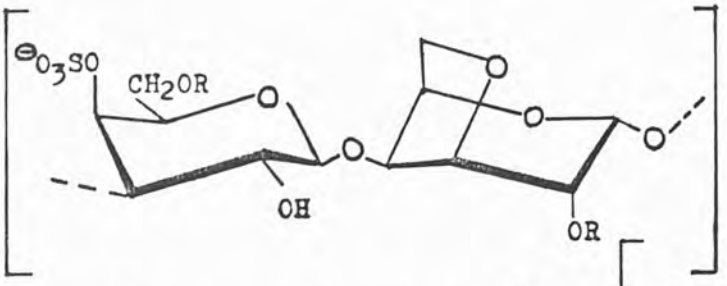
Carrageenan

The major difference between the agars and carrageenans is that the former contains D- and L-galactose units whereas the latter consists entirely of the D-sugar. In other words it differs from agar mainly in that, 3,6-anhydro-D-galactose replaces the 3,6-anhydro L-galactose of agar[82,89] and has a higher content of mainly alkali stable ester sulphate.[83]

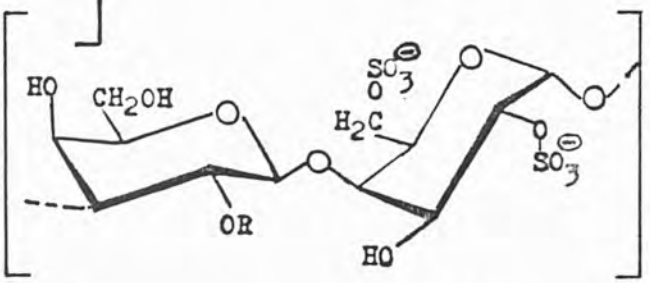
The precipitation by K ions of K-carrageenan[84,85] a sulphated gelling fraction from a whole extract of Chondras crispus or Gigartina species[86] are examples of the fractionation of carrageenan. The material left in solution, the non-gelling fraction known as λ-carrageenan represents a very complex system, which can be further fractionated and which may also contain acetal linked pyruvic acid.[87]

The repeating unit of K-carrageenan consists of chains of alternate (1 ⟶ 3)-linked-D-galactose-4-sulphate and (1 ⟶ 4)-linked-3,6-anhydro-D-galactose.

λ-Carrageenan is a polysaccharide consisting of highly sulphated alternating (1 ⟶ 3) and (1 ⟶ 4)-linked galactose units. Some of the sulphate is alkali labile, indicating its presence on C-3 or C-6 of (1 ⟶ 4)-linked units.



κ-Carrageenan. R=H or $SO_3^{\ominus}$



λ-Carrageenan. R=H or $SO_3^{\ominus}$

Fig. 1.2.

Many of the papers published refer to the whole KCl-soluble fraction as λ-carrageenans although Rees[90] has suggested that the term λ-carrageenan should be restricted to one fraction of this material.

Two important groups of carrageenans can be recognised according to a variety of chemical studies carried out. In the first group the (1 ⟶ 3)-linked units are sulphated in the position 4-, whereas in the second group the sulphate is in the 2- position. Both groups have been subdivided according to the nature of the (1 ⟶ 4)-linked residues. See table 1.1 on next page (19).

TABLE 1.1.

Repeating units of carrageenans

$$-{}^{3}A^{1\beta}-{}^{4}B^{1\alpha}-{}^{3}A^{1\beta}-{}^{4}B^{1\alpha}-{}^{3}A^{1\beta}-$$

A Units	B Units	Name
	D-galactose-6-sulphate	mu (μ)
D-galactose-4-sulphate	D-galactose-2, 6-disulphate	nu (ν)
	3, 6-anhydro-D-galactose	kappa (κ)
	3, 6-anhydro-D-galactose-2-sulphate	iota (ι)
	D-galactose-2-sulphate	xi (ξ)
D-galactose-2-sulphate	D-galactose-2, 6, disulphate	lambda (λ)
	3, 6-anhydro-D-galactose-2-sulphate	theta (θ)

Furcellaran known also as Danish agar, extracted from Furcellaria fastigiata has many similarities with the K-fraction of carregeenan.[91, 92]

At least two types of ι-carrageenan can be found in different species of Eucheuma[93]. Gigartina atropurpurea synthesises Xi-carrageenan.[94, 95]

^{1}H n.m.r. and ^{13}C n.m.r. spectroscopy have been used for structural analysis on carrageenans and agaroses.[80, 81, 101, 102]

X-ray diffraction[90, 96, 97] and deuteration-dichroism[98] studies have been carried out to estimate the conformation of carrageenans and vacuum-ultraviolet circular dichroism[99], and X-ray diffraction studies[100] as well, to determine conformation of agarose.

Porphyran

This type of polysaccharide is mainly found in Porphyra and Laurentia species. It is similar to agarose containing 3, 6-anhydro-L-galactose and 6-O-methyl galactose and

resembles carrageenan in containing galactose-6-sulphate[103].

Porphyran consists mainly of 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-L-galactose.[104]

There are some red algae investigated which present some 'anomalies' among the galactans. For example, residues of D-glucuronic acid have been found in a galactan from Anantheca dentata,[105] which appeared as 4-O-(α-D-glucopyranuronic acid)-L-galactose in a partial hydrolysate.[106] This aldobiouronic acid is identical with that isolated from the proteoglycan of the microscopic red alga Porphyridium cruentum[107]. This same galactan contained 8% of residues of D-xylose.[106] The sulfated galactan of Dilsea edulis also contains residues of glucuronic acid and xylose.[108]

An investigation of the extracellular mucilage produced by Rhodella maculata[109] a microscopic unicellular red seaweed showed that it comprised a sulphated hetero-polysaccharide containing mainly xylose and glucuronic acid together with smaller proportions of 3-O-methyl-xylose, galactose and glucose.

1.6.3. Structural polysaccharides

1.6.3.1. Cellulose

The red algae apart from the Bangiales contain cellulose in their cell walls[44], but the microfibrils are built up of other sugars as well as glucose. The structure of cellulose found in Gelidium amansii was similar to that of cotton except for its colloidal properties[110]. A methylated purified cellulose from the residue after sequential extraction of Rhodymenia pertusa[111] was shown by gas liquid chromatography, mass spectrometry and proton magnetic resonance studies to consist of (1 ⟶4)-β-linked D-

glucopyranose residues.

1.6.3.2. Mannans

Mannans have been reported as a cuticle in the Rhodophyta algae. A β - (1 ⟶4)-linked mannan has been extracted from the cell wall of Porphyra umbilicalis[112,115], while a sulphated α - (1 ⟶3)-linked mannan was found in Nemalion vermiculare Sur.[113,114]

1.6.3.3. Xylans

Cell walls of Porphyra umbilicalis and Bangia fuscopurpurea were mechanically separated.[115] and showed from X-ray diffraction and electron microscope studies that they consisted of chains of (1 ⟶3)-linked xylose in a random network of microfibrils.

A major polysaccharide of Rhodymenia palmata is a water soluble essentially linear xylan made up of (1 ⟶3) and (1 ⟶4)-linked units in purely random sequence[116] with an average one branch point in each molecule linked at C-3 and C-4. Extraction of the residual weed of Rhodymenia gave a (1 ⟶4)-linked xylan[117]. Extracts from Rhodochorton floridulum[117] contain a xylan similar to that one of Rhodymenia but with a higher proportion of branch points. In these extracts a glucan backbone has been suggested since glucose units have been found also.

Others, (1 ⟶3)-and (1 ⟶ 4)-linked xylans have been separated from Porphyra umbilicalis, Laurencia pinnatafida[112] and from Chaetangium fastigiatum.[118,119]

1.7 Carbohydrates of the Cyanophyta (blue-green seaweeds)

Following early indications[120, 121] of a starch-like material in blue-green algae, Hough et al[122] established by methylation analysis that the glucan in an Oscillatoria species was a fairly typical amylopectin with an average chain-length of 23 to 26 units. Starch granules in the ordinary sense are absent,[121] but tiny, roughly cylindrical particles have been clearly demonstrated in Nostoc muscorum.[123]

The material yielded ca. 95% of D-glucose upon acidic hydrolysis, the remainder being firmly-bound protein.

There is considerably doubt concerning the presence of cellulose in Cyanophyta. Histochemical works indicate the presence of some microfibrils, heavily encrusted with amorphous material. These stain with iodine in concentrated aqueous sulfuric acid, zinc chloride or calcium chloride, but β-(1 → 4)-linked xylans and mannans are also stained by these reagents.[124]

No structural studies have been carried out on polysaccharides of blue green algae. Some workers have published only the carbohydrate compositions of O-somatic side-chains (moles per cent) and of extracellular mucilages (molar ratio) from Cyanophyta. In nearly all the species investigated glucose appeared to be the major sugar among other constituents.

1.8 REFERENCES

CHAPTER I GENERAL INTRODUCTION

1. Silva P.C. Classification of Algae "Physiology and Biochemistry of Algae" Edited by Ralph A. Lewin. Academic Press, New York and London, p.827 (1962).
2. Schopf J.W. Evol. Biol. 7, 1 (1974); Biol. Rev. Cambridge Philos. Soc. 45, 319 (1970).
3. Mason T.R. and Von Brunn V. Nature (Lond) 266, 47 (1977). Brasier M.D. Chem. Brit. (Roy. Soc. Chem. Lond) 15, 588 (1979).
4. Sarjeant W.A.S. "Fossil and Living Dinoflagellates" Academic Press, London (1974)
5. Simonsen R. (Ed.) Fourth Symp. on "Recent and Fossil Marine Diatoms", Oslo (Sept. 1976).
6. Prescott, G.W. "The Algae - A Review" Thomas Nelson, London (1969).
7. Percival E. and McDowell R.H. "Chemistry and Enzymology of Marine Algal Polysaccharides" Academic Press, London (1967).
8. Searles R.B. Br. Phycol. J. 13, 361-81 (1978).
9. Biebl R. Seaweeds "Physiology and Biochemistry of Algae" Edited by Ralph A. Lewin. Academic Press, New York and London, p.799-826 (1962).
10. Haxo F.T. and Blinks L.R. J. Gen. Physiol. 33, p389-442, (1950).
11. Percival Elizabeth Br. phycol. J. 14, 103-117 (1979).
12. Thompson E.W. and Preston, R.D. Nature (London) 213, 684-85 (1967).
13. Turvey J.R. Biochemistry of algal polysaccharides. In Internat. Rev. Biochem. Biochem Carbohydrate II. Vol. 16 (D.J. Manners Ed), p.151-77, University Park Press, Baltimore (1978).
14. Percival Elizabeth and Turvey J.R. Polysaccharides of algae. In CRC Handbook of Microbiology (Laskin A.I., Lechevalier H.A. eds) p.532-550. CRC Press Inc. Cleveland, Ohio (1974).

15. Chapman V.J. "The Algae" McMillan, New York (1962) Ragan M.A. and Chapman D.J. "A Biochemical Phylogeny of the Protists" Academic Press, New York (1978).
16. Craigie J.S.,McLachlan, J.C.,Majak W., Ackerman R.G., and Tocher C.S. Can.J.Bot. 44, 1247 (1966).
17. Percival E. and Young M. Phytochem. 10, 807 (1971).
18. Percival E. and Young M. Carbohyd. Res. 20, 217-23 (1971).
19. Love J. Mackie W. McKinnell J.P. and Percival E. J. Chem. Soc. 4177 (1963).
20. Hirst E.L. Manners D.J. and Pennie I.R. Carbohydr.Res. 22, 5 (1972).
21. Suzuki H. Phytochemistry, 13, 1159 (1974).
22. Anderson D.M.W. and King N.J. J.Chem.Soc. 2914 (1961).
23. Bourne E.J. Megarry M.L. and Percival E. J.Carbohydrates Nucleosides Nucleotides 1 (3), 235 (1974).
24. Eddy B.P., Fleming I.D. and Manners D.J. J.Chem.Soc. 2827 (1958).
25. Du Mérac M.L.R., Rev. gén. botan. 60, 689 (1953)
26. Bourne E.J., Percival E. and Smestad B., Carbohydr.Res. 22, 75 (1972).
27. Meeuse B.J.D. Acta Bot.Neerl. 12, 315 (1963).
28. Mackie I.M. and Percival E. J. Chem.Soc. 1151 (1959)
29. Fisher I.S. and Percival E. J.Chem.Soc. 2666 (1957)
30. Carlberg G.E. and Percival E. Carbohydr.Res. 57 223 (1977)
31. Hawthorne D.B., Sawyer W.H., Grant B.R., Carbohydr.Res., 77, 157-167, (1979).
32. Preston R.D. Abstr. 3rd Internat. Seaweed Symp. Galway, Ireland, (1958).
33. Myers A.and Preston R.D. Proc.Roy.Soc. B150, 447 (1959).
34. Hirst Sir Edmund, Mackie W. and Percival E. J.Chem.Soc., 2958 (1965).
35. Mackie I.M. and Percival E. J.Chem.Soc. 3010 (1967).

36. Love J. and Percival E. J. Chem. Soc. 3338 (1964).

37. McKinnell J. P. and Percival E. J. Chem. Soc. 2082 and 3141, (1962).

38. Brading J. Georg-Plant M. M. T. and Hardy J. J. Chem. Soc., 319 (1954).

39. O'Donnell J. J. and Percival E. J. Chem. Soc. 2168 (1959).

40. Percival E. Sulphated Polysaccharides metabolised by the marine Chlorophyceae. In Carbohydrate Sulfates. (Schweiger, R. G., editor) 203-212 A. C. S. Symposium Series No. 77 (1978)

41. Percival E., Wold J. K., J. Chem. Soc. 5459 (1963).

42. Haq Q. N. and Percival E. Proc. 5th Int. Seaweed Symp. (E. G. Young and J. L. McLachlan eds.) Pergamon Press, Oxford, 261 (1966).

43. Kreger D. R. in "Physiology and Biochemistry of Algae" (R. A. Lewin, ed.) p.289-291, Academic Press, New York (1962).

44. Frei E. and Preston, R. D., Nature, London 192, 939, (1961).

45. Nicolai E, and Preston R. D. Proc. Roy. Soc. B140, 244 (1952).

46. Cronshaw J., Myers A and Preston R. D. Biochem. Biophys. Acta, 27, 89 (1958).

47. Nisizawa K, Miwa T, Iriki, Y and Suzuki T, Nature, (London), 187, 82 (1960).

48. Miwa T, Iriki Y and Suzuki T. Mannan and xylan as essential cell wall constituents of some siphoneous green algae. Colloques internationaux de C. N. R. S. 103, 135-43 (1961).

49. Iriki Y, Suzuki T, Nisizawa K and Miwa T., Nature (London) 187, 82-83 (1960).

50. Frei E. and Preston R. D. Proc. R. Soc. London Ser. B 160 293-313 (1964).

51. Atkins E. D. T., Parker K. D. and Preston R. D. Proc. R. Soc. London, Ser. B 173, 209-221 (1969).

52. Atkins E. D. T., and Parker, K. D. J Polymer Sci., Part C 28, 69-81 (1969)

53. Huizing, H.J., Rietema, H., Sietsma J.H. Br.Phycol J. 14, 25-32 (1979)

54. Iriki, Y. and Miwa T. Nature (London) 185, 178 (1960).

55. Mackie W. and Preston R.D. Planta (Berl) 79, 249-53 (1968)

56. Huizing H.J. and Rietema H. Brit.Phycol.J. 10, 13 (1975).

57. Colin H. and Guéguen E. C.r. hebd.Séanc.Acad.Sci.,Paris, 191, 163 (1930).

58. Putman, E.W. and Hassid W.Z. J.Am.Chem.Soc., 76 2221 (1954).

59. Lindberg B. Acta Chem.Scand. 7, 1119-22 and 1218-19 (1953).

60. Majak W. Craigie J.S. McLachlan J.C., Can.J.Bot., 44, 541 (1966).

61. Bidwell R.G.C. Can.J.Bot., 36, 337 (1958).

62. Colin H, and Augier J. C.r. hebd Seanc Acad.Sci., Paris, 208, 1450 (1939).

63. Lindberg B.Acta.Chem.Scand. 9, 1093 (1955).

64. Lindberg B. Acta Chem.Scand. 9, 1097 (1955).

65. Nagashima O.H., Nakamura S., Nisizawa K. Bot.Mag., Tokyo, 82, 462-473 (1969).

66. Peat S., Turvey J.R. and Evans J.M. J.Chem.Soc. 3223-3227 (1959).

67. Meeuse B.J.D. in "Physiology and Biochemistry of Algae" (R.A.Lewin, ed.) pp.289-291. Academic Press, New York (1962).

68. Fleming I.D., Hirst E.L. and Manners D.J. J.Chem.Soc. 2831 (1956).

69. Greenwood C.T. and Thomson J. J.Chem.Soc. 1534 (1961)

70. Peat S., Turvey J.R. Fortsch. Chem.Org.Nat.Stoffe , 23, 1 (1965).

71. Rees, D.A. "Carbohydrate sulphates" in Ann.Reports, 469, (1965)

72. Bull A.T., Lagnado J.R., Thomas J.O. Tipton K.F. Companion to Biochemistry (Longman Group Ltd), 328 (1974).

73. Araki C. Proc. 5th Int. Seaweed Symp. (E.G. Young, J.L. McLachlan, eds.) pp. 3-17 Pergamon Press (1966).

74. Duckworth M. and Yaphe W. Carbohyd. Res. 16, 189-197 (1971).

75. Young K. Duckworth M. and Yaphe W. Carbohyd. Res. 16, 446-448 (1971).

76. Bowker D.M. and Turvey J.R. J. Chem. Soc. 983-988; 989-992 (1968).

77. Turvey J.R. and Williams E.L. Carbohyd. Res. 49, 419-425 (1976).

78. Nunn J.R. and Parolis H. Carbohyd. Res. 9, 265-276 (1969)

79. Groleau D. and Yaphe W. Can. J. Microbiol. 23, 672-79 (1977).

80. Hamer G.K., Bhattacharjee S.S. and Yaphe W. Carbohyd. Res. 54, C7-C10 (1977).

81. Bhattacharjee S.S., Yaphe W. and Hamer G.K. Proc. 9th Int. Seaweed Symp. (Jensen A. SteinJanet R. eds.) p. 379-385 Princeton Science Press (1978).

82. O'Neill A.N. J. Am. Chem. Soc., 76, 5074-6 (1954).

83. Rees D.A. "Carbohydrate sulphates' in Annual Reports, 469 (1965).

84. Smith D.B. and Cook W.H. Arch. Biochem. Biophys., 45, 232-233 (1953).

85. Stancioff D.J. Extraction of hydrocolloid fractions from sea plants. (to Marine Colloids Inc.) U.S. Pat. 3176003 (1965).

86. Cerezo A.S. Carbohyd. Res., 26, 335-340 (1973).

87. Hirase S., and Watanase K. Bull. Inst. Chem. Res. Kyoto Univ. 50, 332-336 (1972).

88. Smith D.B., Cook W.H. and Neal J.L. Archs. Biochem. Biophysics, 53, 192 (1954).

89. Percival E.E. Chem. Ind. 1487, (1954).

90. Rees D.A. Adv. Carbohyd. Chem. Biochem. (Wolfrom M.L., Tipson R.S. eds.) 24, 267-332 (1969).

91. Yaphe W. Can. J. Bot., 37, 751-757 (1959).

92. Painter T.J. Proc.5th Int.Seaweed Symp. (Young E.G.; McLachlan J L. eds.) pp.305-313 Pergamon Press, London, (1966).

93. Dawes C.J. Proc. 9th Int.Seaweed Symp. (Jensen A. Stein Janet R. eds.) pp.199-207, Princeton, Sciences Press (1978).

94. Lawson C.J., Rees D.A., Stancioff D.J. and Stanley N.F. J.Chem.Soc., 2177-2182 (1973).

95. Penman A. and Rees D.A. J.Chem.Soc., 2182-2187 (1973).

96. Arnott S., Scott N.E., Rees D.A. and McNab C.G.A. J.Mol.Biol., 90, 253-267 (1974).

97. Rees D.A. and Welsh E.J. Angew.Chem.Int. (Ed. Engl.) 16, 214-224 (1977).

98. Anderson N.S., Dolan T.C.S., Lawson C.J., Penman A. and Rees D.A. Carbohyd.Res., 7, 468-473 (1968).

99. Liang J.N., Stevens E.S., Morris E.R. and Rees D.A. Biopolymers, 18, 327-333 (1979).

100. Arnott S., Fulmer A., Scott W.E., Moorhouse R. and Rees D A. J.Mol.Biol., 90, 269-284 (1974).

101. Harris M.J. and Turvey J.R. Carbohyd. Res., 15, 57 (1970).

102. Usov A.I., Yarotsky S.V. and Shashkov. Biopolymers, 19, 977 (1980).

103. Turvey J.R. and Rees D.A. Nature, 189, 831 (1961).

104. Turvey J.R. and Williams T.D. Proc. 4th Int.Seaweed Symp. (R.D. DeVirville and J.Feldman, eds.) Pergamon Press, Oxford p.370 (1964).

105. Nunn J.R., Parolis H.and Russell I. Carbohydr.Res.,20, 205, (1971).

106. Nunn J.R., Parolis H.and Russell I. Carbohydr.Res.,29, 281, (1973).

107. Percival E. and Foyle R.A.J. Carbohydr. Res., 72, 165, (1979).

108. Barry V.C. and McCormick J.E. J.Chem.Soc., 2777, (1957).

109. Evans L.V., Callow M.E., Percival E. and Fareed V.S. J.Cell.Sci. 1 (1974).

110. Araki C., Mashi V. Memoirs 45th Anniv.Kyoto Industr. Tech.School,64, (1948).

111. Whyte J.N.C., and Englar J.R. Can.J.Chem., 49, 1302-1305 (1971).

112. Jones J.K.N., J.Chem.Soc., 3292 (1950).

113. Usov A.I., Adamyants K.S.,Yarotsky S.V., Anushina A.A., Kochetkov N.K. Carbohydr.Res., 26, 282 (1973).

114. Augier J. Compt.Rend. 242, 1069 (1956).

115. Frei E. and Preston R.D. Proc.R.Soc.London Ser.B. 160, 314-327 (1964).

116. Bjorndahl H.,Eriksson K.E., Garegg Per J., Lindberg B. and Swan B. Acta Chem.Scand., 19, 2309-2315 (1965).

117. Turvey J.R. and Williams E.L. Phytochemistry, 9, 2383-2388 (1970).

118. Cerezo A.S., Lezerovich A., Labriola R., and Rees D.A. Carbohydr.Res., 19, 289-296 (1971).

119. Cerezo A.S. Carbohydr.Res., 22, 209-211 (1972).

120. Pagen J. Rev.Algol. 11, 1, (1938).

121. Kylin H. Kgl Fysiograf. Sällskap i Lund Förch, 13, 1 (1943).

122. Hough L., Jones J.K.N. and Wadman W.H. J.Chem.Soc., 3393 (1952).

123. Lee Chao and Bowen C.C. J.Bacteriol. 105, 331 (1971).

124. Gaillard B.D.E., Thompson N.S. and Morak A.J. Carbohydr.Res. 11, 509 (1969).

CHAPTER II

GENERAL METHODS

2.1. Physical Techniques

2.1.1. Water: deionised, distilled water was used in all experiments.

2.1.2. Evaporations were carried out under reduced pressure using a "Buchi" rotatory evaporator with water bath between 30-40°C.

2.1.3. Melting points were determined on a Gallenkamp micro-melting point apparatus.

2.1.4. Specific rotations were measured on a Perkin-Elmer 141 polarimeter, using a 1 dm polarimeter tube. All samples were measured in aqueous solution at 25°C using the sodium D light.

2.1.5. Dialysis were carried out in Visking Cellophane tubing against distilled water with a solution of sodium azide acting as a bacteriostat. The tubing was initially boiled with water for a couple of minutes in order to render it free of glycerol.

2.1.6. Resin used was Amberlite or Biodeminrolit unless stated otherwise.

2.1.7. Freeze drying (lyophilisation) was carried out on a Chem-lab freeze drier, at a pressure of 0.1-0.2 torr and -40°C. Samples were first frozen in a cardice-acetone mixture.

2.1.8. Some solutions and hydrolysates were filtered through millipore filters (0.45μ) before quantitative determinations.

2.1.9. Molecular sieves 4A (BDH) were activated by heating at 400° for 4 hours.

2.1.10. Preparative (quantitative) 3 M.M. Whatman and 17 M.M. paper chromatograms were first washed with deionised water.

2.1.11. Ultraviolet and visible absorbances were determined using a Pye Unicam SP500 spectrophotometer.

2.1.12. Gas liquid chromatograms were obtained on a Pye 104 apparatus with N_2 as carrier gas.

2.1.13. Mass spectra were obtained on a Micromass 12F apparatus.

2.2. Purification of Solvents

2.2.1. Methanol was purified as described by Vogel,[1] distilled with the exclusion of moisture and stored over activated 4 A molecular sieves.

2.2.2. Chloroform was distilled from anhydrous sodium sulphate and stored, refrigerated, kept in brown glass bottle over anhydrous sodium sulphate.

2.2.3. Pyridine was distilled from sodium hydroxide pellets and stored over sodium hydroxide or potassium hydroxide pellets. (BDH Analar).

2.3. Acid Hydrolysis

2.3.1. Formic acid method

The sample was dissolved in 90% formic acid (ca. 35 ml for 100 mg) and solid carbon dioxide added to provide an inert atmosphere. The flask was sealed (rubber and wire) and heated for 6-8 hrs in a boiling water bath. The hydrolysate was diluted with water (5 vol.) and heated at 100°C for a further 2 hours in order to hydrolyse the formyl esters. The solution was evaporated in a Büchi evaporator. This was followed by repeated additions of methanol until formic acid could no longer be detected.

2.3.2. Sulphuric acid method (molar)

The sample was dissolved in M-sulphuric acid and a small amount of solid CO_2 was added to give an inert atmosphere. The flask was sealed and placed into a boiling water bath for 4 hours. The hydrolysate was transferred to a separatory funnel and an equal volume of 5% N-dioctylamine in chloroform was added to neutralise the acid. After vigorous shaking for some minutes it

was allowed to settle and the organic layer discarded. This was repeated. The aqueous layer was then shaken with chloroform only and the chloroform layer discarded. The aqueous layer was then evaporated down to dryness under reduced pressure.

2.3.3. 72% Sulphuric acid method[29]

Cold 72% sulphuric acid was added to the sample and the mixture left at room temperature for one hour. Water (10 volumes) was added with cooling and the solution heated for 4 hours in a boiling water bath. After cooling the hydrolysate was neutralised with solid analar barium carbonate.

2.3.4. Triofluoroacetic acid method

The sample was dissolved in 2M trifluoroacetic acid and the sealed tube was heated at 120°C for 2 hours. The hydrolysate was then cooled, diluted with water and evaporated under reduced pressure; dilution with water and subsequent evaporation was confirmed until the hydrolysate was neutral.

2.4. Chromatography

2.4.1. Paper chromatography

Paper chromatography was carried out by the descending technique using Whatman No. 1 and No. 3 papers for qualitative analysis and Whatman No. 3 and No. 17 papers for preparative separations.

All preparative papers were washed with distilled water before being used.

The solvent systems used for this method were as follows:

2.4.1.1. n-butanol:pyridine:water (6:4:3, by vol.)[2]

2.4.1.2. ethyl acetate:acetic acid:formic acid:water (18:3:1:4, by vol.)[3]

2.4.1.3. n-butanol:ethanol:water (40:11:19, by vol.)[4]

2.4.1.4. n-butanol:ethanol:water (3:1:1, by vol)
3% cetyl pyridinium chloride (w/v)[5]

2.4.1.5. n-butanol:acetic acid:water (50:12:25, by vol.)[6]

2.4.2. Ionophoresis

The Shandon high voltage electrophoresis apparatus L24 was used with Whatman No. 3 MM paper and the following electrolytes:

2.4.2.1. Borate with calcium ions[7]

0.01 M sodium tetraborate (borax) in water containing 0.005 M calcium chloride (pH = 9.2) electrophoresis was carried out for 2.0 h at 2 kV.

2.4.2.2. Pyridine/Acetic acid[8]

Pyridine (1 litre) adjusted to pH = 6.8 with 5% acetic acid in water. The electrophoresis was carried out for 2 h at 3000 volts.

2.4.2.3. Borate[9]

0.2 M sodium borate in water adjusted to pH10 with sodium hydroxide. Electrophoresis was carried out for 2 h at 2.000 volts.

2.4.3. Trimethylsilylated glass fibre paper (TGFP) electrophoresis method.[10]

2.4.3.1. Buffers: 1) 0.1 M acetate pH 5 containing 0.02M EDTA
2) 0.2 M sodium borate in water adjusted to pH 10 with sodium hydroxide.

2.4.3.2. Preparation of TGFP:

Glass fibre paper (Whatman GF/81 or GF/A) was cut into strips of 14 cm width and heated at 400°C for 2 hours in an oven to burn out all the organic materials, then immersed for 18 h. in CCl_4 containing 2% dimethyl dichlorosilane, rinsed in toluene and dried. The TGFP strips were then wetted by floating for 18 hours in a tray containing the appropriate buffer to which Tween-20 (0.2% by volume) had been added.

2.4.3.3. Analytical electrophoresis

TGFP strips were blotted only lightly before being subjected to electrophoresis. Before application of the sample it was found advantageous to pre-equilibrate for 10-20 minutes at the running voltage. The sample, usually 1-5 µg in ca. 2 µl of buffer was applied at or near the mid-point of the strip. Separation conditions were: potential gradient 1000-1300 volts, current 40-60 mA, running time 20-40 minutes.

2.4.3.4. Location and quantitation

When the separation was completed the strips were dried in air and the polysaccharides were located either by:

1) dipping first in a saturated toludine blue solution in ethanol[11] for 5 minutes, then in 40% aqueous ethanol for a further 5 minutes and finally washing in ethanol. Dark blue spots appear on pale blue background, or

2) by placing the strips 15 minutes in the fixing solution made of 20 ml 35% formaldehyde in 80 ml ethanol. After drying the strips were sprayed with a solution containing 0.04 g toludine blue in 80 ml acetone and 20 ml water. The excess dye was first rinsed with a dip solution (5% aqueous acetic acid) and finally with water.

2.5. Staining reagents:

In order to locate and identify compounds on paper chromatograms and electropherograms the following staining reagents were used:

2.5.1. Silver nitrate dip.[12]

The paper was dipped sequentially in

1) saturated silver nitrate solution (2.5 ml) and water (10 ml) in acetone 500 ml.

2) sodium hydroxide (20 g) in water (40 ml) and ethanol 960 ml.

3) 10% aqueous sodium thiosulphate.

The papers dried at each stage at room temperature.

2.5.2. Aniline oxalate spray[13]

A saturated solution was prepared by adding aniline oxalate (25 g) in 50% aqueous ethanol (1 lt.) and stirring vigorously during 12-18 hours at room temperature.

The papers were then dried at 105°C.

2.5.3. Glucose oxidase[14]

"Glucostat" kit (Worthington Biochemical Company) was prepared as directed. A pink colour indicating the presence of D-glucose develops at room temperature 5 minutes after dipping the paper chromatogram in this solution.

2.5.4. Galactose oxidase[14]

"Galactostat" kit (Worthington Biochemical Company) was prepared as directed. A pink colour indicating the presence of D-galactose develops at room temperature 5 minutes after having dipped the paper chromatogram in this solution.

2.5.5. Ninhydrin

A freshly made solution of ninhydrin (2 g) in ethanol (100 ml) was used. A blue colour specific for amino acids developed after 5 minutes at 70°C.

2.6. Gas Liquid Chromatography (g.l.c.)

2.6.1. Instrumentation

A Pye 104 gas chromatograph with nitrogen carrier gas and flame ionisation detector with glass column (3 m x 5 mm) was used.

2.6.2. Column

The column used was packed with 3% OV225 (cyanopropyl-methyl-phenyl methyl silicone) coated on 100-120 gaschrom Q.

2.6.3. Gas chromatography linked to mass spectrometry

2.6.3.1. A Pye 104 gas chromatograph with helium carrier gas was coupled to a VG Micromass 12F mass spectrometer with a total ion monitor detector system. For EI spectra the 'ion source' was operated at about 200°C, 70 eV and 20 μA target current under a pressure of 10^{-6} torr. For CI spectra the 'ion source' was operated at about 150°C, 50 eV and 1000 μA under a pressure of 0.4-0.5 torr with isobutane as the reactant gas.

2.6.3.2. A Perkin Elmer Sigma 3 gas chromatograph with helium carrier gas (30 ml/min) coupled to a Kratos MS25 mass spectrometer with a Data System DS 50S computer was used. Isothermic temperature at 170°C. The conditions were:

ionisation potential: 70eV
acceleration voltage: 1.33 Kv
emission current: 1000 μA
ion source temperature: 200°C
injector temperature: 200°C
interphase temperature: 250°C

2.7 Assays and Analyses.

2.7.1 Carbohydrate content

Carbohydrate content was estimated by the phenol sulphuric acid method.[15] A 4% aqueous phenol solution (1 ml) was added to a sugar solution (1 ml) containing between 10 - 100 μg. Then 5 ml of concentrated sulphuric acid was added rapidly by means of an automatic pipette, the stream of acid being directed against the liquid surface rather than against the side of the test tube in order to obtain good mixing. The tubes were allowed to stand at room temperature for 30 minutes and the colour developed was read at 487 nm on a Unicam

SP500 spectrophotometer. Standard graphs plotted absorbance vs. concentration, were prepared for different sugars and mixtures of sugars in the ratios corresponding to those of the particular polysaccharide.

2.7.2 Uronic acid determination was carried out by two different methods.

2.7.2.1. The meta-hydroxydiphenyl method[16]

To a sample solution (0.6 ml) containing from 1.5 to 60 μ g. uronic acid, a 0.0125 M solution of sodium tetraborate in concentrated sulphuric acid (3.6 ml) was added. The solutions were cooled in ice and then mixed and heated at 100° for 5 minutes. After again cooling in ice the m-hydroxy-diphenyl solution (50 μ l, 0.15% solution in 0.5% sodium hydroxide) was added. The solutions were shaken and the absorbance measured at 520 nm on a Unicam SP500 spectrophotometer.

Standard graphs were prepared for the different uronic acids.

2.7.2.2. Modified carbazole reaction[17]

The samples containing (10-70 μ g of uronic acid in 1 ml of water, the test solution, were treated at 55°C and 100°C with and without borate solutions.

(1) Reaction without borate (both 55° and 100°C)

The test solution (1 ml) was previously cooled in ice and then concentrated sulphuric acid (6 ml) was added and the solution cooled again. After the sample was mixed with a Vorter Jr mixer it was heated for 20 minutes at the appropriate temperature and again cooled in ice. After the carbazole reagent (0.2 ml 0.1% solution in ethanol) was added, samples were mixed and allowed to stand for 3 hours for colour development.

(2) Reaction with borate at 100°C

A 0.1M boric acid solution in concentrated sulphuric acid, H_2SO_4-borate reagent, (6 ml) was cooled in an ice bath. Carefully, 0.7 ml of test solution was layered on top of the cold acid. Samples were mixed (all exactly the same time, ca. 4-5 seconds) and again equilibrated in the ice bath. After samples were heated in a boiling water bath for 15 min. and then cooled again. Carbazole reagent (0.2 ml) was added. The solutions were mixed and again heated for 10 minutes and cooled to room temperature. Colour was stable for at least 48 h.

(3) Reaction with borate at 55°C

The same procedure as above was used except heating before the addition of the carbazole reagent was omitted. After adding carbazole reagent the samples were heated for 30 minutes at 55°C. Colour development was then completed and stable for 2 h.at room temperature.

The colours which developed were read at 530 nm on a Unicam SP500 spectrophotometer.

Standard graphs for different acids under each of the conditions were prepared.

2.7.3 Nitrogen and protein content

Nitrogen content was measured by A. Bernhardt (West Germany) by Butterworth Laboratories Ltd. (U.K.) and by The School of Pharmacy (University of London). The protein content was calculated by multiplying the figure for nitrogen by 6.25.[18]

2.7.4. Sulphate estimation

The polysaccharide (10 mg) was digested in a sealed tube with analar nitric acid (1 ml plus a few mg of sodium chloride) for 12 hours at 100°C. Then the opened tube was placed over a bunsen burner and the sample evaporated to dryness.

Then the residual solid was treated with concentrated hydrochloric acid (1 ml) and evaporated again. The solid was treated with water (1 ml) and evaporated to dryness. The tube was then placed in an oven at 110°C for not less than 2 hours. The sample was then ready for sulphate estimation and the following modification of the Jones and Letham method[19] was applied.

To the sulphate solutions (1 ml) containing 30-100 μg of sulphate in microcentrifuge tubes, the reagent 4 chloro-4-aminodiphenyl (1 ml of 0.19% in 0.1 M hydrochloric acid) and a trace of solid hexadecyltrimethyl ammonium bromide (cetavlon) were added. After mixing, the solutions including a blank, were kept for 2 hours and then centrifuged. The surface active agent caused the precipitates to fall completely to the bottom of the tubes. Aliquots of the supernatants (0.2 ml) were removed to volumetric flasks and diluted to 25 ml with 0.1 M hydrochloric acid. The optical densities of the resulting solutions were read at 254 nm on a Unicam SP500 against the blank. Alternatively the blank and each of the solutions can be read against distilled water. In the latter case the results need to be subtracted from that of the blank to obtain the amount of sulphate. A graph of weight of $SO_4^{=}$ against absorption can then be constructed. This should give a straight line.

2.7.5 Degree of polymerization (D.P.)

This was estimated according to the Timell[20] modification of the Peat et al method.[21] Three solutions were prepared as follows:

(1) A blank containing water (0.5 ml) and 2% potassium borohydride solution (0.5 ml).

(2) An aqueous sugar solution (0.5 ml, containing

60-80 μ g.as carbohydrate) and 2% potassium borohydride (0.5 ml).

(3) A 2$\underline{\underline{M}}$ sulphuric acid solution (0.5 ml) containing the same amount of sugar in (2) and 2% potassium borohydride solution (0.5 ml).

These mixtures were left at room temperature for 20 hours. The optical density of each solution was then measured by phenol sulphuric acid method The optical density of solution (1) was used as a blank The D.P. of the carbohydrate material is given by the following relationship.

$$D.P. = \frac{(O.D.)\,N}{(O.D.)N - (O.D.)R}$$

where,

(O.D.)N = optical density of non-reduced solution (3)

(O.D.)R = optical density of reduced solution (2)

This equation is valid only for homopolysaccharides. Otherwise the O.D. may be converted into the corresponding weight of sugar (read off the appropriate standard graph). Then,

$$D.P. = \frac{\text{wt. of non-reduced carbohydrate}}{\text{wt. of non-reduced carbohyr.} - \text{wt. of reduced carbohyd.}}$$

2.8. General Reactions and Preparations

2.8.1. Preparation of Amberlite IR 120(H^+) dry form in methanol.

The resin was washed with water until free of colour. It was then stirred with methanol for 18 hours and then filtered. This procedure was repeated three times, the third time dry methanol was used. The resin was then stored under dry methanol.

2.8.2. Preparation of methanolic hydrogen chloride.

Hydrogen chloride gas was passed into dry

methanol until saturation was reached. The solution was titrated with $\underline{\underline{M}}$ NaOH and diluted with dry methanol as required.

An alternative method of preparation consists in adding carefully acetyl chloride (5 ml) to dry methanol (95 ml), but with this method only a ca. 3% methanolic hydrogen chloride solution is obtained.

2.8.3. Esterification of uronic acids.

2.8.3.1. The sample was dried at 40°C over phosphorus pentoxide in vacuo. The dry sample was refluxed with ca. 3% methanolic hydrogen chloride (volume enough to cover the sample) for 18 hours. A $CaCl_2$ tube was used to keep a dry atmosphere. The solution was then neutralised with dry silver carbonate, filtered and the precipitate washed with dry methanol. The combined filtrate and washings were evaporated to dryness.

2.8.3.2. The sample was dried as above and then dissolved in dry methanol. A small amount of IR 120(H^+)resin (dry form) was added as catalyst. The mixture was refluxed for 18 hours, the resin filtered off and the methanol removed by evaporation.[28]

2.8.4. Reduction of sugars to alditol[22]

The sample (20-25 mg) was dissolved in water (3 ml) or water/methanol (1:1 v/v) and a small amount (spatula tip) of sodium borohydride was added to give approximately a 2% borohydride solution. It was left standing for about 6-8 hours. If the solution was not still alkaline after this time, more sodium borohydride was added and the mixture left for another 6 hours and then shaken with IR 120(H^+) resin to neutrality. After filtration the filtrate was co-distilled with methanol to remove boric acid and finally evaporated to dryness. Fehling's solution was used to check complete reduction.

2.8.5. Alditol acetates[23]

Samples of sugar alditols or of partially methylated alditols, dried in a desiccator, were dissolved in pyridine:acetic anhydride mixture (1:1 v/v) and heated for 30 minutes at 100°C. A small volume of toluene was added, to make evaporation easier, and the sample was evaporated to dryness under reduced pressure (rotavapour) at 40°C. The residue was dissolved in chloroform and analysed by g.l.c. and g.l.c. mass spectrometry.

2.8.6. Esterification of acidic oligosaccharides with diazomethane

2.8.6.1. Preparation of diazomethane:

The method for preparation of diazomethane was similar to that of De Boer and Backer.[24] A solution of potassium hydroxide (3 g) in water (5 ml) was mixed with methyldigol (18 ml) and dichloro methane (5 ml) and heated in an oil bath to 70-75°C. A solution of toluene-p-sulphonyl methyl nitrosoamide (10 g) in dichloro methane (50 ml) was added over a period of 30 minutes with constant stirring, effecting distillation of diazomethane together with dichloromethane. The distillate was collected in a cooled receiver and comprised approximately 65% diazomethane. The deep yellow solution was dried over sodium hydroxide pellets and stored in a refrigerator at a temperature of about 0°C.

2.8.6.2. Esterification

Samples of acidic oligosaccharides were esterified with diazomethane according to Wood's method.[32] 14-15 mg of each sample were evaporated to dryness from their aqueous solution after separation on paper chromatogram and anion-exchange resin column (Zerolit FFIP). A solution of diazomethane (containing 5 mg) in dichloromethane was added and the reaction allowed to stand

6 hours at room temperature. After evaporation to dryness in order to remove the solvent and excess of diazomethane, the samples were ready to be reduced and methylated.

2.8.7 Methylation of oligosaccharides

The Bishop and Perila[25] modification of the Kuhn[26] methylation method was used.

The oligosaccharide (0.5-2 mg) was shaken with methyl iodide (0.2 ml), N-N dimethyl formamide (0.2 ml) and dry silver oxide (0.2 g) at room temperature for 18 hours in the dark. The reaction was begun at 0°C and maintained at that temperature for 30 minutes. The mixture was filtered, the residue washed with dry chloroform and the combined filtrate and washings were dried with anhydrous calcium sulphate. The methylated product was obtained after evaporation to dryness under reduced pressure (0.03 mm) at room temperature.

2.8.8. Desulphation by alkali[27]

A sample of polysaccharide (0.35 g) was dissolved in water (35 ml) and potassium borohydride (65 mg) was added. After 20 hours at room temperature 3 M sodium hydroxide (15 ml) was added, with a further quantity of potassium borohydride (195 mg), and the mixture was heated at 80°C for 7 hours. The solution was transferred to a dialysis sac and dialysed exhaustively against distilled water, concentrated and freeze dried (0.22 g).

2.8.9. Desulphation by methanolic hydrogen chloride

A sample of the polysaccharide (0.26 g) was suspended in 0.08 M hydrochloric acid in methanol (130 ml) and left shaking overnight. The solid was

filtered off and washed with dry methanol and suspended again in methanolic hydrogen chloride solution (130 ml) and again left on a shaker overnight. After a third similar treatment the solid was redissolved in water and freeze-dried; then estimated for sulphate content (GM.2.7.4.).

2.8.10. Preparation of a DE-52 ion exchange cellulose column

The cellulose (200 g diethylamino ethyl D.E.A.E., grade D.E.52 preswollen) was suspended in 0.5 M HCl (2 litres) and deaerated with magnetic stirring under vacuum for about 20 min. After standing for another 20 minutes the supernatant was discarded and the cellulose filtered and washed with water until the filtrate was neutral. The cellulose was then treated with 0.5 M sodium hydroxide (2 l.) in the same way as the acidic suspension. These two operations were repeated three times, and in the last alkali treatment, the cellulose was transferred into a column (internal diameter 3.0 cm and length 39 cm). The material was washed to neutrality with water. The column was equilibrated with 0.5 M KCl solution (2 l.) and washed with water until the washings were chloride free.

2.8.11. Preparation of Sepharose 4B column[30]

2.8.11.1. Packing the column

The sepharose 4B was obtained already in a swollen state, supplied as a "ready-to-use" suspension. Before packing the column, the suspension was degassed under vacuum produced by a water pump until bubbling ceased. The column to be packed was first filled with eluant until the dead space was taken up. Then the gel slurry was poured down the inside wall of the column. All the gel required was poured in a single operation. Throughout packing and during elution of the column

a constant flow rate was maintained. Two to three column volumes of eluant were passed through the column in order to stabilise the bed and equilibrate with eluant buffer ($\underline{\underline{M}}$KCl)

2.8.11.2 Determination of the void volume (Vo) and the total volume (Vt) of the column packed with gel filtration media.

Blue Dextran 2000 (2 mg/ml, Pharmacia Fine Chemicals) and glucose (2 mg/ml) were dissolved in elution buffer and applied to the column (0.5 ml). Elution with $\underline{\underline{M}}$KCl at the desired flow rate, 1 drop every 15 secs., was carried out by means of a peristaltic pump and fractions collected (2 ml) by means of an automatic collector. Carbohydrate of these fractions was estimated (GM 2.7.1.)

From the elution volumes (Ve) of the samples, Vo and Vt could be determined as follows:

Ve blue dextran = Vo (Interstitial volume between the gel granules)

Ve glucose = Vt (Total volume of the packed column known as bed volume)

2.8.12. Thin layer chromatography[31]

2.8.12.1. Apparatus

1) Plastic backed F1500 silica gel from Schleicher and Schull, Dassel, Germany was used as thin layer plate.
2) Five microlitre graduated microsyringes
3) Chromatography tank, Shandon SAA 2144/D6 28 x 28 x 28 cm with cover and aluminium tray.
4) Dipping chamber especially constructed.
5) Hot air oven, with air circulating fan and motor for rotating chromatograms during colour reaction.

2.8.12.2. Solvent system used was ethyl acetate: pyridine: acetic acid: water (6:3:1:1) by volume.

2.8.12.3. Colour reaction

Para-amino benzoic acid (PABA) solution was used as locating reagent. The following solution was prepared To 70 ml of PABA stock solution (50 g PABA in 500 ml methanol) 17.5 ml of orthophosphoric acid (90g/dl) was added, and this mixture made up to 500 ml with methanol. These solutions are stable at room temperature. A dipping rather than a spraying technique is preferred for applying the PABA.

2.8.12.4. Procedure

Two plates were cut from one standard size (20 x 20 cm) plastic-backed layer. Margins were scraped free of silica gel at the sides and top of each layer. Samples (1 mg/ml) applications of 5 μ l were made manually by the microsyringe technique as bands of 1.5 cm long and 0.5-1.0 cm apart one from each other along a line of origin 1.0 cm above the lower edge of the plate. A cold air blower helps to dry the surface of the layer throughout the application of the samples. Each chromatograph was rolled into a cylinder, with the plastic surface facing outwards within a 250 ml. beaker. The beaker was inverted within the chromatography tank so that the lower edge of the layer stands in the appropriate developing solvent. Upward development was performed as many times as required using the same solvent. Between runs the beaker was removed and the plate dried with the cold air blower. After the last run had been completed the pyridine was completely removed by exposing the chromatograms to a current of mild warm air overnight.

The plate held at one edge by a suitable clip was inserted rapidly into the locating reagent contained in the specially constructed dipping chamber. Immersion

should be as brief as possible to avoid zone trailing.

After drying for 10 minutes, and excess reagent wiped from the plastic surface of the layer, the colour reaction was performed by heating the plate in a hot air oven (110-115^{o}C) for three minutes. The reaction was improved by rotating the chromatogram by means of an electric motor. The colours produced are relatively stable, but it is advisable to preserve the chromatogram by storing at -20^{o}C in a polyethylene bag.

2.8.13. Separation of neutral oligosacchardies from acidic oligosaccharides using an anion exchange resin column.

A column containing Zerolit FFIP SRA 69 anion exchange resin in chloride form (300 mg) was washed with 2 $\underline{\underline{M}}$ NaOH ($\sim$ 3 l.) until washings were free from chloride (tested with silver nitrate solution). The excess alkali was removed by washing the column with water until neutral pH was obtained. Then the resin was converted into the formate form by elution with 2$\underline{\underline{M}}$ formic acid ($\sim$ 5 l.), and then with water until neutrality. Sample containing neutral and acidic oligosaccharides was then layered on top of the column and neutral components were eluted with water until the eluant was carbohydrate free. Then acidic oligosaccharides were eluted with gradient 0.5-2 $\underline{\underline{M}}$ formic acid.

2.8.14. Protein assay.

The Bio Rad protein assay was applied. This method is based on the binding of a dye to proteins and when this happens there is a shift in the wavelength of maximum absorption from 465 nm to 595 nm allowing the determination of the protein concentration.[33] The method includes pre-tested dye reagent concentrate and a standard protein solution.

2.8.14.1. Reagent preparation

The dye reagent is provided as a five-fold concentrate. It must be diluted and filtered prior to use. 1 volume of dye concentrate was diluted with 4 volumes of high quality deionised water, filtered through Whatman No. 1 paper and stored in a glass container at room temperature. Diluted reagent must be discarded after 2 weeks. Dye reagent concentrate should be stored at $4^{o}C$.

The Bio-Rad Protein Standard supplied consists of freeze dried bovine gamma globulin sealed under nitrogen. 20 ml of water was added to reconstitute it which gave a concentration of approximately 1.4 mg/ml.

As many samples will require dilution, a physiologically buffered saline solution is recommended for dilution of samples and standard.

2.8.14.2. Assay procedure

Several dilutions of Bio-Rad Protein Standard containing from 0.2 to 1.4 mg/ml were prepared. 0.1 ml of standards and appropriately dilute unknowns were placed in clean and dry test tubes. 5.0 ml diluted dye reagent were added to each tube.

Each tube was shaken, avoiding excessive foaming and after 15 minutes (could be after 5 minutes to 1 hour), samples were measured for absorbance at 595 nm and read against appropriate blank.

A graph of absorbance reading against concentration of standards was plotted and unknowns read from the standard curve.

2.8.15. Partial hydrolysis of polysaccharide.

The freeze dried polysaccharide (1 g) was hydrolysed with 0.25 $\underline{\underline{M}}$ oxalic acid (40 ml) for 2 hours at $100^{o}C$. The hydrolysate was cooled and dialysed against deionised

water (7 x 300 ml). The dialysates were combined, neutralised with $CaCO_3$, centrifuged and concentrated to be estimated for carbohydrate content.

The non-dialysable material (remaining inside the dialysis sac), consisting of soluble and insoluble material, was centrifuged and the precipitate and supernatant separately freeze dried.

2.9. REFERENCES

CHAPTER II. General Methods

1. Vogel A.I. A Textbook of Practical Organic Chemistry 3rd Ed., 169 (1971).
2. Jeanes A, Wise C.S., Dimler R.J., Analyt. Chem. 23, 415 (1951).
3. Adams C.A., Bishop G.T. J.Amer.Chem.Soc., 78, 2642, (1956).
4. Andrews P., Ball D.H., Jones J.K.N. J.Chem.Soc., 4090, (1953).
5. Rees D.A. Nature, Vol.185, No.4709, p.309 (1960).
6. T.J.Painter 5th Int.Seaweed Symp. Pergamon Press (1966).
7. Haug A., Larsen B. Acta Chem.Scand. 15, 1395 (1961)
8. Gross D. Chem.Ind. 1219 (1959).
9. Weigel H. Adv. Carb.Chem. 18, 61 (1963).
10. Jarvis M.C., Threlfall D.R. and Friend J. Phytochemistry, 16, 851 (1972).
11. Ricketts C.R., Walton K.W., Saddington S.M. Biochem J., 533, (1954).
12. Trevelyan W.G., Proctor D.P., Harrison J.S. Nature 166, 444, (1950).
13. Hough L., Jones J.K.N. Wadman W.H. J.Chem.Soc., 1702 (1950).
14. Salton M.R. Nature, 186, 966 (1960).
15. Dubois M., Gilles K.A., Hamilton J.K., Rebers D.A., Smith F. Analyt. Chem. 28, 350 (1956).
16. Blumenkrauts N., Asboe-Hansen G. Analyt. Biochem. 54, 484 (1973).
17. Knutson C.A., Jeanes A., Analyt.Biochem., 24, 470 and 482 (1968).

18. White A., Handler P., Smith E.L. "Principles of Biochemistry" McGraw Hill, 11, (1956).

19. Jones A.S., Letham D.S. Chem.Ind., 662, (1954).

20. Timell T.E. Svensk Paperstidning, 668 (1960).

21. Peat S., Whelan W.J., Roberts J.G. J.Chem.Soc. 2258 (1956).

22. Abdel-Akher M., Hamilton J.K., Smith F. J.Chem.Soc., 73, 4691 (1951).

23. Bjorndal H., Lindberg B.,Svensson S. Acta Chem.Scand. 21, 1801 (1967).

24. De Boer T. J., Backer H.J., Org.Synt Coll. Vol.IV, 250, (1963).

25. Bishop C.T., Perila O. Can.Journ.Chem., 39, 815, (1961).

26. Kuhn R.,Trischmann H., Löw I. Angew.Chem., 67, 32, (1955).

27. Rees D.A. J.Chem.Soc., 1821, (1963).

28. Bollenback G.N. Methods in Carb.Chem. 2, 327 (1963).

29. Saeman J.F., Moore N.G., Millett M.A. Methods in Carb.Chem. 3, 55, (1963).

30. Booklets "Sephadex Gel filtration in theory and practice" Pharmacia Fine Chemicals(1977) Sweden and "Gel Chromatography" Bio-Rad Laboratories, U.S.A. (1971).

31. Menzies I.S., Mount J.N., Wheeler M.J. Annals of Clin. Biochem. 15, 65 (1978).

32. Wood H.B.Jr., Methods in Carb.Chem. 2, 59, (1963).

33. Bradford M.M., Anal.Biochem. 72, 248, (1976).

CHAPTER III

EXTRACTION AND CHARACTERISATION OF THE CARBOHYDRATES OF LESSONIA NIGRESCENS.

3.1. Carbohydrates of the Phaeophyta

3.1.1. Introduction

The last three decades in particular have seen a considerable development on the chemical studies of the brown seaweeds because of the commercial importance of alginates now being used in various industrial processes.

The Phaeophyta have been characterised from a general chemical point of view by their laminaran and alginic acid contents which replace the amylopectin type polysaccharides and the galactan sulphates found in Rhodophyta. The phylum is exclusively adopted to the marine environment and therefore it cannot be expected to contain glycans with affinities to those of terrestial plants. Indeed, cellulose which is present in very small proportion is the only polysaccharide that it has in common with land plants. The polysaccharide sulphated esters are not excluded from the brown seaweeds, being represented in this case by "fucans" typified by the presence of fucose.

Different factors which might affect the carbohydrate constituents have been studied. Thus, seasonal effects, depth of immersion and composition of seawater have been considered to affect the alginic acid, laminaran and "fucan" contents of the seaweed.

Carbohydrates metabolised by the Phaeophyta will be discussed with the division used in Chapter I for the other phyta of seaweeds. This is, low molecular weight carbohydrates (soluble in 80% ethanol); water soluble polysaccharides and cell wall material or structural polysaccharides.

3.1.2. Low molecular weight carbohydrates

D-Mannitol appears to be present in all brown algae already studied. In some species and at some seasons in large quantities (about 25% of the dry weight of some Laminaria species in the autumn).[1] Mannitol could not be found in the brown seaweed Dictyopteris plagiogramma (Montague) Vickers being probably the only exception that has been reported in literature.[2] Mannitol is believed to be a storage material and also a substrate for respiration.[3]

D-volemitol, a seven carbon atom sugar alcohol was found in addition in Pelvetia canaliculata[4] also a brown seaweed. Furthermore, 1-O-D-mannitol-β-D-glucanpyranoside and 1,6-O-D-mannitol-di-(β-D-glucopyranoside) have been reported in several other brown algae[5], while β-glucosides of D-volemitol were found in Pelvetia canaliculata. Laminitol (3-O-β-D-glucopyranosyl-D-glucitol) has been found in Laminaria hyperborea, and related species and trace quantities of sucrose, galactose and mannose have been reported in Cladostephus species.[6]

In Desmarestia firma, the presence of a monouronic acid and its lactone, with the same paper chromatographic and electrophoretic mobilities as mannuronic acid, was also observed.[7]

3.1.3. Water soluble polysaccharides

3.1.3.1. Food reserve material

In the Phaeophyta, the food reserve materials are mannitol and a β-(1 → 3)-linked glucan known as laminaran because it was first discovered in Laminaria species. This is a water soluble polysaccharide containing approximately 20-25 glucose units,[8] with a rotation $[\alpha]_D = -12^\circ$ to -14° in water. Laminaran is greatly reduced during the period of rapid growth in the spring, but in autumn

and winter it may comprise up to 35% of the dry weight of the fronds.[9, 10]

Mannitol has been found to be a constituent of the laminaran from some species.[11] In these species there are two types of chain, G-chains which are terminated at the reducing end with glucose and M-chains which are terminated by mannitol.

Normally up to three units per molecule are branched through position 6, but a proportion of branching-points, that is, units linked through positions 1, 3 and 6 may be replaced by units that are linked through positions 1 and 6 only.

The solubility in cold water increases markedly with increasing number of branching points. According to the solubility in cold water laminaran has been divided into two forms, the "soluble" and "insoluble", although both forms are soluble in hot water. The main difference between the two forms appears to be the presence of branch points, the "soluble" seems to contain more glucose units linked through positions 1, 3 and 6 than the "insoluble".[12] In the laminaran where M chains occur they are linked glycosidically through their reducing terminal D-glucopyranose residue to one of the primary hydroxyl groups of D-mannitol and they may be present to the extent of 40%.

Laminarans are fairly low molecular weight polysaccharides, normally in the region of 4000-5000. Evidence for (1 → 6) linked glucose within the chain has been reported, for laminaran of Eisenia bicyclis which has a rotation of $[\alpha] = -46^{\circ}$, contains no mannitol and is unbranched, it consists of a linear sequence of (1 → 3) and (1 → 6) linkages in a ratio of 2:1 and at least some of the (1 → 6) linkages are consecutive[13]. In a survey

of eight brown algae the average content of cellulose was found to be 4.6% of the dry weight.[14] The lowest content, 0.6% was found in Pelvetia canaliculata which is a prostrate and supple species, and the highest content, 10% was found in the rigid, erect stems of Laminaria hyperborea. Cellulose from Laminaria digitata, L.cloustoni (now hyperborea) and Fucus vesiculosus contains residues of D-glucose only; is completely soluble in cuprammonium hydroxide and gives the characteristic blue colour with iodine in aqueous zinc chloride.[15]

3.1.3.2. Other soluble polysaccharides

1) "Fucan"

The sulphated polysaccharides of brown seaweeds have been described under different names such as fucoidin, fucoidan, ascophyllan, sargassan, pelvetian, glucuronosylo fucans and "fucans". In the present thesis in order to avoid confusion the word "fucan" will be used with inverted commas on the understanding that this polysaccharide comprises other sugars as well as fucose. These polysaccharides have all been found to comprise a family of polydisperse, heteromolecules based on fucose, xylose, glucuronic acid and half ester sulphate. Galactose and/or mannose may also be major constituents.[16]

In the early studies extensive purification was carried out in an attempt to isolate a "fucan" containing only fucose residues. This was never achieved completely and even in the so-called purest samples small proportions of galactose, xylose and uronic acid persisted.[17]

A small amount of protein has been found with all "fucans" analysed indicating that they may be proteoglycans. Indications of linkage through serine and threonine have been found for the "fucan" from Ascophyllum nodosum.[18]

Larsen[19] suggests that the name fucoidan be restricted to the polymeric material extractable with acid, but this is misleading since it depends on acid strength and temperature[20], but Quatrano et al. confuse the names fucoidan and fucan even more.[21] According to polysaccharide nomenclature rules the correct name would be D-glucurono-D-galacto-D-manno-D-xylo-L-"fucan".

This polysaccharide is most abundant in brown seaweeds that grow in the inter-tidal zone, although all species of Phaeophyta contain some "fucan". The proportion varies not only seasonally but also with the particular species. Most prominent among those that grow in the inter-tidal zone are the order Fucales easily recognised by the gas-filled bladders that give them buoyancy when submerged.

The "fucan" from Pelvetia canaliculata comprises from 18-24% of the dry weight of the weed whereas in Durvillea potatorum the proportion of "fucan" is very small, 0-2%[22]. The isolation of "fucan" is not restricted to water or acid extracts of the algae. Subsequent extractions with chelating agents like EDTA, alkali at room temperature or at elevated temperatures will remove additional amount of "fucan".

Sulphated polysaccharides have been isolated from many seaweeds; among them, Ascophyllum nodosum,[18, 23] Fucus vesiculosus,[18, 24] F. spiralis and Pelvetia canaliculata[25], Himanthalia lorea, Bifurcaria bifurcata and Padina pavonia[26] Sargassum pallidum[27], S. linifolium[28], Pelvetia wrightii[29, 30], Lessonia flavicans[31], Dictyota dichotoma[32, 33], Desmarestia aculeata[34], D. ligulata and D. firma[2, 7], Dictyopteris plagiogramma[2], etc.

The yield and fucose, uronic acid and sulphate content of extracts of 21 different species (including 3 species of

laminaria) of brown seaweeds has been reported by Fujikawa and Nakashima[35] and further studies of Nemacystus decipiens (order Chordariales) were carried out.[36] The proportions of the sugars comprising the hydrolysates of "fucans" from 11 different species of brown seaweed were reported by Quillet.[37]

Evidence for more than a single molecular species in "fucan" has also been revealed by free-boundary electrophoresis on material extracted from Ascophyllum nodosum and Fucus vesiculosus.[38, 39]

Fractionation on a column of DE52 cellulose of the extracts from Himanthalia lorea, Bifurcaria bifurcata and Padina pavonia[26], Desmarestia aculeata[34], D. ligulata and D. firma[7]; and Dictyopteris plagiogramma[2] by elution with increasing concentration of potassium chloride resulted in every case in the separation with dilute eluant of a fraction with a high proportion of glucuronic acid and a relatively low fucose and sulphate content. As the concentration of the eluting solution was increased, the proportion of uronic acid decreased and the fucose and sulphate increased. Similar heterogeneity of acid extracted "fucans" from Ascophyllum nodosum and Fucus vesiculosus has been demonstrated by fractional precipitation with ethanol containing magnesium or calcium chloride.[18, 40]

Structure

Where structural studies have been carried out on "fucans" extracted from brown seaweeds, the linkages between the individual sugars appear to be the same.

Methylation, periodate oxidation and partial hydrolysis studies have revealed the essential structural similarity of the "fucans" separated and fractionated from Himanthalia lorea and Bifurcaria bifurcata.[41] The high fucose and high sulphate containing material more closely

resembles the structure of the "fucan" from Fucus vesiculosus[42] and that from Ascophyllum nodosum[38, 43, 44] namely (1 ⟶ 2)and(1 ⟶ 3)-linked fucose residues with sulphate at O-4. The glucuronic acid and xylose residues are not sulphated and appear to be on the periphery of highly branched molecules. Partial hydrolysis revealed D-glucuronosyl (1 ⟶ 3)-L-fucose as a structural feature of the macromolecule.[41]

The galactose present in the fucan from Desmarestia aculeata appears to be present as end group and (1 ⟶ 3)-linked units.[34]

The fucans are clearly a family of complex highly branched polysaccharides and different groups of workers have advanced a variety of structures for the macromolecules including a glucuronic acid backbone for the "fucan" from Ascophyllum nodosum[23]. Later work[18] however, on dilute acid extracted "fucan" complexes from this weed indicated a variety of structures for the different fractions. Glucuronosyl-mannose as a repeating unit in a backbone for the polysaccharide from Sargassum linifolium has been advanced[45], but other conclusions of their results are possible and further study should be necessary before the structures can be accepted.

Site of sulphate

Confirmation that C-4 on fucose carries half ester sulphate was achieved by the isolation of fucose 4-sulphate from partial hydrolysates of the "fucan" from Pelvetia wrightii.[46] Partial hydrolysis of "fucan" extracted from Sargassum linifolium[45] gave a number of sulphated fucose and galactose oligosaccharides and residual material consisting of glucuronic acid and mannose. The presence of sulphated mannose, galactose and xylose in the "fucans" from Sargassum pallidum and Pelvetia wrightii have also been reported.[47] However, no other workers have

reported any sulphated residues other than fucose and galactose and in attempts to apply Pavlenko et al's[47] methods to other "fucans", Percival and Rahman[2] revealed only sulphated fucose.

"Fucans" are located in the cell wall and intercellular region of the weed, and evidence from electron-microscope studies indicate that they are laid down in the vicinity of the cell walls. While a certain proportion of the "fucan" of Fucus and Ascophyllum species is extracted by water and dilute acid the residual weed always contains some "fucan", supporting the fact that at least some of the molecules are bound in the cell wall structure. Evidence of this was indicated by ^{14}C studies[48] on Fucus vesiculosus "fucans".

Apart from other functions this polysaccharide almost certainly protects the seaweed from desiccation and high salt concentrations. Lestang and Quillet[49] showed that "fucan" extracted from Pelvetia canaliculata had a very strong affinity for magnesium so that when the fronds are in contact with seawater the ester sulphate groups are largely associated with magnesium ions. They point out that magnesium ions are highly hydrated and will therefore retain water in fronds. In support of this conclusion is the fact that Pelvetia canaliculata which grows on the higher parts of the shores and is therefore frequently exposed, has a high "fucan" content (18-27% of the dry weight of the weed), whereas Desmarestia and Durvillea species which grow below tide mark contain very little or no "fucan".[7]

"Fucan" has been suggested as source of the rare sugar L-fucose[50] and it has also been tested as a blood anticoagulant.[51, 51a]

2) Alginic acid

Alginic acid, a polyuronide found in brown seaweeds was first obtained nearly a century ago by E C C Stanford, a chemist who was concerned with the better utilisation of seaweed than burning it for the recovery of iodine. He made a number of suggestions[52] for the commercial use of the product but only since 1930 industry has developed the production and uses of alginic acid. The principal producing countries are the United States, Britain, France, Norway, Japan and very recently, China.

Alginic acid has been found in all species of Phaeophyta that have been examined, but it is not present in any other plant tissues.

The proportion of alginate shows wide variations from one species of alga to another and in some species changes with the seasons and location of growth[12]. In general the alginic acid forms a smaller proportion in the periods of rapid growth than in the colder months when little growth is taking place.[1]

An indication of the variation is the nearly 50% of dry weight found in some samples of Durvillea antarctica[53] and 15% in Padina pavonia.[26]

The alginate cannot be isolated from seaweed without some chemical changes. Methods used have tried to minimize the breakdown of the alginic acid molecule and the reactions used only change the cations associated with it. Solubilisation of alginate requires exchange of the Ca^{+2} and/or Sr^{+2} ions for an alkali metal, the product thus obtained always contains traces of sulphated polysaccharide and protein, but can be purified by precipitation as the calcium salt from which the acid is recovered by leaching out the calcium.

The fact that the alginate can be extracted by such relatively mild conditions indicates that it is not covalently linked to insoluble cell wall constituents. However some degradation of the polymer takes place during extraction.

Alginic acid is a linear polymer (1 ⟶ 4)-linked β-D-mannuronic acid and α-L-guluronic acid. See Figure 3.1.

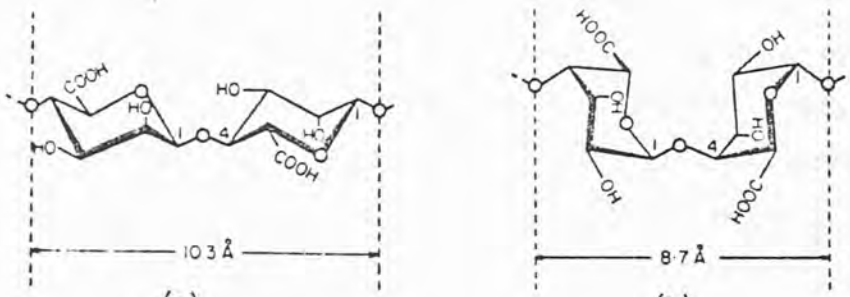


Fig. 3.1. Parts of Alginic Acid Molecule
(a) Mannuronic acid residues; (b) Guluronic acid residues

The proportion of these two acids varies widely in the alginates in different algal species.[12] Tables of M/G ratios (Man Ac/Gul Ac) have been published for alginates from many different species. The values range from 0.4 for alginate from Laminaria hyperborea stems to 2.4 for alginate from new fronds of Laminaria digitata. Periodate and bromine oxidation of alginic acid followed by hydrolysis yielded both threaric and erythraric acid showing that both uronic acids are (1 ⟶ 4) linked[54].

Several methods have been used to determine the sequence of the units in the molecule. Partial hydrolysis with dilute acid[55, 56] was carried out and it was found that part of the alginate was solubilised and that the insoluble residue could be fractionated into a mannuronic rich fraction and a guluronic rich fraction. The soluble

fraction contained nearly equal amounts of the two uronides (MG-blocks) and was thought at first to be built up of alternating units, but later kinetic study[57] of acid hydrolyses determined that MG-blocks are arranged in a more random fashion and further studies confirmed this.

Enzymic studies using lyases[58,59] found that MG-blocks preparations have in them both mannuronic followed by mannuronic residues (MM sequences) and guluronic followed by guluronic (GG sequences) as well as linkages between the two different units.

In a study of methods of determining the proportions of M and G residues in an alginate it was found[60] that using proton magnetic resonance (p.m.r.) it was possible to distinguish the signals from H-1 and H-5 in the guluronic residues and from H-1 in mannuronic acid when the homopolymeric blocks obtained by partial hydrolysis of the alginate were examined. By the use of high resolution[61] p.m.r. it was possible to distinguish signals of H-5 of guluronic residues with a mannuronic neighbour (GM sequence) from those with a guluronic neighbour (GG sequence). In conjunction with the assignments for H-1 it was possible to obtain the proportion of each of the following sequences: MM; GG; MG and GM. ^{13}C nuclear magnetic resonance (n.m.r.) to show these sequences and the triplet sequences with M units in the middle has also been used[62] as well as ^{13}C n.m.r. signals for any two corresponding ring carbon atoms.[63,64] These methods required slight depolymerization with dilute acid to diminish viscosity, but are essentially non-destructive. A method based upon circular dichroism (C.D.) spectroscopy[65] requires only milligrams of alginate and depolymerization is unnecessary, but calibration and correction for an influence of monomeric sequence are needed.

A polarimetric method for determining the M/G ratios has also been developed[66]. Following the characterisation of brucine-L-guluronate[67] it was shown that the difference in $[\alpha]_D$ values of the brucine salts of D-mannuronic and L-guluronic acid is sufficient to provide the basis of a polarimetric method for determining the composition of a mixture. However the presence of a drextrorotary contaminant would tend to lower the mannuronic acid content and increase the guluronic acid content. Therefore it is necessary that the samples of alginates for analysis be free as far as possible from such contaminant.

3.1.4. Structural polysaccharides

3.1.4.1. Cellulose

A small proportion of cellulose seems to be a general structural polysaccharide in the brown algae. In most cases the X-ray diagram of Cellulose I (the native cellulose of terrestial plants) is shown after the microfibrillar material has been freed from encrusting materials of the cell wall.[68] Evidence for β-(1 ⟶ 4)-linked glucose units has been obtained by the isolation of cellobiose and periodate studies excluded the presence of (1 ⟶ 3)- and (1 ⟶ 6)-linkages.[69]

3.2 Investigation of the carbohydrates of the brown seaweed Lessonia nigrescens.

Lessonia nigrescens is a member of the brown algae Phaeophyta and belongs to the order Laminariales, family Lessoniaceae.

A study of Lessonia in Southern Chile and Argentina (South America) indicates that this genus is represented there by three species Lessonia nigrescens Bory, L. flavicans Bory and Lessonia vadosa Searles[70].

Plants of these three species have been confused

with each other in the literature, but appear clearly separable. Lessonia nigrescens is distinguished from the others by its massive, solid holdfast and numerous stems bearing long, narrow fronds. In Lessonia flavicans and L. vadosa a single stem arises from a branched holdfast but the latter has more narrow fronds and lacunate cortex.

The three species are distinguishable from each other in details of fronds morphology, anatomy and ecology.

The genus was named by J. B. Bory de Saint-Vincent (1825) when he described the single species Lessonia flavicans from the Islas Malvinas (Argentina). He provided later (1826) a more complete generic description and added two species to the genus, Lessonia nigrescens from Cape Horn (Southern Chile) and a southern Australian species which was later transferred to the genus Myriodesma as M. quercifolia (Bory).

Lessonia nigrescens grows specially in southern Chile and Argentina, although many samples have been collected from the coast of northern Chile, in particular in Bahia Herradura, Coquimbo. This alga grows normally on exposed shores, although it may occur from low water to a maximum depth of 38 m. It can reach up to 4 m. tall, up to 20 terete, dichotomously branched stems arising from a conical to hemispherical mound of tightly fused haptera. Stems to 4 cm diameter, terminating in linear 1.0 - 3.5 cm. broad fronds. Frond margins entire or minutely denticulate. Cortex solid parenchymatous.[70] (See Figures 3.2 and 3.3, p. 66 and p. 67 respectively) Lessonia flavicans can reach 4 m. tall also, but it has only a single dichotomously to subdichotomously branched stem arising from the holdfast; haptera of holdfast is

highly branched, occasionally forming an adventitious secondary stem. Base of stem to 5 cm diameter, branches bearing fronds 3-41 cm. broad and 17-100 cm. long, margins of fronds entire to coarsely toothed Cortex of fronds and stems a solid, parenchymatous tissue, which it makes it quite different from Lessonia nigrescens.[70] (See Figures 3.4 and 3.5, p. 68 and p.69)

The only previous chemical studies on the carbohydrate of this genus Lessonia have been on Lessonia flavicans, water soluble polysaccharide[31] and on Lessonia nigrescens where the presence of alginic acid[71] and of several aminoacids has been reported[72, 73]

The present sample of Lessonia nigrescens was harvested in Chile, South America, and supplied to us by Alginate Industries Ltd. as dried material.

Figure 3.2 Lessonia nigrescens, brown seaweed

Lessonia Nigrescens

Figure 3.3 Fronds of Lessonia nigrescens

Figure 3.4 Lessonia flavicans, brown seaweed

Figure 3.5 Fronds of Lessonia flavicans

3.3. EXPERIMENTAL

3.3.1. Sequential extraction

As stated in the Introduction, Lessonia nigrescens is distinguished from other species by its massive and solid stems. As a result,the only successful way to grind the stems was by means of a small manual grinding mill. Fronds were ground without difficulty to a fine powder with liquid air in a mortar.

Dry fronds and stems (it being understood that a proportion of hold fasts is included with the stems) were investigated separately and the extraction procedure illustrated in flow charts I and II was followed. See p. 86 and 88.

Experiment 3.1. Ethanolic extraction.

The seaweed ground to a fine powder (30 g. each, fronds and stems) was extracted three times with 80% ethanol at room temperature and another three times with boiling ethanol. After each extraction the residue was centrifuged off. After the third boiling ethanolic extraction the extract was virtually devoid of carbohydrate. The concentrated light green ethanolic solutions, after filtration to separate light green precipitates[(1) and(3) from the cold and hot ethanol extracts respectively], were partitioned between toluene and water with about 5% n-butanol to break the emulsion. This procedure gave colourless ethanol-water layers and dark green toluene layers (due to chlorophyll). The latter layers were discarded and the aqueous-ethanol solution poured into ethanol. White substances, [(2) and (4)] precipitated from the cold and hot extracts respectively. See the procedure outlined in flow chart I where full details of the different products are given.

Experiment 3.2. Formaldehyde treatment

The residual dry weed (2) (24.9 g. of stems and 23.8 g. of fronds) was immersed in a 40% formaldehyde solution and left overnight. Then formaldehyde was discarded and the seaweed spread over sheets of filter paper to be air dried.

Experiment 3.3. Aqueous extraction

After air drying, the residual weed was extracted with aqueous calcium chloride (2%) solution. Once at room temperature and then at 70°C as is shown in flow chart II, p.88. After each extraction the solid was centrifuged off, the extracts taken down to small volume, dialysed until chloride free (tested with silver nitrate solution) and then freeze dried to a white powder (white powders (3) and (4)). The weight of each product is shown in Table 3.1, p.91

Experiment 3.4. Acid extraction

The residual weed (4) (see flow chart II, p.88 was extracted with dilute hydrochloric acid (pH=2). first at room temperature and then at 70°C. After each extraction the mixture was centrifuged off and the extracts dialysed against deionised water until no chloride was present. The extracts were then freeze-dried to white powders (5) and (6). Weights are shown in Table 3.1 p.91

Experiment 3.5. Alkali extraction

Residual weed (6) (see flow chart II, p. 88 continued), 17.23 g. of stems and 16.76 g. fronds, was extracted three times with 3% Na_2CO_3 at 50°C for 6 hours each time.

The third extract was tested for carbohydrate [GM 2.7.1].

In both fronds and stems there was negligible carbohydrate [ca. 3% on weight]. This indicates as unnecessary a fourth extraction. The combined extracts separately for fronds and stems, were poured into ethanol

(4 volumes) with constant stirring and a white gelatinous precipitate was produced and this was filtered through muslin. The precipitate was washed twice with ethanol and centrifuged. Washings and filtrate were combined, concentrated and poured again into ethanol following the same procedure as above. Finally the combined filtrates were concentrated to small volume and estimated for carbohydrate.

The precipitates were combined as well, redissolved in water, dialysed and freeze-dried. A white powder [(7) in flow chart II, p. 89] was obtained. This product was dissolved in water up to a 1% solution and then a 2% aqueous calcium chloride solution was added slowly with stirring until precipitation of calcium alginate was complete. This was centrifuged, dispersed in water and freeze-dried. A white powder (9) of Ca alginate was obtained. The supernatant was dialysed till chloride free and freeze-dried. A white powder (8) was obtained which for simplicity will hereinafter be called "fucan".

3.3.2. Investigations of the different extracts.

Ethanolic extract

Experiment 3.6.

The combined precipitates (1), (2), (3) and (4) (ca. 232 mg and ca. 369 mg from fronds and stems respectively, see flow chart I, p. 86) were redissolved in water and treated with Biodeminrolit, (carbonate form), filtered and the solution taken to a small volume. Mannitol was precipitated by six sequential additions of ethanol. Crystalline needles were removed by filtration after each addition, and combined and recrystallised from ethanol; dried and weighed. Melting point and mixed melting point were measured. Crystals were examined under the microscope and compared with a standard sample.

Experiment 3.7.

The combined extracts (1) and (2) from fronds and stems (see flow chart I) were analysed separately by paper chromatography. Spots with $R_{Glc} = 0.89$ in solvent [GM 2.4.1.1.] and $R_{Glc} = 0.37$; $R_{Glc} = 077$, $R_{Glc} = 0.99$ in solvent [GM 2.4.1.2.] were obtained from both fronds and stems.

Experiment 3.8

All the above extracts were combined and concentrated to a single syrup (200 mg) and separated into 4 different fractions on 3 mm paper chromatogram with the following R_{Glc}=0.42; 0.49; 0.64 and 1.0 mobilities in solvent [GM 2.4.1.2.].

Experiment 3.9

The substances with R_{Glc}=0.49 and 0.64 in the above fractionation were hydrolysed [GM 2.3.2] and the hydrolysates analysed by paper chromatography in solvents [GM 2.4.1.1. and 2.4.1.2.], sprays (GM2.5.1 and 2.5.2] and by ionophoresis [GM 2.4.2.2.].

Aqueous extract

Experiment 3.10

The different aqueous extracts were estimated for carbohydrate content [GM 2.7.1.] uronic acid content [GM 2.7.2.1 and 2.7.2.2. (1), (2) and (3)] sulphate content [GM 2.7.4], nitrogen and protein content [GM 2.7.3.]. Results are shown in Table 3.2. p. 92

Experiment 3.11

Separate aliquots (50 mg each) of the white powders (3) and (4) (see flow chart II, p. 88)separated from the cold and hot aqueous extracts, from both fronds and stems were hydrolysed separately [GM 2.3.1]. The hydrolysates were examined on paper chromatograms in solvents [GM 2.4.1.1. and 2.4.1.2.] and sprays [GM 2.5.1; 2.5.2; 2.5.3 and 2.5.4.].

Experiment 3.12

The hydrolysates of both fronds and stems of the hot aqueous extracts (solids (4), flow chart II, p.88) were separated on 3MM paper chromatogram, solvent [GM 2.4.1.1.] into neutral and acid materials. The neutral fractions were reduced [GM 2.8.4.] converted into the acetate derivatives [GM 2.8.5.] and analysed on g.l.c. [GM 2.6.1. and 2.6.2.]. Results are shown in Table 3.4 p. 94

Experiment 3.13

Hot aqueous extract, stems (solid (4), flow chart II, p.88) was dissolved in water (2 mg/ml) and layered on to a Sepharose 4B column (16.5 x 1.3 cm), eluted with MKCl [GM 2.8.11.2]. The fractions were estimated for carbohydrate content and the maximum absorbance gave the approximate molecular weight of the polysaccharide in comparison with standard dextrans of different molecular weights run separately on the same column and under the same conditions. See figure 3.6 p. 97

Acid Extract

Experiment 3.14

The different extracts were analysed for carbohydrate, uronic acid, sulphate and protein contents as outlined in Experiment 3.10. Results are shown in Table 3.2. p.92

Experiment 3.15.

The acid extracts from both fronds and stems were investigated through the same procedure outlined in Experiment 3.11.

Experiment 3.16

The hydrolysates of both fronds and stems of the hot acid extracts (solids (6), see flow chart II, p. 88) were separated on 3MM paper chromatogram, solvent [GM 2.4.1.1.], into neutral and acid materials. The

total acid material was estimated by phenol sulphuric acid method [GM 2.7.1] as glucuronic acid. The combined neutral sugars were reseparated on 3MM paper with solvent [GM 2.4.1.2.] and the appropriate areas corresponding to the individual sugars eluted with water from the paper strips, filtered through millipore filter (0.45 μ) and analysed for carbohydrate content [GM 2.7.1.]. Relative proportions of the monosaccharide constituents and the uronic acid content were obtained from standard graphs. Results can be seen in Table 3.3, p.93.

Experiment 3.17

An aliquot of the hydrolysates of extract (6) for both fronds and stems were separated into acid and neutral fractions as outlined in Experiment 3.16. The combined neutral sugars were investigated as in Experiment 3.12. Results are shown in Table 3.4. p.94

Alkali Extract

Experiment 3.18

A portion of the "fucan", separately for fronds and stems, was dissolved in water to estimate carbohydrate content [GM 2 7.1.] and another portion was analysed for constituents sugars as in Experiment 3.11.

Experiment 3.19

A sample of the "fucan" was hydrolysed [GM 2.3.1.] and the hydrolysate was treated as in Experiment 3.12 above. Relative molar proportion of constituent monosaccharides sugars are shown in Table 3.5a, p. 99. Total recovery from fronds and stems, after sequential extraction can be seen in Table 3.6., p. 100.

3.3.3. Direct extraction of alginic acid (see flow chart III. p.101

Experiment 3.20

A portion of the dry alga (5 g. fronds and stems separately) ground to a fine powder was soaked in a 1.8% solution of formalin and the mixture stirred for some minutes and then left for half an hour. The supernatant was decanted off and water (150 ml) was added and the pH of the mixture was found to be 6.5.

To this mixture solid Na_2CO_3 (1.5 g) and a 10% NaOH solution (1.5 ml) were added and the mixture was kept at 60°C for 2 hours under stirring. The residue (1) was filtered off and the solution neutralised (pH = 6.5 - 7.0) with dilute hydrochloric acid and diluted to 800 ml.

Calcium chloride solution (100 ml) was added under stirring so that the final concentration of calcium chloride in the solution became 2%. The gelatinous calcium alginate precipitated was centrifuged off and then washed with dilute $CaCl_2$ solution. Then suspended in water and freeze-dried to a white solid of Ca alginate A (1.83 g stems and 1.27 g fronds, (see flow chart III, p.101). The supernatant was dialysed for three days and then freeze-dried, "fucan" A was obtained (0.14 g stems and 0.20 g fronds).

The residue (1) was extracted three times with a 3% Na_2CO_3 solution (150 ml) at 70°C for 3 hours each time. The combined solutions were neutralised (pH = 6.55) with dilute HCl and diluted to 500 ml. This solution was treated as previously described and gave a white solid of calcium alginate B , (0.22 g for stems and 0.44 g fronds) as well as a white solid, "fucan" B (0.20 g stems and 0.27 g fronds).

3.3.4. Examination of the calcium alginate from the alkaline extract

Experiment 3.21

Conversion of calcium alginate to sodium alginate

The combined calcium alginates A and B (Exp. 3.20) 2.05 g stems and 1.71 g of fronds, were suspended in

0.5 M HCl (100 ml) solution in a sintered funnel, the base of which was filled with water. The suspension was occasionally stirred and after 3 hours the hydrochloric acid was filtered off. The filtrate was tested for calcium ions with an oxalate solution and the process repeated twice. No calcium was found in the last filtrate and the solid was washed with water till the filtrate was nearly neutral using tropolein as indicator. The solid was then suspended in water under vigorous stirring and titrated with 0.1 M NaOH solution until pH = 7 was reached by which time all the alginic acid had dissolved. The solution was dialysed for two days and freeze-dried to a white solid (1.826 g stems and 1.448 g fronds).

Experiment 3.22

Portions of dry alga (93.4 g fronds; 4.88 g stems) each ground to a powder, were separately extracted as in experiment 3.20 and the corresponding calcium alginates were converted into soluble sodium alginates as in experiment 3.21. Yields can be seen in Table 3.8 p.107.

Experiment 3.23

The viscosity of 1% aqueous sodium alginate solutions (25 ml) of the different extracts (experiments 3.21 and 3.22) were measured at 25°C in an Oswald capillary viscometer. Results in Table 3.9 p.109.

Experiment 3.24

Portions of sodium alginate A (ca. 55 mg) from fronds and stems, see flow chart III, p.101, were separately hydrolysed [GM 2.3.1.] and the hydrolysates analysed on paper chromatography, solvents [GM 2.4.1.1. and 2.4.1.2.] locating reagents [GM 2.5.1 and 2.5.2.] and by ionophoresis [GM 2.4.2.1.]

Experiment 3.25

A sample of Na Alginate (31.5 mg) extracted from stems, having viscosity 267.6 cps (see Table 3.9), was shaken twice with deuterium oxide and freeze dried. Then analysed by ^{1}H n.m.r. at 296°K, referenced to external T.M.S. Another freeze dried sample of the same Na Alginate (60.7 mg) was dissolved in deuterium oxide and analysed through ^{13}C n.m.r. method at 353°K, referenced to external T.M.S. at 305°K. Both methods were carried out on a 400 MHz apparatus.

3.3.5. Investigation of "fucan" extracted by direct alkali method.

Experiment 3.26

Samples of "fucan" A and B (see flow chart III, p.101), Na alginates A and B and mixture of "fucan" and alginate were analysed through TGFP electrophoresis method [GM 2.4.3.] and their behaviour compared with "fucans" previously investigated in these laboratories and with a standard sample of soluble starch.

Experiment 3.27.

"Fucan" A and B combined, separately for fronds and stems, were hydrolysed [GM 2.3.1.] and analysed for sugars by paper chromatography using the usual solvents and locating reagents. The hydrolysate of stems was separated by preparative paper chromatography into neutral sugars and uronic acid.

The latter was esterified [GM 2.8.3.1.], reduced [GM 2.8.4.] and hydrolysed [GM 2.3.1.] and tested for glucose by paper chromatography, solvent [GM 2.4.1.1.] and glucose oxidase reagent [GM 2.5.3.].

Experiment 3.28

Samples of "fucan" from fronds extracted by sequential alkaline method and direct alkaline method

were hydrolysed [GM 2.3.1.] The hydrolysates converted into the corresponding alditol acetates [GM 2.8.4 and 2.8.5] and then analysed by g.l.c. [GM 2.6.1 and 2.6.2.] and by g.l.c. - m.s. [GM 2.6.3.]. Relative molar proportion of sugars are shown in Table 3.5a, p. 99.

Experiment 3.29

A solution of 4 mg/ml of fucan A (flow chart III, p.101 from fronds was layered on top of a Sepharose 4B column [16.5 x 1.3 cm] and eluted as outlined in Experiment 3.13. to estimate its molecular weight. The same sample was analysed for protein content [GM 2.8.1.4.] 1 ml of the fractions collected was analysed for carbohydrate contents and another 1 ml for protein assay. See Figure 3.8 p. 106.

Experiment 3.30

Aliquots of "fucans" A and B, were analysed for sulphate content [GM 2.7.4.], for nitrogen and protein content [GM 2.7.3.] and carbohydrate content [GM 2.7.1.]. See results on Table 3.7, p. 100.
A portion of "fucan" B, 40.6 mg of fronds (see flow chart III, p.101) was hydrolysed with 12 NHCl and the hydrolysate analysed by paper chromatography for amino acids, with solvent [GM 2.4.1.2.] and staining reagent [GM 2.5.5.].

Experiment 3.31

A sample of "fucan", 50.2 mg, from fronds, was dried in a vacuum pistol drier at 45°C. The dried sample was esterified under anhydrous conditions [GM 2.8.3.1.], reduced with $NaBD_4$ [GM 2.8.4.], hydrolysed [GM 2.3.1.], reduced again, now with $NaBH_4$ and the reduced sample converted into the alditol acetate derivative [GM 2.8.5] and analysed on g.l.c. [GM 2.6.1.] and g.l.c. - m.s. [GM 2.6.2. and 2.6.3.].

Experiment 3.32

Seaweed residue [fronds and stems], 1 g. after sequential extraction; 1 g of fronds, residual weed from direct alkaline extraction and 0.4 g each of fronds and stems from direct alkaline extraction [this means after Experiment 3.5; Experiment 3.22 and Experiment 3.20 respectively] were hydrolysed with 90% HCOOH [GM 2.3.1.] and with 72% H_2SO_4 [GM 2.3.3.]. The hydrolysates were analysed by paper chromatography for sugars, solvents [GM 2.4.1.1. and 2.4.1.2.] and locating reagents [GM 2.5.1. and 2.5.2.].

3.3.6. Direct hot aqueous extraction of "fucan" (see flow chart IV)

Experiment 3.33

Stems and fronds were ground to powder and mixed approximately in equal proportions. The combined powder 771.0 g. was treated with a 25% solution of formaldehyde, stirred for 30 minutes and then left overnight. The formaldehyde layer was discarded and the residue spread over sheets of filter paper in a fume cupboard, with a current of cold air to eliminate the maximum amount of formaldehyde.

The residual weed was dried in air and then extracted 4 times with 2% $CaCl_2$ solution (2 l) at 70°C, 5 hours each time. After each extraction the mixture was centrifuged and the extracts evaporated down to a smaller volume [GM 2.1.2.], dialysed to get rid of inorganic contaminants and low molecular weight carbohydrates and then combined to be finally freeze-dried and weighed. A white solid, "fucan" (20.32 g.) was obtained. See flow chart IV, p. 114.

3.3.7 Investigation of this "fucan", hot aqueous extract.

Experiment 3.34

A portion of the "fucan" (10 mg) was analysed

for carbohydrate [GM 2.7.1.], uronic acid [GM 2.7.2.2. 2) and 3)] sulphate [GM 2.7.4.] nitrogen and protein [GM 2.7.3] ash and moisture content. See results in Table 3.10 p. 113.

Experiment 3.35

A sample of the "fucan" was hydrolysed [GM 2.3.1] and the hydrolysate examined for sugars on a paper chromatograph [GM 2.4.1.], solvent [GM 2.4.1.1., 2.4.2.2,. and 2.4.2.3.] and sprays [GM 2.5.1., 2.5.2., 2.5.3. and 2.5.4.]. A similar sample was hydrolysed with 12 N HCl and the hydrolysate examined for amino acids on a paper chromatograph, solvents [GM 2.4.1.1. and 2.4.2.2.] and spray [GM 2.5.5.].

Experiment 3.36

Desulphation by a) acidic method [GM 2.8.9.] and by b) alkaline method [GM 2.8.8.] of portions of the "fucan" was attempted. The sulphate content of the products was determined [GM 2.7.4] and compared with that of the original "fucan". Hydrolysates of the derived polysaccharides were examined by paper chromatography.

Experiment 3.37

A sample of "fucan" extracted from Himanthalia lorea, also a brown seaweed previously investigated in these laboratories by G. Carlberg and A. Rahman was subjected to desulphation under the alkaline method [GM 2.8.8.]. The results were compared with "fucan" from Lessonia nigrescens. See Table 3.16 p. 127

Experiment 3.38 Fractionation of the "fucan" on DE 52 cellulose column.

500 mg of the "fucan" were dissolved in 10 ml of water and layered on to a DE 52 cellulose column [GM 2.8.10]. The column was eluted with ca. 1 litre of

each of the following solutions: water: 0.2 M KCl; 0.5 M KCl and M KCl. The recovery of the different fractions is tabulated in Table 3.12, p. 117.

Experiment 3.39.

The carbohydrate [GM 2.7.1.], the sulphate [GM 2.7.4] and the uronic acid contents [GM 2.7.2.2., 2) and 3)] were determined for each of the fractions. Specific rotation [GM 2.1.4] of these fractions was also determined. See Table3.11, p. 116.

After hydrolysis [GM 2.3.1.], the monosaccharide constituents of the hydrolysates of the different fractions were characterised by paper chromatography and by g.l.c. of the corresponding alditol acetates. Results are shown in Table 3.12, p.117 in comparison with other "fucans" extracted from brown seaweeds.

Experiment 3.40

A portion of 0.2 M KCl and 0.5M KCl (2 mg/ml) fractions were separately layered on to a Sepharose 4B column [16.5 cm x 1.3 cm], eluted with M KCl [GM 2.8.11.2] and molecular weights estimated as in Experiment 3.13. See Figure 3.10 p.120.

Experiment 3.41

150 mg of "fucan" were hydrolysed [GM 2.3.1.] and the hydrolysate separated on 3 MM paper chromatogram, solvent [GM 2.4.1.1.] into neutral and acidic components. The neutral sugars were then separated in the same way with solvent [GM 2.4.1.2.] and both fractions, neutral sugars and uronic acids, separately investigated.

Experiment 3.42

Separated monosaccharides sugars from the "fucan" were freeze dried and weighed and the molar proportion determined; purity of these sugars was analysed on paper chromatography with solvents [GM 2.4.1.1. and 2.4.1.2.] and locating reagents [GM 2.5.1. and 2.5.2.] A portion

of these sugars was converted to the alditol acetate [GM 2.8.4. and 2.8.5.] and separately analysed on g.l.c. [GM 2.6.1. and 2.6.2.]

Experiment 3.43. Separation attempt of glucose from galactose.

A sample of the mixture of glucose and galactose separated from the "fucan" hydrolysate, was spotted on a sheet of 3 MM paper chromatogram. 5 volumes of glucose oxidase reagent [Worthington Biochemical Company] were spotted over 1 volume of the mixture. The paper then run for 18 hours in solvent [GM 2.4.1.1.] Glucose was converted into gluconic acid and hence remains near the starting line in the paper chromatogram. The mobility of galactose was unchanged. It was eluted from the paper with water, freeze-dried and weighed and then converted into the alditol acetate [GM 2.8.4. and 2.8.5.] and analysed on g.l.c. [GM 2.6.1. and 2.6.2.] to check its purity.

Experiment 3.44

A solution of 2 mg/ml in M KCl of "fucan" was layered on to a Sepharose 4B column and its molecular weight estimated as stated in Experiment 3.13. See Figure 3.11. p.123.

Experiment 3.45 Detection of cell-wall in the "fucan".

A certain amount of "fucan" was dissolved in water (25 mg/2 ml) and filtered through a glass fibre millipore filter. The filtrate was evaporated down to dryness [GM 2.1.2.] and then hydrolysed [GM 2.3.1.]. The residue remaining on the glass fibre filter was hydrolysed in the same way. Both hydrolysates were analysed on paper chromatography, solvent [GM 2.4.1.1.] and locating reagent [GM 2.5.3.] for glucose detection. Carbohydrate content was also estimated for both hydrolysates. [GM 2.7.1.].

Experiment 3.46

A portion of the fucan was hydrolysed [GM 2.3.1.] and the hydrolysate converted to the alditol acetate [GM 2.8.4. and 2.8.5.] analysed then by g.l.c. [GM 2.6.1. and 2.6.2.]. The proportion of sugars was determined and results are shown on Table 3.15 p.122.

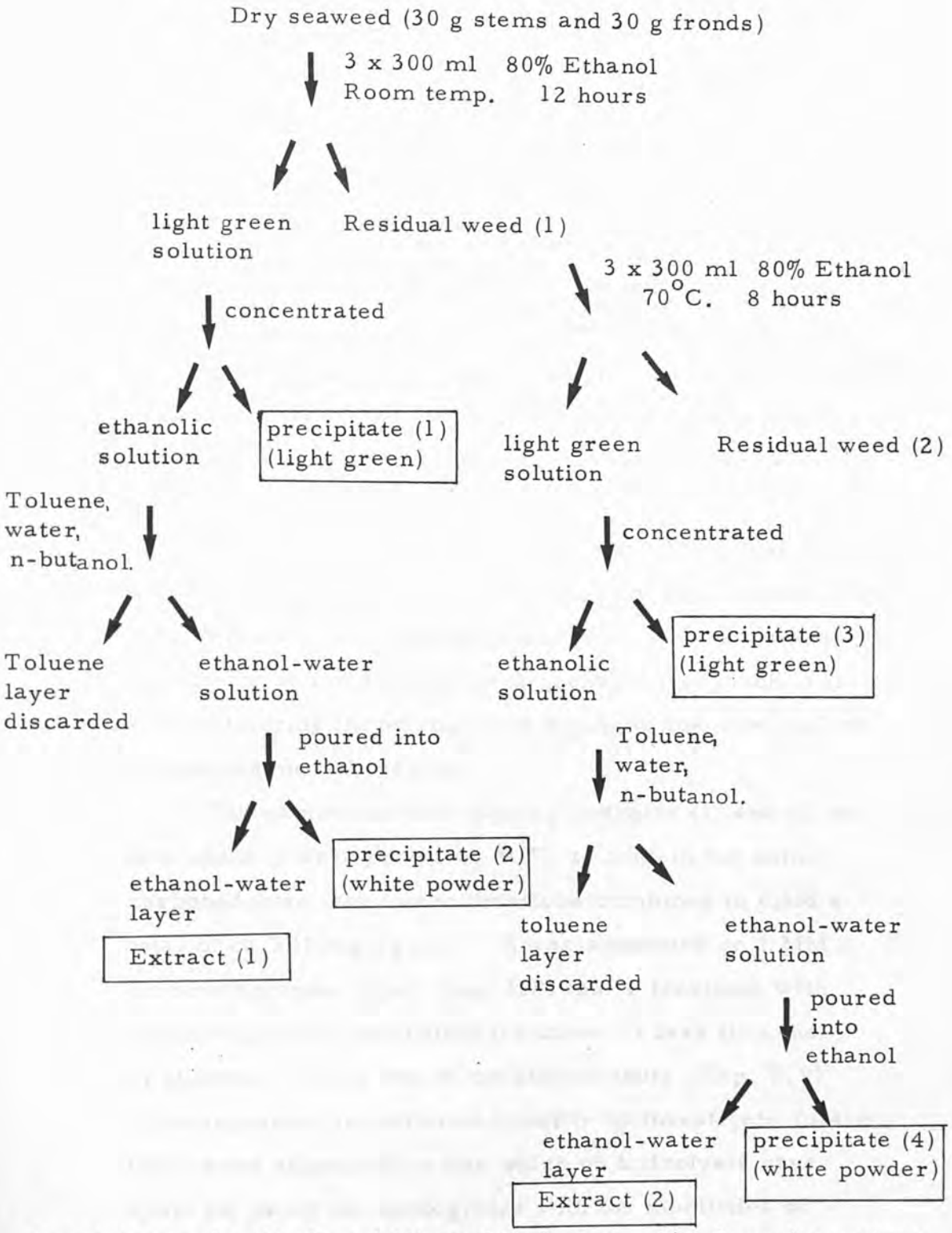
3.4. RESULTS AND DISCUSSION

The majority of the chemical investigations carried out on the carbohydrates of seaweeds belonging to the Phaeophyta have been on extracts of the whole seaweed. In only a few instances has the alginate extracted from different parts of the weed been investigated. As an example, alginate was extracted and analysed[1] from different parts of Ascophyllum nodosum, Laminaria digitata and L. hyperborea plants. It was found that the percentage of mannuronic acid varied always being higher in the new frond tissue. In a more recent study[2] a progressive increase in the proportion of guluronate residues is observed through the series; frond, stem, unattached haptera, attached haptera. While workers have used different extractants for the "fucans", apart from exudates, such as that from Macrocystis pyrifera, and studies of zygotes of species of Fucus[3], they have always been on extracts from whole plants.

After a preliminary hydrolyses of the dried weed (fronds and stems) of Lessonia nigrescens and the identification by paper chromatography (P.C.) of fucose, galactose, mannose, xylose and mannitol in the hydrolysate, it was decided to extract the fronds and stems separately and to investigate the respective extracts. If differences occurred in the extracts from the two parts of the plant it was hoped that it would be possible to deduce something of the function, particularly of the "fucans", that the different polysaccharides play in the life of the plant.

Each of the parts (30 g. of each) was subjected to sequential extraction with ethanol, cold and hot water, acid and finally alkali (flow charts I and II).

Flow chart I (Ethanolic extraction)



Precipitates (1), (2), (3) and (4) combined gave: 369.3 mg from stems
231.7 mg from fronds

Pure mannitol (after re-crystallisation): 139.4 mg from stems (0.46%)
80.6 mg from fronds (0.27%)

Extracts (1) and (2), stems and fronds combined, gave a syrup of ca. 200 mg.

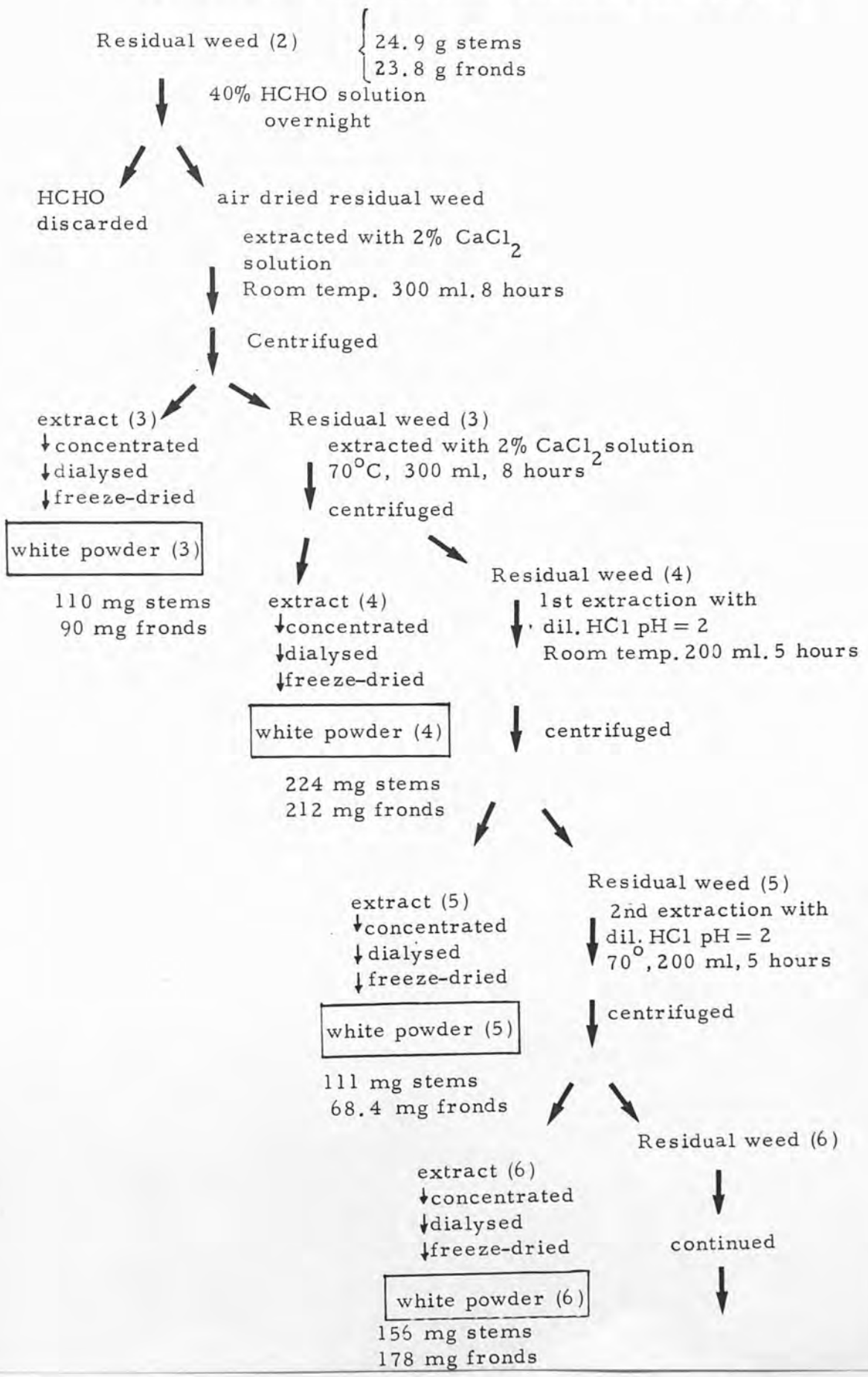
3.4.1. Ethanolic extracts

From the ethanolic extracts on concentration, white solids were precipitated, 369.3 mg from stems and 231.7 mg from fronds. Microscopic examination indicated the presence of inorganic salts. Dissolution in water and shaking with Biodeminrolit (Exp.3.6.) removed these and from the solution, mannitol was removed and recrystallised from ethanol, 139.4 mg from stems and 80.6 mg from fronds (a total of 0.37% on the weight of dry seaweed). It had melting point and mixed melting point with an authentic sample of mannitol of 165°C. It had the correct mobility on P.C. and on gas liquid chromatography as the alditol acetate and the same crystalline form as mannitol.

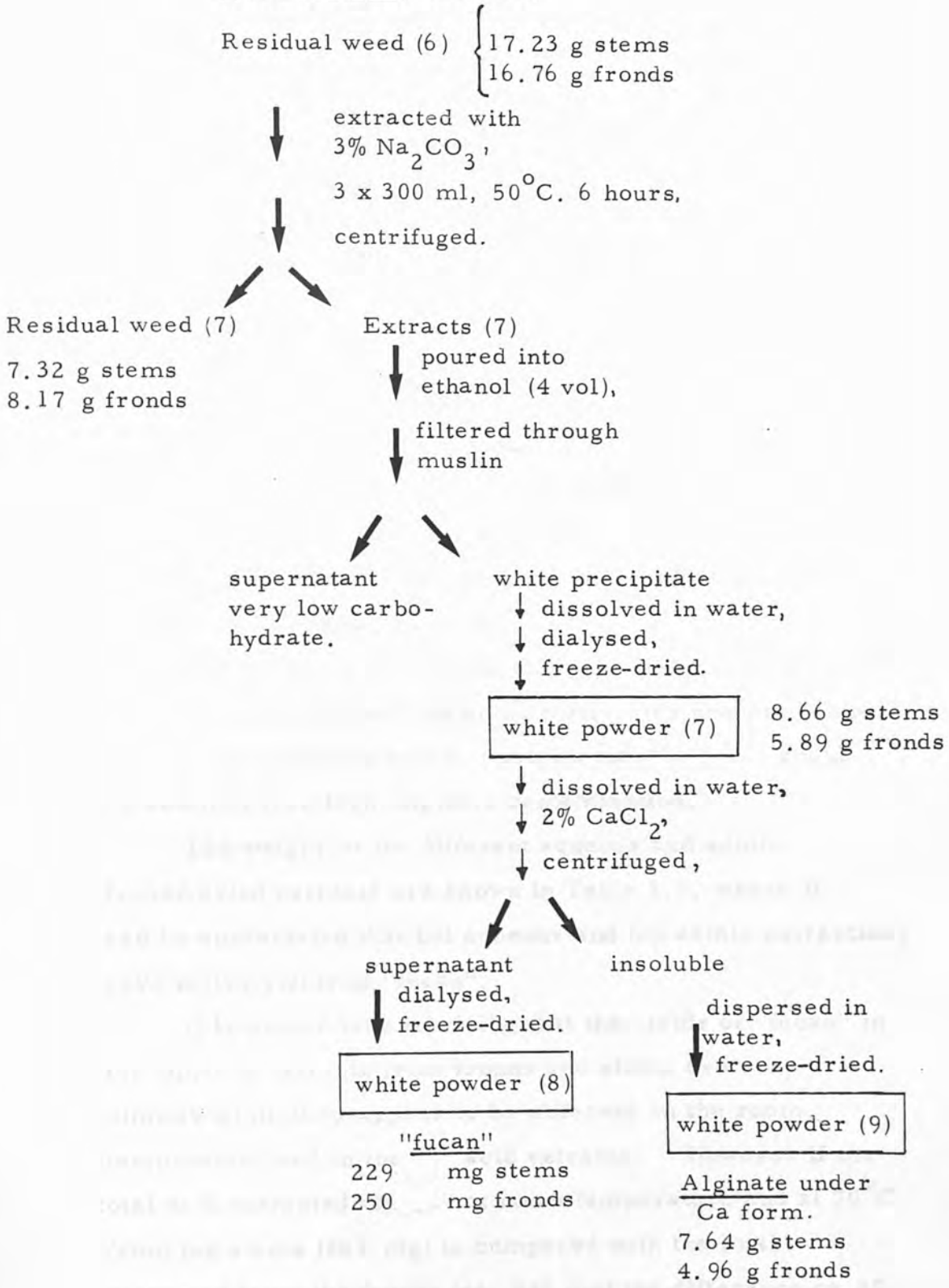
Mannitol is a common constituent of the Phaeophyta and yields of 3 - 25% of the dry weight of the seaweed have been reported.[4] In the present instance the recovery appears to be low but it is very probable that much of it was lost during the washing and drying of the weed before it reached our laboratory.

The various mother liquors (extracts (1) and (2) on flow chart I) were shown by P.C. to contain the same carbohydrates, they were therefore combined to yield a total of ca. 200 mg syrup. It was separated on 3 MM chromatographic paper (Exp.3.8) into 4 fractions with chromatographic mobilities the same or less than that of glucose. Only two of the slower spots (Exp. 3.9) were separated in sufficient quantity to investigate further. Both were oligouronic acids which on hydrolysis gave spots on paper chromatograms with the mobilities of fucose (major), xylose, mannose, galactose and glucuronic acid.

Flow chart II (aqueous, acidic and alkaline extractions).



Flow chart II (continued)



The presence of the glucuronic acid was confirmed by its ionophoretic mobility at pH. 6.8 (Py/AcOH). The presence of these oligouronic acids in the ethanolic extracts could be due to degradation of the "fucan" but it is also possible that they are precursors of the macromolecules.

During the ethanolic extraction ca. 16.2% of material was lost from the stems and ca. 20% of material from the fronds (see Table 3.6, p.100 and Flow charts I and II).

3.4.2. Aqueous and Acidic Extracts

The residual weed (residual weed (2), flow chart II), from fronds 23.8 g and stems 24.9 g respectively, was soaked in formaldehyde solution after the ethanolic extraction in order to polymerise phenolic constituents which otherwise contaminate the subsequent extracts.

The presence of calcium chloride in the extracting solution was to convert the soluble alginates present in the weed to the insoluble calcium salt so that the fucan could be obtained free from alginate contamination.

The weight of the different aqueous and acidic freeze dried extracts are shown in Table 3.1, where it can be appreciated that hot aqueous and hot acidic extractions gave better yields of "fucan".

It is somewhat surprising that the yields of "fucan" in the aqueous extracts from fronds and stems are very similar while they appear to be different in the room temperature and in the 70° acid extracts. However if the total acid extracted "fucan" at room temperature and at 70°C from the stems (267 mg) is compared with the total extracted from the fronds (ca. 246 mg) the difference, ca. 8%, is not very great.

TABLE 3.1

Weight of fronds and stems extracts

Extract	Weight (mg)
White powder (3), fronds, 2% $CaCl_2$ aqueous solution extraction, room temperature.	90
White powder (3), stems, 2% $CaCl_2$ aqueous solution extraction, room temperature	110
White powder (4), fronds, 2% $CaCl_2$ aqueous solution extraction, 70°C	212
White powder (4), stems, 2% $CaCl_2$ aqueous solution extraction, 70°C	224
White powder (5), fronds, HCl pH = 2 extraction, room temperature	68.4
White powder (5), stems, HCl pH = 2 extraction, room temperature	111
White powder (6), fronds, HCl pH = 2 extraction, 70°C	178
White powder (6), stems, HCl pH = 2 extraction, 70°C	156

The constituents of the various aqueous acid extracts are shown in Table 3.2 where it can be seen that the highest carbohydrate content, for both fronds and stems, is obtained with the hot aqueous extract.

TABLE 3.2.

Constituents of the aqueous and acidic sequential extracts.

Extract	Carbohydrate content % a)	Uronic acid content % a)	Sulphate content % a)	Protein content % a)
Fronds, cold water	36	9 (25) b)	10(27.7)	8
Stems, cold water	47	19 (40.4)	9 (19.1)	7.3
Fronds, hot water	63	16 (25.4)	13 (20.6)	11.5
Stems, hot water	62	20 (32.2)	11 (17.7)	10.3
Fronds, cold acid	40	12 (30.0)	9 (22.5)	12.9
Stems, cold acid	44	13 (29.5)	10 (22.7)	11.8
Fronds, hot acid	46	17 (36.9)	7 (15.2)	6.3
Stems, hot acid	53	29 (54.7)	6 (11.3)	5.8

a) % calculated on weight of material.

b) Figures in brackets calculated on carbohydrate content.

All figures are within $\pm$ 5%.

Since the hot water and hot acid extracts were obtained in highest yield (see Table 3.1 p.91) and had the highest carbohydrate contents (see Table 3.2. p.92) it was decided to confine the immediate investigations to these two extracts. The approximate relative proportions of the monosaccharides in the hydrolysate of the hot acid extracts were determined in the first instance by separation and elution from a paper chromatogram (Exp. 3.16 and see Table 3.3.).

TABLE 3.3.

Approximate relative proportions of the constituent monosaccharides from the hydrolysates of the hot acid extracts. Calculated with phenol sulphuric acid method.

Extract		Gluc. acid	Fucose	Xylose	Mannose	Galactose*
Fronds (hot acid)		3.8	7.2	1.0	0.58	1.31
	%	(27.4)**	(51.8)	(7.2)	(4.2)	(9.4)
Stems (hot acid)		2.2	5.5	1.0	0.60	1.24
	%	(20.9)**	(52.2)	(9.5)	(5.7)	(11.7)
Leaving glucuronic acid out						
Fronds			(71.4)	(9.9)	(5.7)	(13.0)
Stems			(66.0)	(12.0)	(7.2)	(14.8)

* It may contain traces of glucose, which is difficult to separate quantitatively from galactose by paper chromatography technique with the common solvents used.

** The figures in brackets correspond to % on weight of material analysed.

The percentages of uronic acid in these hydrolysates are very much lower than the figure derived by the carbazole method on the whole polysaccharide. Controlled experiments have shown that a considerable amount of glucuronic acid is destroyed during acid hydrolysis (see p.164, Chapter IV).

Attempts to estimate the proportions of the neutral sugars and uronic acid by this method in the aqueous extracts gave unrepeatable results. Consequently it was decided to convert the neutral monosaccharides in the hydrolysates of both the aqueous and acid extracts into their respective alditol acetates and determine their proportions by gas liquid chromatography. See Table 3.4. where percentages were calculated from the chromatograms considering the total area under the peaks as 100%.

TABLE 3.4

Approximate relative molar proportions of the constituent monosaccharide sugars in the extracts. Calculated from g.l.c. traces of the alditol acetate derivatives.

Extract	Sugar present	Proportion	%
Fronds, hot water	Fucose	3.20	40.6
	Xylose	1.00	12.7
	Mannose	1.58	20.1
	Galactose	1.63	20.7
	Glucose	0.47	5.9
Stems, hot water	Fucose	5.17	57.6
	Xylose	1.00	11.1
	Mannose	1.43	16.0
	Galactose	0.89	10.0
	Glucose	0.48	5.3
Fronds, hot acid	Fucose	6.70	81.7
	Xylose	1.00	12.2
	Mannose	0.50	6.1
	Galactose	trace	–
	Glucose	nil	–
Stems, hot acid	Fucose	4.65	63.0
	Xylose	1.00	13.9
	Mannose	0.70	9.7
	Galactose	0.97	13.4
	Glucose	nil	-

Reasonable agreement is found in Tables 3.3 and 3.4 for the hot acidic extracts estimated by the two different methods, apart from the galactose in the fronds,

which appeared to be absent in the g.l.c. determination. This is very difficult to account for since by separation on a paper chromatogram it was found in similar proportions to that from the stems.

Fucose is the main sugar (more than 50%) in "fucans" from both fronds and stems, although glucuronic acid (identity proved later, p.102), mannose, galactose and xylose also were present in each of the extracts. The presence of a low percentage of glucose in the "fucans" extracted by hot water in both fronds and stems was later explained [Experiment 3.45] by the presence of microscopic fragments of cell walls in these extracts.

This is the first time that "fucans" from fronds and stems of a particular alga have been extracted separately and it is interesting to see that those extracted by water and by acid from the two parts are essentially similar in their monosaccharide composition. However, apart from the cold acid extract all the other extracts from the stems have higher proportions of uronic acid than comparable ones from the fronds (see Table 3.2. p. 92) It is possible that in the stems the "fucans" are more stable molecules and perhaps fulfil a structural function. The "fucans" from whole plants, which have relatively high uronic acid contents are found in Sargassum linifolium (23%), Desmarestia aculeata (34%) and Bifurcaria bifurcata (26%).

The presence of protein in all these extracts indicates that they may be proteoglycans. Since covalent linkage between the "fucan" and the protein has not been proved the polysaccharides will be discussed as separate entities.

The hot aqueous extract from stems (solid (4), flow chart II p. 88) was estimated for molecular weight by

elution from a Sepharose 4B column (Exp. 3.13). The pattern is shown in Fig. 3.6 p. 97. This pattern indicates poly-dispersity of the molecule with a considerable portion with a relatively high molecular weight comparable with standard Dextran T. 500 (500.000 molecular weight). The approximate molecular weight, 670.000, was obtained from the selectivity curve for Sepharose 4B using Dextrans T. 40, T. 70, T. 250, T. 500 and T. 2000 as standards. See Figure 3.7 p. 98.

The following formula was applied[6]

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$ where V_e = elution volume of sample
V_o = void volume
V_t = total bed volume (glucose elution volume)

In this case from Exp. 3.13 V_e = 22 ml; V_o = 14 ml and V_t = 34 ml which gave a value of K_{av} = 0.4.

It will be seen later that all the "fucans" from this seaweed extracted by different means and conditions present nearly the same elution pattern and also the same molecular weight distribution.

3.4.3. Alkaline Extracts

Calcium alginates were obtained by precipitation with calcium ions (2% $CaCl_2$) (see flow chart II continued), as white freeze dried powders in a yield of 23.5% (7.64 g) and 16.5% (4.96 g) for stems and fronds respectively. These figures are referred to the initial 30 g of dried seaweed.

From the mother liquors after precipitation of the calcium alginate and dialysis, "fucans" were obtained as white powders after freeze drying; 229.5 mg from stems and 250.3 mg from fronds. Carbohydrate estimation gave 62% for stems extract and 42% for frond

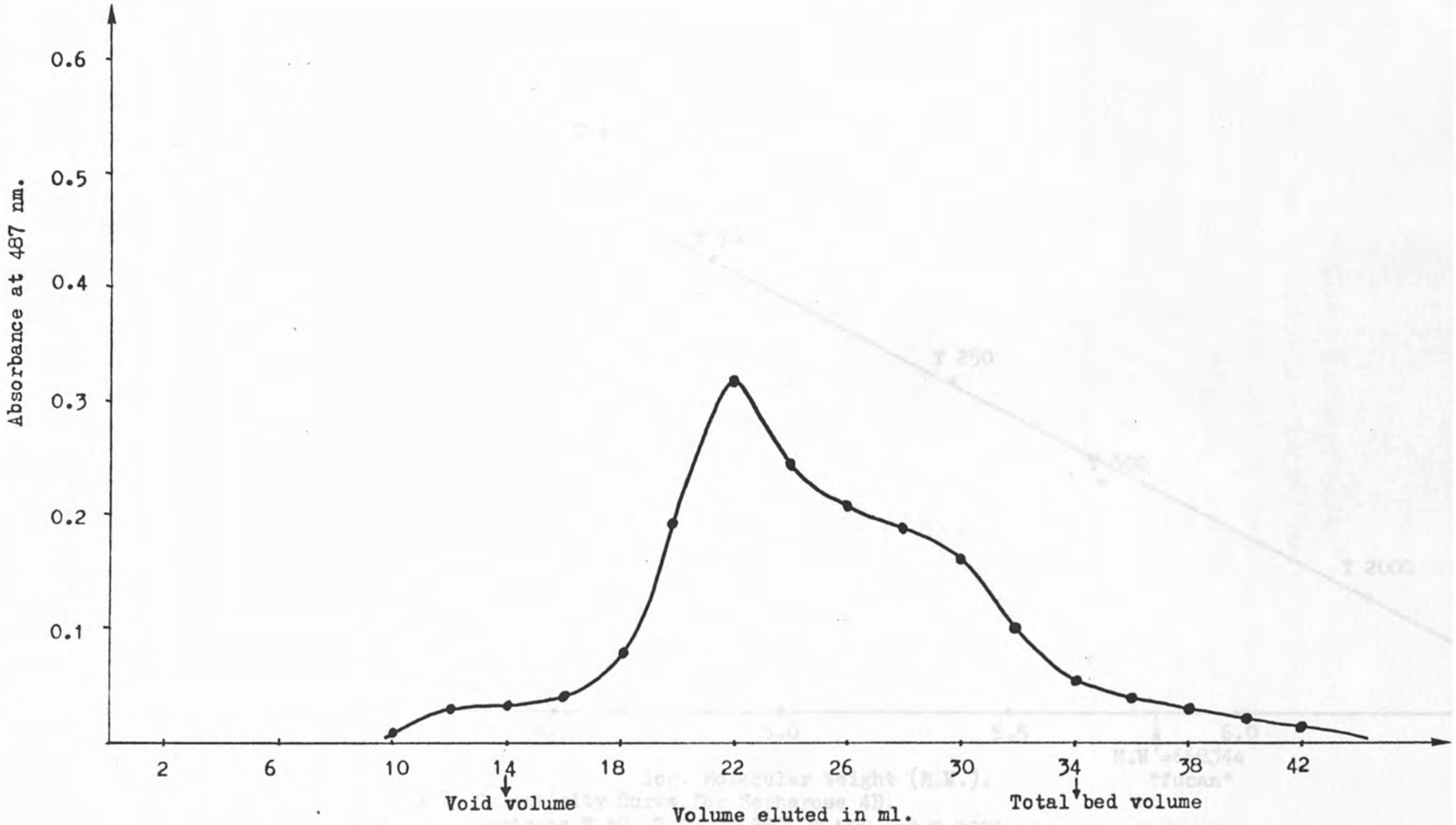


Fig. 3.6 Elution profile of "fucan" on Sepharose 4B column.
Hot aqueous sequential extract, 2% $CaCl_2$, 70°C.

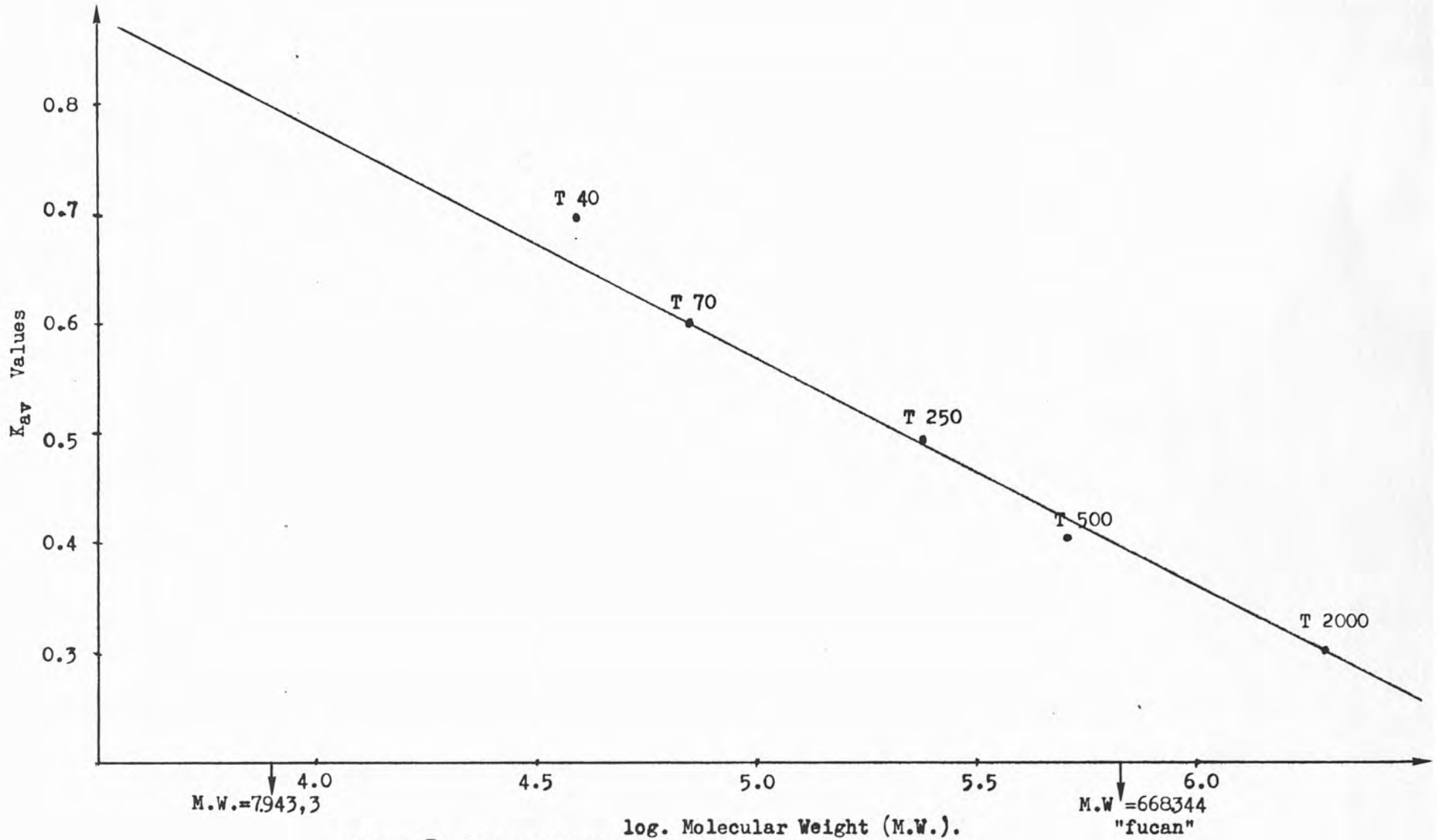


Fig. 3.7 Selectivity Curve for Sepharose 4B.
Dextrans T 40, T 70, T 250, T 500 and T 2000.

extract.

Paper chromatography studies of the respective hydrolysates of these alkali extracted "fucans" showed, as was the case of the earlier extracts, that they comprised the same sugars, being fucose (the major), xylose, galactose, mannose and glucuronic acid.

The approximate molar proportions of neutral monosaccharide sugars from fronds extracts are shown in Table 3.5a,where the 57% of fucose proves the fact that this is again the main sugar in the polysaccharide. The presence of these sugars was confirmed by gas liquid chromatography (g.l.c.) linked to mass spectrometry with electron impact (E.I.) and chemical ionization (C.I.) methods as their alditol acetates derivatives.

TABLE 3.5a

Approximate relative molar proportion of monosaccharide sugars from fronds sequential and direct alkaline extracts.

Sugar	Fronds			
	Sequent. extract		Direct extract	
	molar prop.	%	molar prop.	%
Fucose	4.5	57	11.0	55
Xylose	1.0	13	1.0	5
Mannose	0.7	7	2.8	14
Galactose	1.7	21	4.6	23
Glucose	trace	-	0.6	3

For total recovery from fronds and stems by sequential extraction see Table 3.6. According to the recoveries (see flow chart I) ca. 16.2% by weight was lost from stems and ca. 20% from fronds during ethanolic extraction and the remaining 28% and 31% respectively was lost during the subsequent extractions.

TABLE 3.6

Total recovery from stems and fronds after sequential extraction.

	Stems	Fronds
Initial dry seaweed	30 g	30 g
Mannitol	0.139	0.081
Syrup	ca. 0.101	ca. 0.101
Water soluble "fucan"	0.334	0.302
Acid soluble "fucan"	0.267	0.246
Alkaline soluble "fucan" and alginate	8.660	5.890
Residual weed	7.326	8.173
Total	16.827 g (56.1%)	14.793 g (49.3%)

9.5 g carbohydrate was separated from the stems and 6.6 g from the fronds which is reasonable for a brown seaweed.

3.4.4. "Fucans" and alginate extracted by direct alkaline method.

"Fucans"

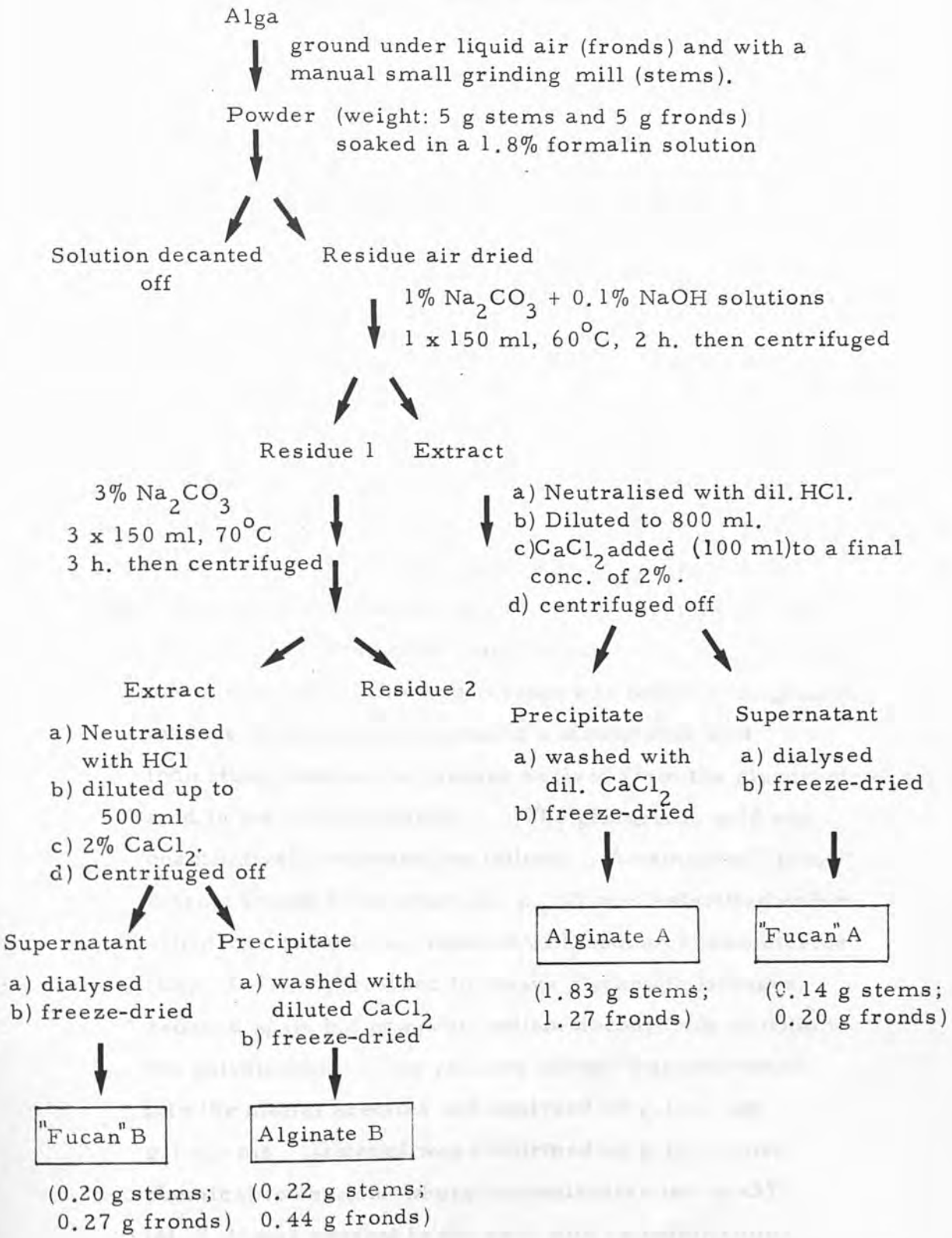
By direct alkali extraction of the weed (see flow chart III, p.101) two "fucans" (A and B) were isolated from the mother liquors after removal of alginate. Carbohydrate, sulphate and protein content from fronds extracts were estimated. See Table 3.7.

TABLE 3.7

Properties of "fucans" A and B from direct alkaline extraction.

	Carbohydrate content %	Sulphate content %	Protein content %
"Fucan" A	63-64	13.3	14.9
"Fucan" B	37-39	9.3	28.1

Flow chart III Direct extraction of alginic acid



It can be seen that "fucan" B has a very high protein content and a low carbohydrate content compared with "fucan" A.

A comparison of the relative proportions, which were determined by g.l.c. and g.l.c.-ms (E.I. and C.I.), of the monosaccharides in the "fucans" present in the hydrolysate of the two alkaline extracts, sequential and direct methods, from fronds (Exp. 3.28) is given in Table 3.5a,p. 99 As would be expected there are differences in the relative proportions of the monosaccharides; however, fucose (more than 50%) is the major sugar in both extracts.

The identity of the uronic acid as glucuronic acid was proved as follows. A hydrolysate of stems extract (combined A and B) which was devoid of glucose was separated by preparative paper chromatography into neutral and acidic fragments in the appropriate solvent (Exp. 3.27). The acidic fraction was esterified, reduced and hydrolysed. This hydrolysate was tested with glucose oxidase reagent which revealed a strong pink spot indicating presence of glucose derived from the glucuronic acid in the polysaccharide. The glucuronic acid was quantitatively estimated as follows. A sample of "fucan" A from fronds (flow chart III, p. 101) was esterified under anhydrous conditions, reduced with sodium borodeuteride (Exp. 3.31), hydrolysed to cleave glycosidic linkages, reduced again but now with sodium borohydride to obtain the polyalcohol. The reduced sample was converted into the alditol acetates and analysed by g.l.c. and g.l.c.-ms. Glucitol was confirmed by g.l.c.- ms, chemical ionization, where the molecular ion at 437 ($M^+ + 2$) was present in the peak with retention time corresponding to glucitol acetate. This is undeniable proof of glucuronic acid.

Relative molar proportions of the monosaccharides obtained by this method can be seen in Table 3.5b.

Table 3.5b

	Method 1: By direct hydrolysis	Method 2: By reduction followed by hydrolysis
	Molar prop. (see Table 3.5a)	Molar prop.
Fucose	1	1
Xylose	0.09	0.71
Mannose	0.25	1.02
Galactose	0.42	0.71
Glucose	0.05	0.74

If it is assumed that the two methods released identical proportions of fucose from the original polysaccharide (i.e. molar prop. of fucose in each hydrolysate is 1) then the relative amounts (relative to fucose) of xylose, mannose, galactose and glucose increased by factors 7.9, 4.1, 1.7 and 14.8 respectively, when the polysaccharide was reduced prior to hydrolysis. It could then be suggested that ca. 93% of the glucose estimated by method 2 was derived from glucuronic acid.

Glycosidic linkages from neutral sugars units are more readily hydrolysed than those from uronic acid. This suggests then that glucuronic acid is linked to at least xylose and mannose. Subsequent partial hydrolyses studies confirmed this to be so for mannose.

Attempted fractionation of the "fucans" extracted directly by alkali from fronds by electrophoresis on trimethyl silylated glass fibre paper (TGFP)

It has been stated that some polysaccharides which were considered as homogeneous on the basis of different fractionation studies can be separated into two or more components when investigated by glass fibre paper electrophoresis.[8] Complexes between the polysaccharide and the glass fibre are avoided,[9] which is an advantage considering that this could happen frequently using cellulose paper. Another advantage seems to be that any polysaccharide can be detected without the risk of any reaction between the locating reagent and the fibre.

The purpose of treating the glass fibre paper with trimethyl silylating agent is to overcome the high rate of endosmotic flow in unmodified glass fibre.[10]

In an attempt to develop a set of techniques to separate

plant cell wall polysaccharides easily at the required level of resolution on the analytical and preparative scale, Jarvis et al[11] have reported that electroendosmosis could be almost eliminated by treating the glass fibre paper with a trimethyl silylating agent. The TGFP would float indefinitely on water but could be wetted in buffer to which a surfactant had been added. Endosmotic flow through this support material was negligible at pH = 5 and too slight to cause any problems at pH = 9 under the conditions used.

Application of this technique in an attempt to fractionate "fucans" extracted directly by alkali from fronds resulted in only one fraction which migrated on the glass fibre paper ca. 12 cm (after 40 minutes running) with buffer pH5 and ca. 5 cm (after 30 minutes running) with buffer pH 10. This showed no evidence of a mixture of polysaccharides.

"Fucans" extracted from the brown seaweeds Desmarestia firma and Dictyopteris plagiogramma had a mobility of 8.2 cm and 7.9 cm respectively (40 minutes running)with buffer pH5 and 4.4 cm and 4.0 cm respectively (30 minutes running) in buffer pH10. Alginate samples of Lessonia nigrescens extract did not migrate from the starting line at these pHs. The same happened to a standard sample of starch."Fucan"mixed with alginates or with starch were held back to a less migration distance and alginate and starch remained on the starting line.

It is of interest to note that "fucan" from Dictyopteris plagiogramma showed traces of another fraction on the starting line, which is in agreement with Dr M.A. Rahman's studies[12], but the stationary fraction could well be contamination by alginate in the

"fucan" rather than another "fucan" as he reported.

The "fucan" from Lessonia nigrescens was removed from the TGFP by cutting the appropriate paper strips and eluting with water. Hydrolysates of these "fucans" run in P.C. were found to comprise the usual sugars and uronic acid of the original material.

A sample of the direct alkali extracted "fucan" from fronds (Exp. 3.22) was analysed through a Sepharose 4B column to estimate its molecular weight. See Figure 3.8 p.106 As with the sequential alkali extracted "fucan" the pattern shows a polydisperse molecule with a considerable portion with a high molecular weight. 670,000 (calculated from Figure 3.7 p 93 with $K_{av} = 0.4$), comparable with Dextran T500 (500,000 molecular weight). At the same time the fractions collected were analysed for protein content (Exp. 3.29). The elution pattern for the protein was very similar to that for the carbohydrate (see Figure 3.8 p.106) indicating a very close relationship; possibly covalent linkage between the two polymers.

3.4.5. General conclusions on the "fucans".

It is surprising that the fucans separated from the stems and from the fronds are so similar. The only differences that emerge are the somewhat higher quantity of "fucan" that can be isolated from the stems and that the proportions of uronic acid in the latter is higher than that of the "fucans" in the fronds. This would support the idea that, apart from the other functions cited for the "fucan" in the introduction, certain of the molecules do play a part in the skeletal structure of the plant.

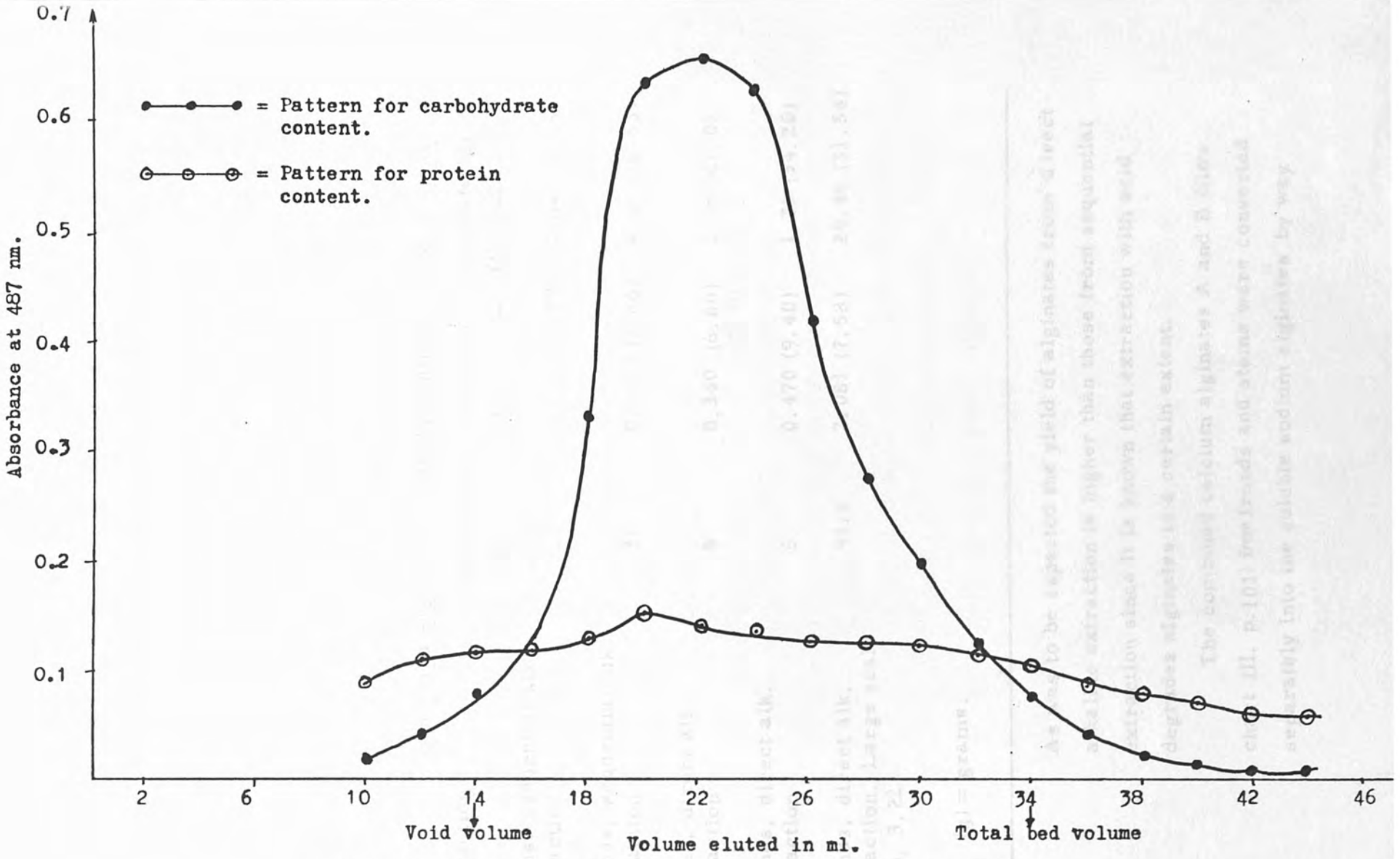


Fig. 3.8 Elution profile of "fucan" on Sepharose 4B column. Direct alkaline extract.

3.4.6. Alginates

Direct alkaline extraction of alginic acid shown in flow chart III gave a yield of 41% (2.05 g) of the dry weight of seaweed of Ca alginates A and B for stems and a 34.2% (1.7 g) for fronds extracts. As comparable results see Table 3.8 where yield on a large scale for fronds and from sequential alkaline extractions are reported.

TABLE 3.8

"Fucans" and Alginates yields from different extractions.

Extraction	Initial weight (g)	Fucan (g) yield. (%)	Alginate (g) yield.(%)
Stems, sequential alk. extraction	30	0.830 (2.77)	7.64 (25.46)
Fronds, sequential alk. extraction	30	0.798 (2.66)	4.96 (16.53)
Stems, direct alk. extraction	5	0.340 (6.80)	2.05 (41.0)
Fronds, direct alk. extraction	5	0.470 (9.40)	1.71 (34.20)
Fronds, direct alk. extraction. Large scale (Exp. 3.22)	93.4	7.081 (7.58)	29.46 (31.54)

(g) = grams.

As was to be expected the yield of alginates from direct alkaline extraction is higher than those from sequential extraction since it is known that extraction with acid degrades alginates to a certain extent.

The combined calcium alginates A and B (flow chart III. p.101) from fronds and stems were converted separately into the soluble sodium alginates by way

of free acids as stated in Experiment 3.21.

Studies of the hydrolysates of these alginates on paper chromatograms for both fronds and stems separately showed $R_{Mann.Ac.} = 0.77$ and 1.2 (in solvent 6:4:3 =n-BuOH:Py:H_2O, whose mobilities are slightly faster than those recorded for guluronic acid ($R_{Mann.Ac.} = 0.62$) and mannuronic acid ($R_{Mann.Ac.} = 1.0$) respectively.

Ionophoresis of the hydrolysates in a borax-$CaCl_2$ buffer, pH 8.9 (Exp. 3.15) revealed the presence of spots which had $M_{Mann.Ac.} = 0.7$ and 1.08 corresponding to Haug and Larsen[20] who reported 0.85 and 1.0 respectively under the same conditions.

The relative viscosities of 1% aqueous solutions of sodium alginate of the different extracts (Exp. 3.21 and 3.22) were measured. The viscosities and the yields of the extracts can be seen in Table 3.9 p. 109

From this table it can be seen that all the extracts have a very low value if they are compared with viscosity values between 500 and 3000 c.p.s. of alginates extracted from seaweeds by methods which avoid degradations.[7]

Even though direct alkaline extraction of the weed usually has considerably less degrading effect on the alginate molecule it can be seen in Table 3.9 that this is not the case for Lessonia alginates. All the extracts had a very low viscosity. Only in the case of the direct alkaline extract of the stems in which the "fucan" had not been removed is the viscosity even reasonably high.

TABLE 3.9

Percentages of yields and viscosities of alginic acid isolated from brown seaweed Lessonia nigrescens.

	Direct alkaline extraction yield %	Sequential extraction yield %	Viscosity (25°C) (η rel.)
Fronds	34.2	16.5	39.2[a]
Stems	41.0	25.4	75.0[a]
Fronds (exp. 3.22 large scale)	31.5		23.2[b]; 37.3[c]
Stems (exp. 3.22)	36.3		620[d]; 267.6[e]

a) Viscosity was measured only for alginic acid from direct alkaline extraction.

b) Alginate A, from 0.1% NaOH and 1% Na_2CO_3 extract.

c) Alginate B, from 3% Na_2CO_3 extract.

d) Viscosity of solution recently extracted, without separating the "fucan".

e) After separating "fucan" from d).

The gel strength of samples of sodium alginate depends to some extent on the relative proportions of mannuronic to guluronic acid in the alginate molecules. In order to determine the proportion of these two acids a ^{13}C n.m.r. analysis was carried out (Exp. 3.25) and figures for $G_4/M_4 = 0.597$ $G_1/M_1 = 0.48$ and $G_5/M_5 = 0.60$ were estimated. An estimative average gives a ratio $G/M = 0.54$.

The ^{1}H n.m.r. spectrum of this sample of alginate was rather featureless or with very broad peaks which made assignments almost impossible. The effect of viscosity of the solution is probably the main reason for this non-clear spectrum.

The ^{13}C n.m.r. spectrum of this sodium alginate was clearer and therefore easier to interpret despite its considerable complexity, displaying numerous peaks (see Figure 3.9).

Grasdalen et al[22] published very similar results to the present work. They investigated a sample of whole alginate prepared from old tissue of Ascophyllum nodosum also a brown seaweed, which had been partly degraded by very mild acid hydrolysis to decrease the viscosity of the solution. The assignments of their ^{13}C n.m.r. spectrum were made by reference to the whole alginate sample and to spectral data for alginate fractions enriched in L-guluronate and D-mannuronate, (G-residues and M-residues) respectively.

Whole alginates contain blocks of contiguous M- and contiguous G-residues having a certain distribution of lengths, interspersed between regions enriched in alternating sequences.[23] The termination of homopolymeric blocks gives rise to asymmetric triads MMG and GMM, having M as the central unit and GGM and MGG having G as the central unit. At the same time four symmetric triads, MMM, GMG, GGG and MGM were also identified.

Although the peaks in Figure 3.9 do not coincide exactly with those in the spectrum of Grasdalen et al, their position relative to one another is so similar that it is considered justifiable to assign them in the same order as in the cited publication. The assignments for the different carbon atoms in the central residue of each of the triads is given in Table 3.9a. p.112.

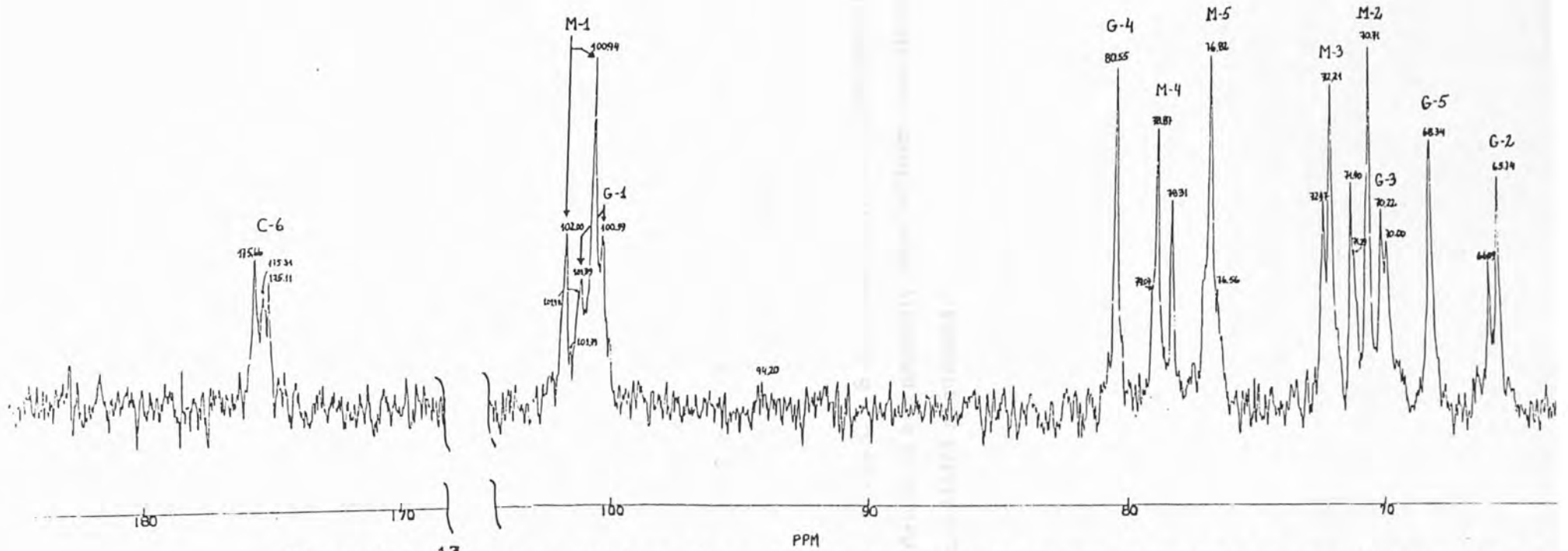


Figure 3.9 ^{13}C n.m.r. spectrum of a solution in D_2O of the sodium alginate from Lessonia nigrescens. Signals designated by M and G refer to those of mannuronate and guluronate residues respectively. 400 MHz apparatus.

TABLE 3.9a.

Assignments of peaks in the ^{13}C n.m.r. spectrum of sodium alginate from Lessonia nigrescens (p.p.m.).

Sequence	Carbon atoms					
	C - 1	C - 2	C - 3	C - 4	C - 5	C - 6
MMM	100.94	71.10	72.21	78.87	76.82	175.11
MMG	102.00	71.29		79.07	76.40	
GMM	100.70	70.71		78.31	76.95	
GMG	101.79	71.40	72.47	78.31	76.56	175.85
GGG	101.39	66.09	70.00	80.55	67.95	175.31
GGM	100.59	65.74		80.55	68.34	
MGG	101.48	66.09		80.55	67.95	
MGM	100.45	65.74	70.22	80.55	68.34	175.66

The C - 6 resonancesdue to asymmetric triads have not been identified but probably most of them are similar to those from GGG and MMM sequences.

3.4.7. Hot aqueous extraction of "fucan" from Lessonia nigrescens.

In view of the similarity of the "fucans" from the fronds and stems in previous extracts (sequential and direct alkaline methods) it was decided that a hot dilute calcium chloride extraction of a mixture of fronds and stems would give a good yield of the desired "fucan" for future structural studies.

A mixture of fronds and stems (771 g) was then extracted as outlined in flow chart IV and Expt. 3.33; 20.3 g of "fucan" were obtained, 2.64% of the initial weight of material.

The percentage composition of this water soluble extract is shown in Table 3.10.

TABLE 3.10

Composition of "fucan" from hot aqueous extraction. [a]

Carbohydrate	60%
Sulphate	9% (15%) [b]
Uronic acid	26% (43.3%) [b]
Protein	11.4%
Moisture	10%
Ash	12%

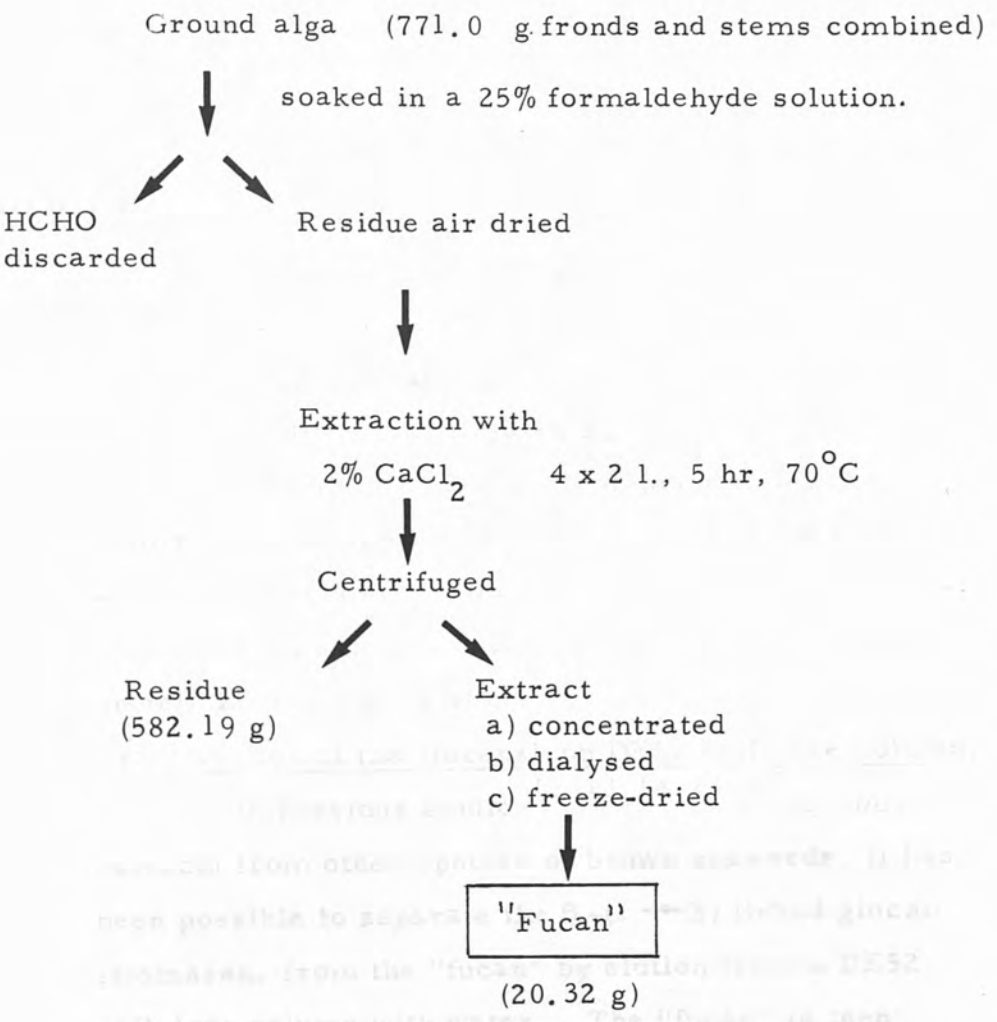
a) All these values are calculated on weight of the "fucan".

b) Figures in brackets calculated on carbohydrate content.

The accuracy of these results are within ± 5%

It must be remembered that this contains the material previously extracted by both cold and hot water. The figures in Table 3.10, apart from a higher uronic acid content are in agreement with those of the previous extracts (see Table 3.2).

Flow chart IV Direct hot aqueous extraction.



Paper chromatography of a hydrolysate in different solvent systems and with different locating reagents revealed the same constituents as those found in previous extracts: fucose, xylose, mannose, galactose and glucuronic acid. Traces of glucose were also found when a paper chromatogram was developed with glucose oxidase reagent.

Samples of "fucans" from cold water extract, sequential alkaline extract, direct alkaline extract and direct hot aqueous extract were hydrolysed with 12N HCl, and the hydrolysates analysed by paper chromatography were found to comprise several amino acids among which serine, alanine and glycine were recognised according to their mobilities compared with standard samples, proving again the presence of protein in all these extracts.

3.4.8. Fractionation of the "fucans" on DE52 cellulose column.

In previous studies[12,13,14,15] on aqueous extracts from other species of brown seaweeds, it has been possible to separate the β-(1→3) linked glucan laminaran, from the "fucan" by elution from a DE52 cellulose column with water. The "fucan" is then recovered from the column by elution with increasing concentration of aqueous potassium chloride. The fractions eluted with the lowest concentrationsof potassium chloride usually containing the highest proportion of glucuronic acid and lowest fucose and sulphate and as the concentration of potassium chloride increases these proportions are reversed.

In the case of the hot aqueous extract from Lessonia nigrescens (Exp. 3.33), the solute in the aqueous eluate which accounted for 28% of the material applied to the column, contained only 3% carbohydrate and paper chromatography of a

hydrolysate revealed only a trace of glucose. It seems that this sample of Lessonia nigrescens is devoid of laminaran. It is of course possible that this was lost or destroyed during the drying of the seaweed in Chile.

With the increasing concentration of potassium chloride (see Table 3.11), the sulphate content increases from 8% to 17%; whereas the contrary was observed with uronic acid content which is in agreement with previous studies. The carbohydrate contents of respective KCl fractions varied very slightly. A good recovery from the column, 97.14% was obtained and each of the fractions of the polysaccharide had a negative rotation.

TABLE 3.11

Fractionation of the "fucan", hot aqueous extract, on a DE-52 cellulose column. (500 mg applied to the column.)

Fraction	Recovery mg; %	Carbohyr. content %	Uronic Acid content %	Sulphate content%	$[\alpha]_D^{25^\circ}$ degrees
Water	138.2;27.6	2.8-2.9[a]	-	-	+2.1
0.2MKCl	167.7;33.5	58-62[b]	28-30[c]	8.0	-30.7
0.5MKCl	149.8;30.0	55-60[b]	20-25[c]	13.2	-34.8
1.0MKCl	30.0; 6.0	51-52[b]	10-13[c]	17.7	-41.0

total recovery: 485.7 mg (97.1%)

[a] Carbohydrate content based on a glucose standard graph

[b] Carbohydrate content based on a mixture standard graph Fu:Glc.A:Gal:Xy:Mann = 5:2:1.5:1.0:0.5

[c] Uronic acid content based on a standard glucuronic acid graph. Carbazole method with borate, 55°C and 100°C.

[d] All figures are calculated on weight of material.

TABLE 3.12

Fractionation of "fucans" extracted from different brown seaweeds on DE-52 cellulose columns by elution with increasing concentration of aqueous potassium chloride.

Species		$[\alpha]_D$ degrees	Recovery %	Carbohydrate %	Sulphate %	Uronic acid %	Molar poportions of sugars Fuc.	Xyl.	Mann.	Galact.	Gluc.
Himanthalia lorea[14]											
KCl fraction	0.3 M	-87	28	50-52	2.5	18.8	2.5	1.0	NR	+	NR
	0.5 M	-58	16	45-47	21.0	8.9	10.0	1.0	NR	+++	NR
	1.0 M	-132	33	40-42	29.0	4.0	14.0	1.0	NR	++	NR
Bifurcaria bifurcata[14]											
KCl fraction	0.3 M	-84	42	48-50	4.6	19.5	2.8	1.0	NR	+	NR
	0.5 M	-54	8	43-45	22.7	11.5	5.5	1.0	NR	++	NR
	1.0 M	-100	20	40-42	30.0	2.6	13.0	1.0	NR	+++	NR
Padina pavonia[14]											
KCl fraction	0.3 M	-94	31	50-52	2.5	20.4	2.0	1.0	NR	+	NR
	0.5 M	-51	20	45-47	11.0	9.1	3.0	1.0	NR	++	NR
	1.0 M	-112	38	40-42	17.0	4.7	12.5	1.0	NR	+++	NR
Lessonia nigrescens											
KCl fraction	0.2 M	-30.7	33.5	58-62	8.0	28-30	3.5	1.0	2.9	0.6	0.5
	0.5 M	-34.8	30.0	55-60	13.2	20-25	3.6	1.0	1.8	3.5	0.5
	1.0 M	-41.0	6.0	51-52	17.7	10-13	4.3	1.0	0.6	7.4	0.5

NR - none reported.

Hydrolysates of the different fractions were characterised by paper chromatography with different solvent systems and locating reagents. The four sugars and glucuronic acid were detected.

Part of the hydrolysates were converted to the alditol acetate derivatives and analysed through gas liquid chromatography.

The proportions of the sugar constituents are shown in Table 3.12 in comparison with those of other species of brown seaweed previously investigated.

It can be seen that in the other examples cited the molar proportion of fucose increases as the concentration of KCl in the eluant is increased but with the "fucan" of Lessonia nigrescens the increase of fucose is comparatively small and the proportion of galactose increases approximately twelvefold and that of mannose decreases almost fivefold.

If percentages of these sugars are considered from the peak area of g.l.c. chromatograms assuming the total areas as a 100%, the following figures are obtained.

TABLE 3.13

	0.2 M KCl fraction	0.5 M KCl fraction	M KCl fraction
% Fucose	41.0	35.0	31.0
% Xylose	12.0	10.0	7.0
% Mannose	34.0	17.0	4.0
% Galactose	7.0	34.0	54.0
% Glucose *	6.0	5.0	4.0

* Shown at a later date to be due to cell wall fragments (p. 124)

Again the change in fucose is comparatively small, galactose increases about eightfold and mannose decreases about ninefold.

From all these results it appears that the adsorption of the "fucan" on DE52 cellulose column depends or is influenced by the uronic acid and sulphate present in the polysaccharide. The adsorption of these columns is undoubtedly enhanced with increasing amounts of acidic groups in the molecules. At the same time the sulphate groups appear to be held more tightly than the carboxyl so that in the heterogeneous mixture of molecules in this "fucan" those with lower sulphate (see Table 3.11) are eluted by the more dilute KCl eluant.

Previous studies in homologous series of linear polysaccharides have stated that the lower molecular weight materialsare held less firmly than the higher molecular weight materials[16,17]. It can also be mentioned that the shape of the polysaccharide molecule also influences adsorption. For example, in fractionation of soluble starch and dextrans, the linear components were retained in the column more strongly than the branched components.[18]

From Figure 3.10 it canbe seen that the 0.2 M KCl fraction has a molecular weight of ca. 8,000 (calculated from Figure 3.7 p.98 , K_{av} vs. log MW) and the 0.5 M KCl fraction a molecular weight comparable to that of Dextran T40 (40,000). Although the size of the "fucan" molecules plays some part in the elution pattern, the overriding factor is the acidity.

This "fucan" (hot aqueous extract), 150 mg, (90 mg carbohydrate), was hydrolysed and the hydrolysate separated by paper chromatography into neutral and acidic fractions (see Experiment 3.41). Both fractions were freeze dried and weighed, 31 mg of acidic fraction and 36.5 mg of neutral fraction were

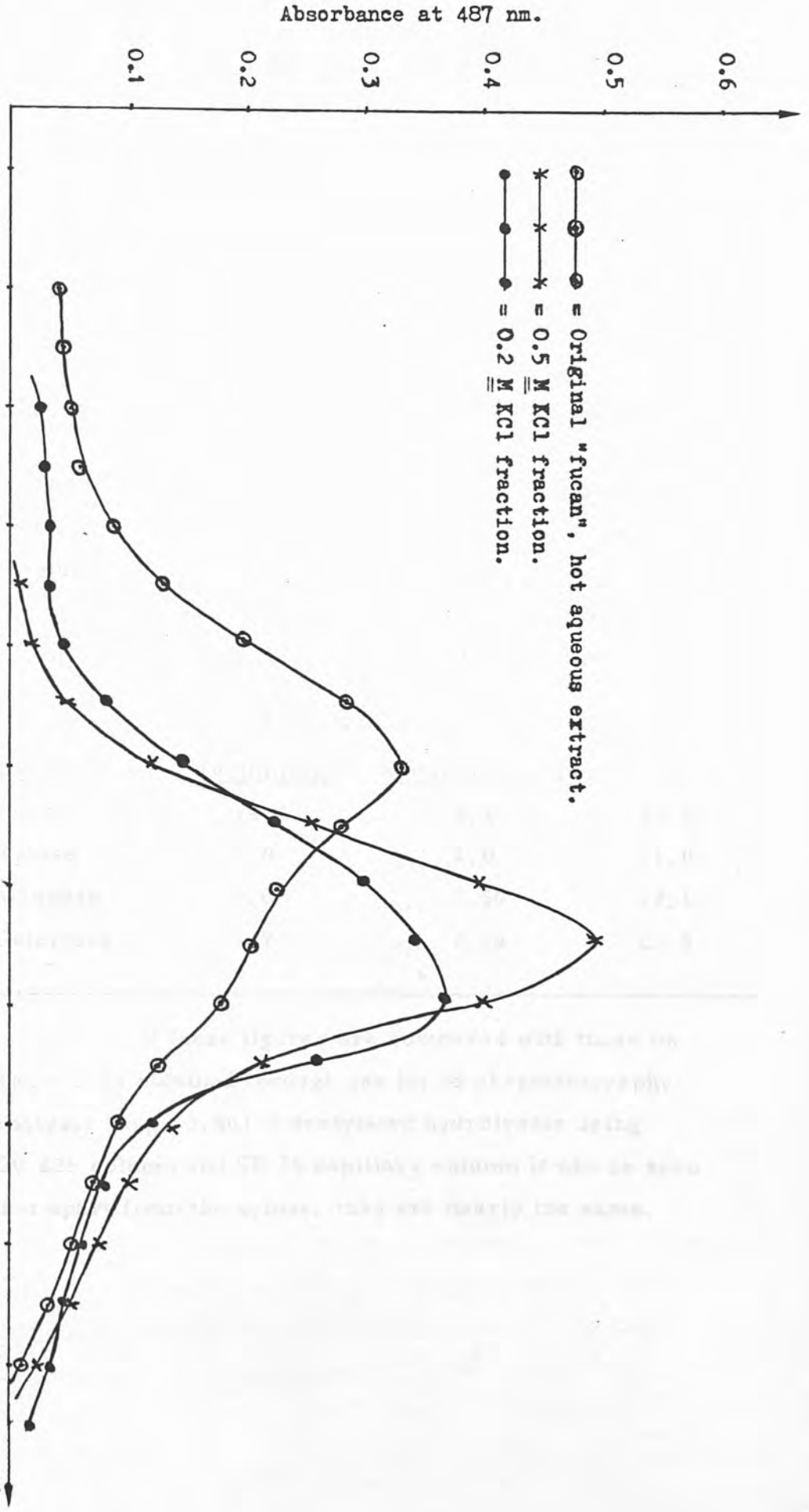


Fig. 3.10 Elution profiles on Sepharose 4B column of "fucan" and fractions obtained from DE - 52 cellulose column.

obtained. On the paper chromatogram used for this separation a product remained on the starting line. This product was eluted from the paper and concentrated. Phenol sulphuric acid method for carbohydrate estimation gave 4.8 mg. This product was rehydrolysed with $\underline{\underline{M}}H_2SO_4$ and the hydrolysate was found to comprise fucose and less xylose, mannose and galactose. Glucuronic acid was present in small amount as well.

The neutral sugars were separated, freeze-dried and weighed. Purity of each of these sugars was proved through g.l.c. (alditol acetates derivatives) and through H.P.L.C. compared with standard sugars. Galactose was found to be contaminated with traces of glucose.

The weight, molar proportion and percentage for these sugars were as follows (Table 3.14).

TABLE 3.14

Sugar	Weight (mg)	Molar proportion	%
Fucose	14.4	4.4	48.4
Xylose	3.0	1.0	11.0
Mannose	5.6	1.56	17.1
Galactose	7.7	2.14	23.5

If these figures are compared with those on Table 3.15 obtained through gas liquid chromatography analysis (Exp. 3.46) of acetylated hydrolysate using OV 225 column and SE 30 capillary column it can be seen that apart from the xylose, they are nearly the same.

TABLE 3.15

Approximate relative molar proportion of monosaccharide sugars from "fucan" hydrolysate (hot aqueous extraction)

Sugar	Column OV 225		Column SE 30 (capillary)	
	molar prop.	%	molar prop.	%
Fucose	6.0	43	7.0	48
Xylose	1.0	7	1.0	7
Mannose	2.9	20	2.5	17
Galactose	3.5	25	3.6	25
Glucose	0.7	5	0.4	3

The galactose fraction separated from the neutral sugars on the previous page was freed from glucose (Exp. 3.43) by converting the latter into gluconic acid with glucose oxidase. It was co-spotted on a paper chromatogram with the glucose oxidase. Preliminary experiments on synthetic mixtures showed that if too large an excess of the oxidase was used then the galactose was also destroyed, so this had to be avoided. The isolated galactose glucose mixture (7.7 mg) were spotted on a 3 MM paper and 5 volumes of glucose oxidase was slowly spotted on top. After development of the paper the galactose was eluted with water from the appropriate strip and the eluate freeze-dried (6.7 mg) P.C. revealed only galactose. It follows that 1 mg glucose had been removed and that the galactose is present in 1.9 molar proportions (20.4 %) and glucose in 0.28 molar proportions (3%) on the whole "fucan".

Molecular weight estimated for this "fucan" (Exp. 3.44) was approximately 500,000 for the portion of highest molecular size and a similar pattern to that of previous extracts was obtained. See Figure 3.11 p. 123

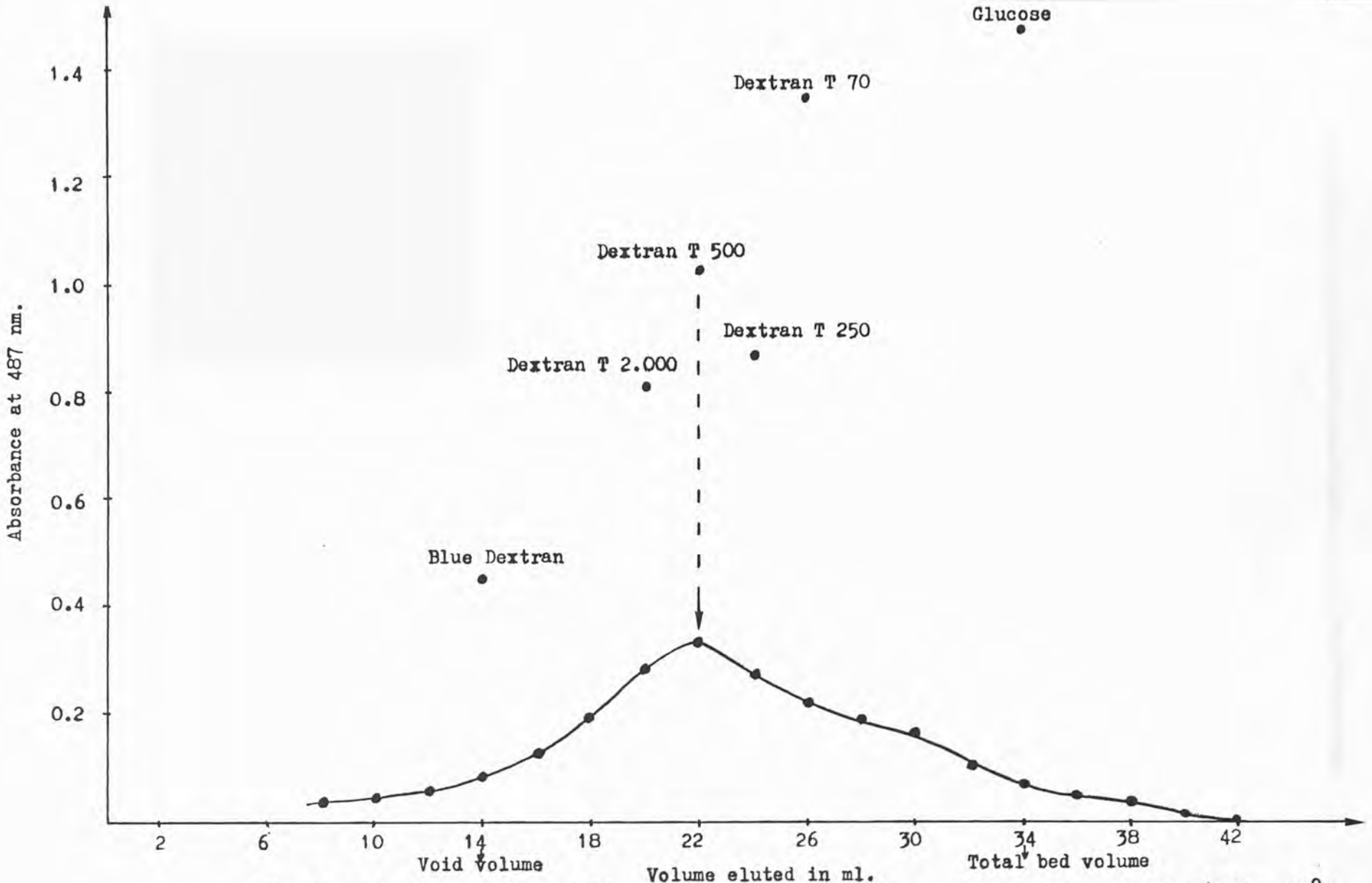


Fig. 3.11 Elution profile of "fucan" on Sepharose 4B column. Hot aqueous extract, 2% $CaCl_2$, 70°C.

The approximate molecular weight calculated from selectivity curve for Sepharose 4B (Figure 3.7) is ca. 668,000.

It was difficult to account for the traces of glucose which occurred in some of the extracts. Examination of these apparent solutions under the microscope revealed what appeared to be minute specks of cell wall. To confirm this a sample of the "fucan" (25.5 mg) was dissolved in 2 ml of water (Exp. 3.45) and filtered through glass fibre millipore filter; the hydrolysates of the filtrate and the residue together with the glass fibre filter were separately analysed with glucose oxidase reagent. The filtrate supposed to be only "fucan" was devoid of glucose which had appeared in previous experiments, whereas the residue showed a strong pink spot corresponding to glucose. Carbohydrate content was estimated for both hydrolysates and the "fucan" was found to have a 55.7% and the residue, assumed to be cell wall had only 5.6%. Altogether they came to 61.3% which is in reasonable agreement with the 60% found previously for initial "fucan" as shown in Table 3.10. p. 113.

3.4.9. Attempted desulphation of "fucan" extracted from Lessonia nigrescens and a sample of "fucan" extracted from Himanthalia lorea also a brown seaweed.

"Fucan" from Lessonia nigrescens (hot aqueous extract) was subjected to desulphation by both acidic and alkaline methods (Exp. 3.36). The sulphate contents of the products were determined and compared with the sulphate content of the original "fucan". The acidic method gave a product with only 2% sulphate by weight.

A hydrolysate run on a paper chromatogram showed the same constituents as previous

analyses. A molecular weight study of this desulphated fucan gave the pattern shown in Figure 3.12. Alkaline desulphation was also attempted in three different experiments for "fucan" from Lessonia nigrescens and a molecular weight study of the desulphated product was also done. The pattern is shown in Figure 3.12.

If these two last patterns are compared with that of the original "fucan" (see Figure 3.11) it can be concluded that both desulphated products have been considerably degraded, the molecular weight of both products being ca. 8000. Even though in the alkaline method potassium borohydride was added to minimise degradation by the alkaline peeling reaction[5], it can be seen that part of the molecule has been degraded and cleavages of glycosidic linkages have occurred.

A sample of "fucan" extracted from Himanthalia lorea (previously studied in our laboratories) was twice subjected to desulphation, but as can be seen in Table 3.16 neither in this case nor in that of Lessonia nigrescens was desulphation achieved.

These results for "fucan" from Himanthalia lorea disagree with those obtained by Dr M A Rahman[12] who found 86% recovery by weight, 21.7% sulphate on original "fucan" (which is similar to the figure in Table 3.16) and 15% sulphate on the desulphated sample.

Hydrolysates of these desulphated "fucans" were analysed by P.C. and found to comprise the same constituents as original "fucans".

It can be concluded at least for the "fucan" from Lessonia nigrescens that sulphate groups are not linked to carbon atoms which are adjacent to carbon atoms carrying hydroxyl groups trans to the sulphate

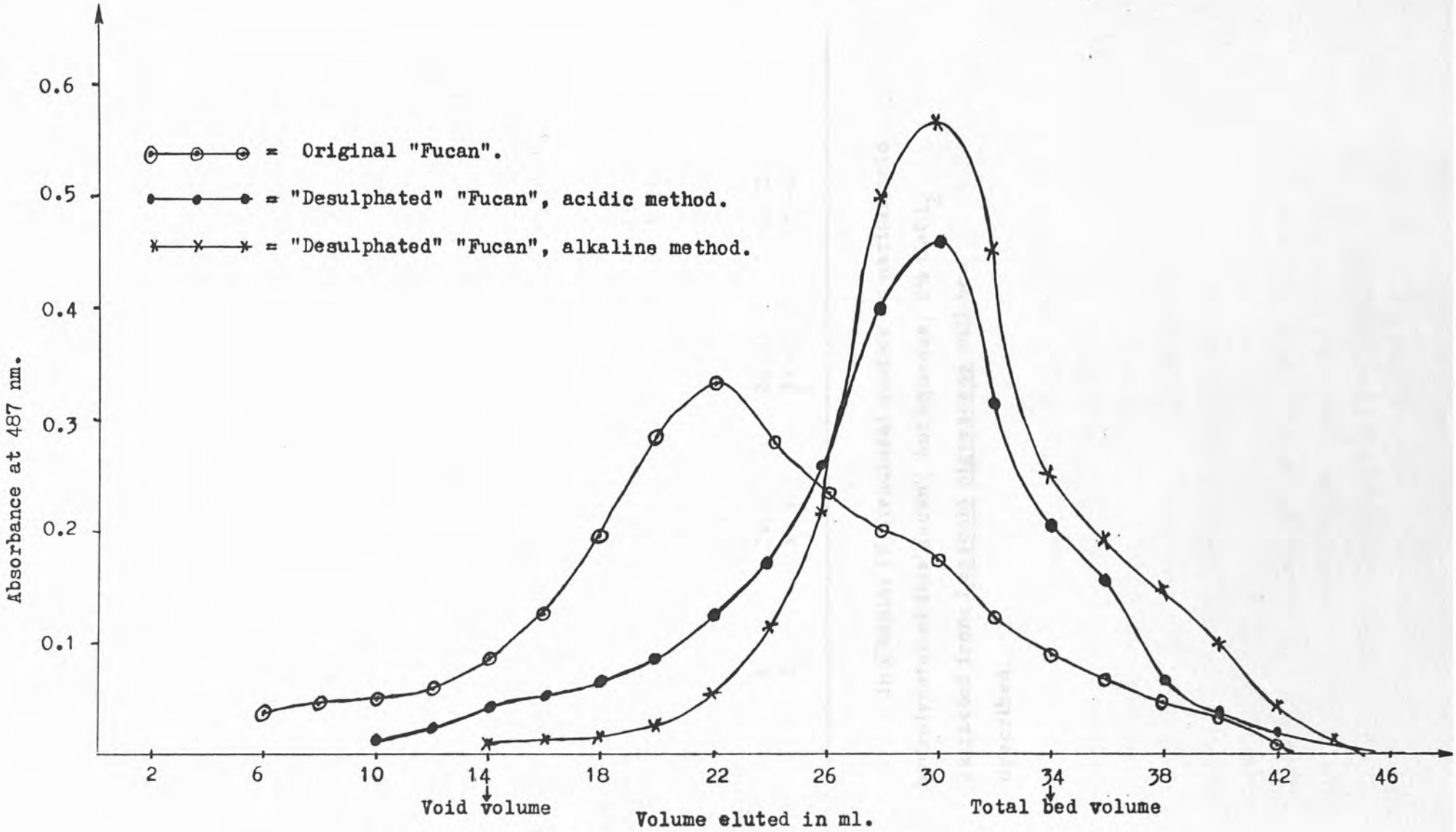


Fig. 3.12 Elution profile of "fucan" and attempt of desulphation of this direct hot aqueous extract. Sepharose 4B column.

residues otherwise the ester sulphate groups would have been cleaved by alkali as has been established from experiments on model monosaccharide sulphates[19] and in polysaccharides, for example that from green seaweed Ulva lactuca.[21]

In general terms sulphate residues in "fucans" are very stable to alkali.

TABLE 3.16

Lessonia nigrescens and Himanthalia lorea "fucans" comparison of % recovery and % sulphate after alkaline desulphation attempts.

"Fucan"	attempt No.	% recovery	original "fucan" % sulphate	derived polysacch. % sulphate
Lessonia nigrescens	1	57.1	9-12	10-11
	2	57.0	9-10	9-10
	3	61.4	10-11	9-11
Himanthalia lorea	1	30.6	24-25	24-25
	2	39.1	21-22	20-21

In Chapter IV, structural studies on partial acid hydrolysates of this "fucan", hot aqueous, 2% $CaCl_2$ extracted from Lessonia nigrescens will be described.

3.5. REFERENCES

3.5.1. CHAPTER III Introduction

1. Black W.A.P. J.Mar Biol.Ass. U.K., 29, 45 (1950).
2. Rahman M.A. PhD Thesis, University of London 1979.
3. Bidwell R.G.S. Can.J.Bot. 45, 1557 (1967).
4. Lindberg B.and Paju J. Acta Chem.Scand., 8, 817 (1954).
5. Lindberg B. and McPherson J. Acta Chem.Scand. 8, 1875 (1954).
6. Fanshawe R.S. and Percival E. J.Soc.Fd.Agric. 9, 241 (1958).
7. Carlberg, G.E. Percival E and Rahman M.A. Phytochem. 17, 1289-1292 (1978).
8. S mith F.and Unrau A.M. Chem. and Ind., 636 (1959).
9. Black W.A.P and Dewar E.T. J.Mar.Biol.Ass.U.K., 28, 673 (1949).
10. Powell J.H. and Meuse B.D.J. Econ.Bot. 18, 164 (1964).
11. Peat S., Whelan W.J. and Lawley H.G. J.Chem.Soc., 724 and 729 (1958).
12. Percival E.and McDowell R.H. "Chemistry and Enzymology of Marine Algal Polysaccharides" (Academic Press, London), 70 (1967).
13. Handa N. and Nisizawa K. Nature (London), 192, 1078 (1961).
14. Black W.A.P. J.Mar.Biol.Ass.U.K. 29, 379 (1950).
15. Percival E.G.V. and Ross A.G. J.Chem.Soc. 3041 (1949).
16. Percival E. Br.Phycol.J., 14, 103-117 (1979).
17. Percival E.G.V. and Ross A.G. J.Chem.Soc. 717-720 (1950)
18. Medcalf D.G. and Larsen B. Carbohydr.Res. 59, 531-537; 539-546 (1977).
19. Larsen B. Physiological and biochemical methods (Hellebust, J.A. and Craigie J.S., eds.) Cambridge University Press, Cambridge p.152-156 (1978).

20. Black W.A.P., Dewar E.T. and Woodward F.N. J.Sci.Food Agric., 3, 122-129 (1952).

21. Quatrano R.S., Brawley S.H. and Hogsett W.E. Determinants of spatial organisation. (Subtelny.Konisberg., eds.) p.77-95 Academic Press (1979).

22. Madgwick J.C., and Ralph B.J. Proc.6th Int.Seaweed Symp.(Magalef R. ed.) Dirección general de pesca marítima, Madrid. p.539-574 (1969).

23. Larsen B, Haug A and Painter T.L. Acta Chem.Scand., 20, 219-230 (1966).

24. Bernardi G., Springer G.F. J.Biol.Chem., 237 75-80 (1962).

25. Quillet M. Bull.Lab.maritime de Dinard,45, 63-68 (1959).

26. Jabbar Mian A. and Percival E. Carbohydr.Res., 26, 133-146 (1973)

27. Ovodov Y.S. Khomenko V.A., and Guseva T.F. Khim. Prir. Soedin., 9, 107-108 (1973).

28. Abdel-Fattah A.F., Hussein MM-D, and Salem H.M. Phytochem., 12, 1995-1998 (1973).

29. Anno K., Terahata H.,Hagashi Y.,and Seno N. Agr.Biol.Chem., 30, 495-499 (1966).

30. Pavlenko A.F. and Ovodov Y.S. Khim.Prir.Soedin., 697-699, (1974).

31. Villarroel L.H. and Zanlungo A.B. Rev.Latinoamer Quim. 6,127-130 (1975).

32. Abdel-Fattah A.F., Hussein MM-D and Fouad S.T. Phytochem. 17, 741-743 (1978).

33. Hussein MM-D, Fouad S.T. and Abdel-Fattah A.F. Carbohydr.Res. 72, 177-181 (1979).

34. Percival E. and Young M., Carbohydr.Res., 32, 195-201, (1974).

35. Fujikawa T., and Nakashima K. J. Agric. Chem. Soc. Japan, 49, 455-461 (1975).

36. Fujikawa T., and Wada M. Agric. Biol. Chem., 39, 1109-1114, (1975).

37. Quillet M. Colloques internat. du centre Nat. de la recherche. 103, 145-158, (1961).

38. O'Neill A.N. J. Am. Chem. Soc. 76, 5074 (1954).

39. Larsen B. and Haug A. Acta Chem. Scand., 17, 1646, (1963).

40. Medcalf D.G., Schneider T.L. and Barnett R.W. Carbohyd. Res., 66, 167-171 (1978).

41. Jabbar Mian A. and Percival E. Carbohyd. Res., 26, 147-161 (1973).

42. Conchie J. and Percival E.G.V. J. Chem. Soc., 827-832, (1950).

43. Coté R.H. J. Chem. Soc., 2248-2254 (1959).

44. Percival E. Carbohydr. Res. 17, 121-126 (1971).

45. Abdel-Fattah A.F., Hussein MM-D., Salem H.M. Carbohydr. Res. 33, 19-24 (1974).

46. Anno K., Seno N., and Ota M. Proc. Int. 6th Seaweed Symp. (R. Margalef ed.) Dirección general de Pesca Marítima, Madrid, p. 421-426 (1969).

47. Pavlenko A.F., Belogortseva N.I., Kalinovskii A.I. and Ovodov Y. S. Khim. Prir. Soedin. 573-577 (1976).

48. Bidwell R.G.S., Percival E. and Smestad B. Can. J. Bot., 50, 191-197 (1972).

49. Lestang G.D. and Quillet M. Physiol. Veg. 12, 199-227, (1974).

50. Schweiger R.G. J. Org. Chem. 27, 4267, (1962).

51. Springer G.F., Wurzel H.A., McNeal G.M. Jr., Ansell N.J. and Doughty M.F. Proc. Soc. exp. Biol. Med. 94, 404 (1957).

51a. Flowers H.M. Chemistry and Biochemistry of D-and L-Fucose. Adv. in Carbohydr. Chem. and Biochem. Vol. 39, p. 279-345 (1981).

52. Stanford E.C.C. Chem.News. London 96, 254 (1883).

53. South G.R. Proc. 9th Int. Seaweed Symp. (Jensen A., Stein Janet R. eds.) Science Press, Princeton, p.133-142 (1978).

54. Drummond D.W., Hirst E.L. and Percival E. Chem.Ind., 1088 (1958).

55. Haug A., Larsen B. and Smidsrod O. Acta Chem.Scand., 20, 183-190 (1966).

56. Haug A., Larsen B. and Smidsrod O. Acta Chem.Scand., 21, 691-704 (1967).

57. Simoneson C.I.,Popa V.I.,Rusan V.,and Liga D. Cellulose Chem.Technol. 10, 587-594 (1976).

58. Min K.H., Sasaki S.F., Kashiwabara Y., Umekawa M. and Nisizawa K. J.Biochem.(Tokyo) 81, 555-562 (1977).

59. Boyd J.and Turvey J.R. Carbohydr.Res.,66, 187-194 (1978).

60. Penman A.and Sanderson G.R. Carbohydr.Res., 25, 273-282 (1972).

61. Grasdalen H., Larsen B. and Smidsrod O. Carbohydr.Res., 68, 23-31 (1979).

62. Grasdalen H., Larsen B. and Smidsrod O. Proc. 9th Int.Seaweed Symp. (Jensen A., SteinJanet R. eds.) Science Press Princeton, p.309-317 (1978).

63. Grasdalen H., Larsen B. and Smidsrod O. Carbohydr.Res., 56, C11, (1977).

64. Grasdalen H., Larsen B. and Smidsrod O. Carbohydr.Res., 89, 179, (1981).

65. Morris E.R., Rees D.A. and Thorn D. Carbohydr.Res., 80, (1980).

66. Siddiqui I.R. Carbohydr.Res., 67, 289-293,(1978).

67. Siddiqui I.R. Carbohydr.Res., 63, 312-314, (1978).

68. Ross A.G., Percival E.G.V. Nature (London) 162, 895 (1948).

69. Percival E.G.V., and Ross A.G. J.Chem.Soc., 895. (1949).

70. Searles R.B. Br.Phycol.J. 13, 361-381 (1978).

71. Vargas Cobeña R.H. Bol.Soc.Quim.Perú, 40, 1, 55-64 (1974).

72. Silva F., Etcheverry H and Quilhot W. Botánica Marina 8, 2-4; 244-251 (1965).

73. Landsberger W., Schwencke J., and Abarca F. Botánica Marina, 12, 1-4; 218-227 (1969).

3.5.2. REFERENCES CHAPTER III Results and discussion

1. Haug A., Larsen B. and Baardseth E. Proc. 6th Int Seaweed Symp. (Margalef R. ed.) 6, 443-451 (1969).

2. Stockton B., Evans, L.V., Morris E.R., Powell D.A., and Rees D.A. Botánic.Marina. XXIII, 563-567 (1980).

3. Quatrano, R.S.,Hoggett W.S. and Roberts M. Proc. 9th Int.Seaweed Symp. (Jensen A., Stein Janet R. eds.) 9, 113-123 (1979).

4. Black W.A.P., J.Mar.Biol.Ass. U.K. 33, 49 (1954)

5. Whistler R.L. and BeMiller J.N. Advan.Carbohydr.Chem. 13, 289 (1958).

6. Gel Chromatography. A Laboratory Manual, published by Bio-Rad Laboratories, California, U.S.A. 15-19 (1971).

7. Percival E. and McDowell R.H. Chemistry and Enzymology of Marine Algal Polysaccharides. Academic Press Inc. (London) Ltd. p.117 (1967).

8. Lewis B.A. and Smith F. J.Am.Chem.Soc. 79 3931 (1957).

9. Bjorndal H., Hellerquist C.G., Lindberg B. and Svensson S., Angew Chem.Int. Ed.Engl. 9, 610 (1970)

10. Barrett A.J. and Northcote D.H., Biochem.J., 94, 617 (1965)

11. Jarvis M.C., Threlfall D.R. and Friend J. Phytochem., 16, 849-52 (1977).

12. Rahman M.A., PhD Thesis, University of London (1979).

13. Carlberg G.E., Percival E.and Rahman M.A., Phytochem, 17, 1289-92 (1978).

14. Mian A.J. and Percival E., Carbohydr.Res., 26,133 and 147 (1973).

15. Carlberg G.E., PhD Thesis, University of London (1974).

16. Heri W.J.Neukom H, and Deuel H. Helv.Chim.Acta, 44, 1939 (1961).

17. Laurent T.C., Arch.Biochem.Biophys., 92, 224 (1961).

18. Neukom H., Deuel H, Heri W.J. and Kundig W. Helv.Chim. Acta, 43, 64 (1960).

19. Percival E G.V., Quart.Rev., 3, 369-384 (1949)

20. Haug A. and Larsen B., Acta Chem.Scand., 15, 1395 (1961)

21. Percival E., and Wold J.K. J.Chem.Soc., 5459-5468 (1963).

22. Grasdalen H., Larsen B. and Smidsrod O., Carbohydr.Res. 89,179-191 (1981).

23. Haug A., Larsen B. and Smidsrod O., Carbohydr.Res. 32, 217-225 (1974).

CHAPTER IV

STRUCTURAL STUDIES OF THE "FUCAN" EXTRACTED WITH HOT AQUEOUS, 2% $CaCl_2$, FROM Lessonia nigrescens BROWN SEAWEED.

4.1. EXPERIMENTAL

Experiment 4.1

"Fucan" (1 g.), was partially hydrolysed with 0.25 M oxalic acid, 40 ml 100°C, 3 hours, dialysed and neutralised [GM 2.8.15]. Yields of fragments and polymeric, soluble and insoluble, residues inside dialysis sac are shown in flow chart V. p. 146.

Experiment 4.2

"Fucan" (1 g.), was partially hydrolysed with 0.125 M oxalic acid, 40 ml, 100°C, 3 hours. Under nearly the same procedure as above [GM 2.8.15], but with some modification in the way of neutralisation. Instead of using $CaCO_3$, the fragments were shaken with IR-120 (H^+) resin and then treated with ammonia solution until neutrality. Yields are shown in brackets in flow chart V. p. 146.

Experiment 4.3

"Fucan" (1 g.), was partially hydrolysed with M trifluoro-acetic acid, 1 hour at 70°C. Once cool, it was dialysed (4 x 300 ml). Dialysates were concentrated and neutralised with IR-45 (OH) resin.

Experiment 4.4.

The hydrolysates obtained in Exp. 4.1, 4.2 and 4.3 were analysed by paper chromatography solvents [GM 2.4.1.1, 2.4.1.2, 2.4.1.3, 2.4.1.4 and 2.4.1.5] and locating reagent [GM 2.5.2]. The mobilities of the oligosaccharides obtained from the oxalic acid hydrolyses were identical. These mobilities in the different solvents are given in Table 4.1, p. 147.

Experiment 4.5

The separation of the derived oligosaccharides was attempted by HPLC chromatography (Waters ALC 202), column Lichrosphere Si 100 (Merck) 60 x 0.2 cm; solvent used 40% water in acetonitrile with a flow pressure of 700-800 psi and a chart speed of 10 mm/hour. Results were compared with a standard partial hydrolysate of starch.

Experiment 4.6

A large scale partial hydrolysis of a freeze-dried sample of "fucan", (8 g., ca. 4.8 of carbohydrate) was carried out with 0.25 M oxalic acid for 3 hours at 100°C [GM 2.8.15]. By this time there was still some insoluble material.

The hydrolysate (including the insoluble material already mentioned) was dialysed against deionised water (10 x 500 ml) and then the combined dialysates concentrated down to 500 ml and neutralised with solid $CaCO_3$. After filtration, the carbohydrate content was estimated [GM 2.7.1] as 3.0 g.

The non-dialysable materials (remaining inside the dialysis sac), consisting of polymeric soluble and insoluble residues, were centrifuged, freeze-dried and weighed, 1.2 g. and 1.63 g. were found respectively. Procedure and yields are shown in flow chart VI. p. 149.

4.1.1. Investigation of the dialysate fragments and soluble and insoluble polymeric residues from large scale partial hydrolysate.

Experiment 4.7

Dialysate fragments were analysed for carbohydrate [GM 2.7.1] 3.0 g. (based on carbohydrate) and separated into acidic and neutral fractions on a column [41 x 3 cm] containing Zerolit FFIP SRA 69 anion exchange resin.

The neutral sugars, monosaccharides and oligosaccharides together, were eluted with deionised water and the acidic oligosaccharides with increasing concentration of formic acid. The carbohydrate content of both fractions was determined (neutral 1.8 g and acidic 0.63 g) i.e. 81% recovery. See flow chart VI The neutral fraction was concentrated (ca. 20 ml). After removal of the formic acid by repeated distillations with methanol the acidic oligosaccharide fractions (0.5 M and 2 M formic acid) were also concentrated separately.

4.1.1.1. Characterisation of neutral oligosaccharides.

Experiment 4.8

The separation of the neutral fraction into mono, di, tri, tetra, etc. saccharides was attempted on a Bio-Gel P-2 resin, 400 mesh column (90 x 1.7 cm) by elution with 0.1 M NaCl. Flow rate 10 drops/min and collection of 2.5 ml fractions which were estimated for carbohydrate [GM 2.7.1].

Experiment 4.9

Since the previous experiment was unsuccessful, the separation of monosaccharides from oligosaccharides was carried out on two sheets of No. 17 Whatman chromatography paper with solvent 6:4:3 = nBuOH:Py:H_2O [GM 2.4.1.1] running for 22 hours. By this means 6 neutral oligosaccharides were also separated.

The appropriate areas corresponding to the individual neutral oligosaccharides were eluted with water from the paper strips, filtered through millipore filter (0.45 μ) and analysed for carbohydrate content [GM 2.7.1]. Weights of these 6 neutral oligosaccharides, calculated on carbohydrate content as galactose can be seen in Table 4.2. p. 152

The separated neutral oligosaccharides were analysed by paper chromatography in solvents 6:4:3 = n-BuOH:Py:H_2O ; 18:3:1:4 = EtOAc:AcOH:HCOOH:H_2O and

40:11:19 = nBuOH:EtOH:H_2O [GM 2.4.1.1; 2.4.1.2. and 2.4.1.3]. Single spots were produced for each oligosaccharide, locating reagent [GM 2.5.2], with mobilities R_{Glc} = 0.04; 0.09; 0.12; 0.27; 0.40 and 0.76 in solvent 18:3:1:4 = EtOAc:AcOH:HCOOH: water [GM 2.4.1.2.], R_{Glc} = 0.04; 0.10; 0.18; 0.24; 0.43 and 0.77 in solvent 6:4:3 = nBuOH:Py:H_2O [GM 2.4.1.1.] See photograph in Figure 4.1 p.151 and R_{Glc} = 0.05; 0.10; 0.15; 0.29; 0.42 and 0.82 in solvent 40:11:19 = nBuOH:EtOH: H_2O [GM 2.4.1.3]. See Table 4.2. p.152.

Experiment 4.10

The specific rotation [GM 2.1.4], Table 4.3 and the degree of polymerization [GM 2.7.5] were determined for each neutral oligosaccharide. The absorbance of non reduced and reduced sugars was compared against a standard galactose graph. The approximate size corresponding to each neutral oligosaccharide can be seen in Table 4.3. p.152.

Experiment 4.11

A portion of each neutral oligosaccharide was hydrolysed with $\underline{\underline{M}}H_2SO_4$ [GM 2.3.2] and half of the hydrolysates analysed for sugars on paper chromatograms, solvents 6:4:3 = nBuOH:Py:H_2O and 18:3:1:4 = EtOAc:AcOH: HCOOH:H_2O [GM 2.4.1.1 and 2.4.1.2] 24 hours and 18 hours running respectively. The hydrolysate of each oligosaccharide gave a single spot with R_{Glc} = 0.86 and 0.88 for the respective solvent which was identical with the mobility of galactose run on the same papers; aniline oxalate [GM 2.5.2.] used as locating reagent, but with silvernitrate as locating reagent [GM 2.5.1] traces of other sugars were found.

The rest of the neutral oligosaccharide hydrolysates were reduced separately [GM 2.8.4], converted into the alditol acetate derivatives [GM 2.8.5]

and analysed on g.l.c. [GM 2.6.1 and 2.6.2]. A peak corresponding to galactose was given by each hydrolysate.

Experiment 4.12

Each neutral oligosaccharide (ca. 2 mg) was methylated following the Bishop and Perila modification of the Kuhn methylation method [GM 2.8.7]. Samples were shaken with methyl iodide (0.2 ml), N-N dimethyl formamide (0.2 ml) and dry silver oxide (0.2 ml) at room temperature for 18 hours in darkness; then the mixture was filtered and the residue washed with dry chloroform. The combined filtrate and washings were dried. The methylated oligosaccharides were obtained after evaporation to dryness under reduced pressure at room temperature. The methylated samples were then hydrolysed [GM 2.3.1] reduced [GM 2.8.4] and converted into the alditol acetate derivatives [GM 2.8.5] to be analysed on g.l.c. [GM 2.6.1 and 2.6.2] and g.l.c.-m.s. [GM 2.6.3].

Experiment 4.13

Neutral oligosaccharides 1, 2 and 3 (R_{Glc} = 0.04; 0.09 and 0.12 respectively, Table 4.2) were treated separately with both α and β-galactosidase [GM 2.8.12] to determine whether they have α-or β-linkages or at least which is predominant. Substrates, including the neutral oligosaccharides already named and standard melibiose and lactose samples, were mixed with α and β enzymes, incubated at 30°C for 30 minutes to allow reaction and then heavily applied (1 mg/ml) to plastic backed F 1500 silica gel thin layer plates. Upward development was performed using solvent 6:3:1:1 = Ethyl Acetate:Pyridine: Acetic Acid: Water and locating reagent 4-amino-benzoic acid (PABA). The colour reaction was induced by heating the plates in a hot air oven (110-115°C) for 3 minutes.

Results can be seen in Figures 4.3 and 4.4 p. 156 and 157 respectively.

Experiment 4.14

Neutral pentasaccharide 2 and tetrasaccharide 3 (see Table 4.3) were analysed by ^{1}H n.m.r. in a 400 MHz apparatus at 343°K with TSP as external reference and at 296°K with HDO reference.

Experiment 4.15

Molecular weight of the heptasaccharide 1 was estimated through a Sepharose 4B column following procedure outlined in Experiment 3.13, Chapter III. SeeFigure 4.2 p.154.

4.1.1.2. Characterisation of the Acidic Oligosaccharides.

Experiment 4.16

Fractions of 0.5M, 1M and 2M formic acid solutions containing acidic oligosaccharides were collected separately from the Zerolit FF1P, SRA69 anion exchange resin column (see Experiment 4.7) and estimated for carbohydrate content [GM 2.7.1]. A total of 630 mg carbohydrate was collected. See flow chart VI p. 149

Experiment 4.17

Ionophoresis at pH 6.8 and 10 [GM 2.4.2.2. and 2.4.2.3.] were carried out for each of the above fractions. At pH10 only a long band for each fraction was produced and at pH 6.8 three spots with mobilities $M_{Glc.A.}$ = 0.4; 0.7 and 0.9 were found for each fraction.

Experiment 4.18

Each of the above acidic fractions was analysed by paper chromatography in solvents 6:4:3 = nBuOH:Py:H_2O [GM 2.4.1.1] running 29 hours; 18:3:1:4 = EtOAc:AcOH: HCOOH:H_2O [GM 2.4.1.2.] 26 hours running; 40:11:19 = n-BuOH:EtOH:H_2O [GM 2.4.1.3] 3 x 24 hours running, dried and put to run again, and finally solvent 50:12:25 = n-BuOH:AcOH : H_2O [GM 2.4.15] 3 x 50 hours running, taken and dried between each run. The best separation was found for the

0.5 M formic acid fraction in solvent 18:3:1:4 = EtOAc. :AcOH : HCOOH : H_2O with distinct spots whose mobilities were $R_{Glc\ A.}$ = 0.05; 0.19; 0.35; 0.52; 0.80 and 1.01. The spot with 0.80 mobility was the largest, fairly elongated and probably contained more than a single oligosaccharide.

Experiment 4.19

An aliquot (0.1 of the volume; ca. 14 mg carbohydrate) of the 0.5 M formic acid was spotted on a strip of 3 MM paper chromatogram (6 cm width) to determine whether these 6 oligosaccharides could be separated preparatively. The paper was developed for 2 x 20 hours (taken and dried) in solvent 18:3:1:4 =EtOAc : AcOH : HCOOH : H_2O [GM 2.4.1.2]. See Figure 4.7 p. 163.

Accordingly the remainder (ca. 130 mg carbohydrate) of the 0.5 M formic acid eluant from the Zerolit FFIP SRA 69 column (see Experiment 4.7) was separated on two sheets of 3 MM paper chromatograms. The papers were developed 2 x 20 hours, as above in solvent 18:3:1:4 = EtOAc. :AcOH: HCOOH: H_2O. Eight acidic oligosaccharides with the mobilities of R_{Glc} = 0.03; 0.18; 0.27; 0.37; 0.54; 0.67; 0.84 and 1.0 were separated. The carbohydrate content of each was determined. See Table 4.6 p.162.

Experiment 4.20

The chromatographic purity of each of the above separated oligosaccharides was tested on a paper chromatogram, developed for 2 x 20 hours, in solvent 18:3:1:4= EtOAc :AcOH:HCOOH:H_2O and located with aniline oxalate reagent. [GM 2.5.2]. Each gave a single spot with the appropriate mobility.

Experiment 4.21

The specific rotation [GM 2.1.4.] and degree of polymerization [GM 2.7.5] were estimated for each acidic

oligosaccharide. Results for specific rotations can be seen in Table 4.6. p. 162.

Experiment 4.22

Acidic oligosaccharides 2, 3, 7 and 8 (R_{Glc} = 0.18; 0.27; 0.84; and 1.00 respectively) were esterified separately with MeOH/HCl method [GM 2.8.3.1] and reduced with $NaBH_4$. Both procedures were repeated. The derived neutral oligosaccharides were hydrolysed with $\underline{\underline{M}}$ H_2SO_4 [GM 2.3.2]. Half of these four hydrolysates were again reduced with $NaBH_4$ [GM 2.8.4] and converted into the alditol acetate derivative [GM 2.8.5] and analysed on g.l.c. [GM 2.6.1 and 2.6.2]. The relative molar proportions of the constituents sugars of the four oligosaccharides calculated from peak areas are shown in Table 4.7 p. 164.

The other half of the four hydrolysates were analysed for their constituent sugars by paper chromatography solvent [GM 2.4.1.1.] and specifically for glucose with glucose oxidase reagent [GM 2.5.3] to determine that the glucuronic acid had been reduced.

Experiment 4.23

Acidic oligosaccharides 1, 4, 5 and 6 (R_{Glc} = 0.03; 0.37; 0.54 and 0.67 respectively. See Experiment 4.19) were esterified separately with diazonmethane [GM 2.8.6.1 and 2.8.6.2]; ca. 15 mg carbohydrate solution of each sample were evaporated to dryness. A solution of diazomethane (containing not less than 5 mg) in dichloromethane was added. After standing for 6 hours at room temperature samples were evaporated down to remove the solvent and excess of diazomethane. Then reduced with $NaBH_4$ [GM 2.8.4]. Both procedures were carried out three times.

Half of each of the derived four neutral oligosaccharides were hydrolysed with $\underline{\underline{M}}$ H_2SO_4 [GM 2.3.2] and a portion of each of the hydrolysates analysed for sugars by paper chromatography, solvents 6:4:3 = n-BuOH:Py:H_2O and 18:3:1:4 = EtOAc :AcOH:HCOOH: H_2O [GM 2.4.1.1. and 2.4.1.2] and staining reagents aniline oxalate [GM 2.5.2] and glucose oxidase [GM 2.5.3]. The latter specifically for glucose derived from the reduced glucuronic acid.

The rest of the hydrolysates were then reduced [GM 2.8.4] converted into the alditol acetates [GM 2.8.5] and analysed on g.l.c. [GM 2.6.1. and 2.6.2.] See Table 4.7 for molar proportion of constituents calculated from peak areas.

Experiment 4.24

The other half of the above four neutral oligosaccharides were methylated following the Bishop and Perila modification of the Kuhn method [GM 2.8.7] as described for the original neutral oligosaccharides (see Experiment 4.12); then hydrolysed with $\underline{\underline{M}}$ H_2SO_4 [GM 2.3.2], reduced to the polyalcohols [GM 2.8.4] and converted into the methylated alditol acetate derivatives [GM 2.8.5] and analysed on g.l.c. [GM 2.6.1 and 2.6.2] and g.l.c.-m.s. [GM 2.6.3].

Experiment 4.25

Two standard samples, one a mixture of glucuronic acid and xylose (1:1) and the other glucuronic acid only, were separately esterified with MeOH/HCl [GM 2.8.3.1] and reduced with $NaBH_4$ [GM 2.8.4]. Both procedures were repeated, then the derived materials were hydrolysed with $\underline{\underline{M}}$ H_2SO_4 [GM 2.3.2].

Half of the hydrolysates were analysed for sugars on paper chromatogram, solvents [GM 2.4.1.1. and 2.4.1.2], locating reagents [GM 2.5.1 and 2.5.2] and particularly with glucose oxidase reagent [GM 2.5.3] to

estimate to what extent had glucuronic acid been converted into glucose.

Half of the mixture hydrolysate was reduced [GM 2.8.4] and converted to the alditol acetate derivative [GM 2.8.5] to be analysed on g.l.c. [GM 2.6.1 and 2.6.2]. A relative molar proportion of glucuronic acid:xylose = 1:2 was found.

Experiment 4.26

In order to check the loss of some glucuronic acid after esterification and reduction, 7.8 mg of glucuronic acid were accurately weighed and esterified and reduced following the procedure outlined in Experiment 4.25. The hydrolysate was then estimated for carbohydrate content [GM 2.7.1] and 4 mg were found.

Acidic oligosaccharides 4 and 6 [R_{Glc} = 0.37 and 0.67 respectively, see Table 4.6) were estimated for carbohydrate [GM 2.7.1] after they had been esterified [GM 2.8.6.1 and 2.8.6.2] and reduced with $NaBH_4$. Initial material had been estimated for carbohydrate also. Initial and final carbohydrate content are shown in Table 4.8, ca. 50% loss. p. 165.

4.1.1.3 Characterisation of soluble and insoluble residues.

Experiment 4.27

Soluble and insoluble residues were analysed for carbohydrate content [GM 2.7.1], uronic acid content [GM 2.7.2.2.(2).], protein content [GM 2.7.3] and ash content. Results can be seen in Table 4.9. p. 170.

Experiment 4.28

30.7 mg of soluble and 68.6 mg of insoluble residues were hydrolysed with 90% HCOOH [GM 2.3.1] and the hydrolysates analysed by paper chromatography, solvents [GM 2.4.1.1. and 2.4.2.2] and staining reagent [GM 2.5.2]. The hydrolysates were then reduced

[GM 2.8.4], converted into alditol acetates derivatives [GM 2.8.5] and analysed on g.l.c. [GM 2.6.1 and 2.6.2]. Approximate molar proportion of sugars are shown in Table 4.10. p. 171.

Experiment 4.29

A portion of the soluble residue was estimated for molecular weight, layering the sample on top of a Sepharose 4B column and eluted as outlined in Experiment 3.13 on previous Chapter III, p. 74.

Experiment 4.30

100 mg of the insoluble residue were dispersed in 50 ml of water and boiled up under reflux for 1 hour, then centrifuged. Residual solid was freeze dried and weighed (61 mg). The supernatant was filtered through millipore glass fibre filters and estimated for carbohydrate content [GM 2.7.1] and protein content. The solution was then freeze dried and weighed (16 mg).

Experiment 4.31

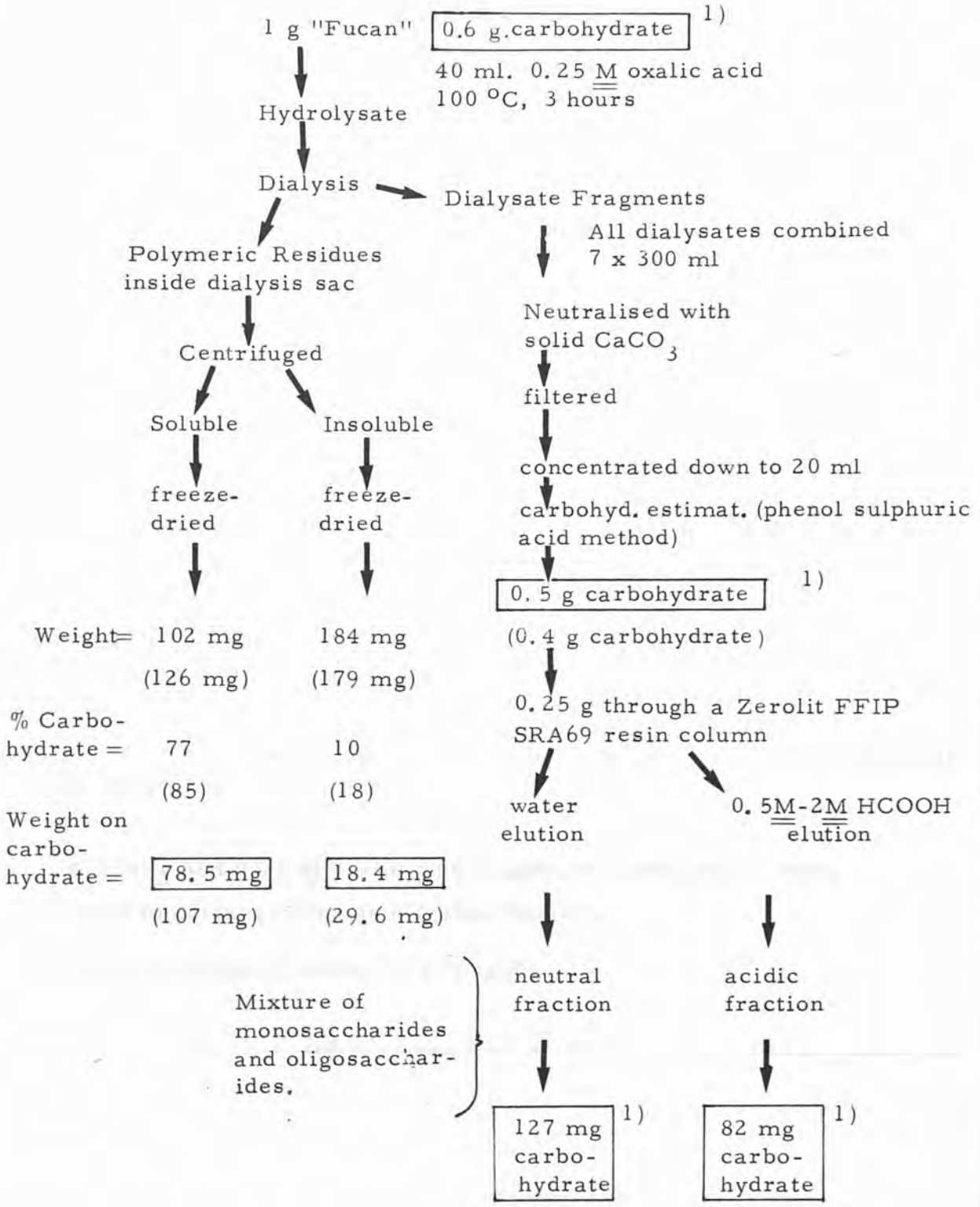
A portion of the soluble residue, 30 mg, was shaken three times with D_2O and freeze-dried after each shaking. Then analysed on ^{1}H n.m.r. at 296°K, DSS as external reference. ^{13}C n.m.r. for another sample of this soluble residue, 60 mg at 343°K with TSP as external reference, was also carried out. Both techniques were carried out on a 400 MHz apparatus.

4.2. RESULTS AND DISCUSSION

The "fucan" extracted with hot aqueous 2% $CaCl_2$ from Lessonia nigrescens in the previous chapter has now been subjected to partial acid hydrolysis studies. It contains approximately 60% carbohydrate, 9% sulphate, 26% uronic acid, 11% protein, 12% of ash and about 10% of moisture by weight (see Table 3.10, Chapter III, p.113). This means that about 43% of the carbohydrate is glucuronic acid and the carbohydrate carries 15% of sulphate. A formic acid hydrolysate contains the following percentages of monosaccharides: fucose 43, xylose 7, mannose 20 and galactose 25 (see Table 3.15, Chapter III, p.122.

Preliminary partial hydrolysis studies with 0.25 $\underline{\underline{M}}$ and 0.125 $\underline{\underline{M}}$ oxalic acid (see Flow chart V) and $\underline{\underline{M}}$ trifluoroacetic acid (see Experiment 4.3) for different periods of time revealed that 0.25$\underline{\underline{M}}$ oxalic acid for 3 hours at 100^{o} gave the best yield of oligosaccharides, although the two concentrations of oxalic acid gave the same overall pattern of oligosaccharides on paper chromatograms in a variety of solvents (see Table 4.1).

Flow chart V Small scale partial hydrolysis of "Fucan"
Hot aqueous, 2% $CaCl_2$, extract.



1) Read from standard graph
Fu:GlcA:Gal:Xy:Mann = 5:2:1.5:1:0.5

In brackets are shown yields for partial hydrolysis with 0.125 M oxalic acid.

TABLE 4.1

Mobilities in different solvents for 0.125 M and 0.25 M oxalic acid partial hydrolysis dialysate fragments.*

Solvent	Time running	R_{Glc}
6:4:3 ($Bu:Py:H_2O$)	22 hours	0.04; 0.10; 0.24; 0.33; 0.49; 0.59; 0.73.
6:4:3 (n-$BuOH:Py:H_2O$)	2 x 20 hours**	0.04; 0.10; 0.17; 0.24; 0.31; 0.43; 0.56; 0.75.
18:3:1:4 (EtO Ac:AcOH:HCOOH : H_2O)	20 hours	0.04; 0.09; 0.18; 0.25; 0.33; 0.43; 0.63; 0.76; 0.89.
40:11:19 (n-$BuOH:EtOH:H_2O$)	36 and 3 x 18 hours**	0.1; 0.18; 0.28; 0.39; 0.44; 0.49; 0.55; 0.66; 0.74.
3:1:1 (n-$BuOH:EtOH:H_2O$;3% Cetylpyridinium Chloride)	35 hours	0.09; 0.25; 0.41; 0.66; 0.80.
50:12:25 (n-$BuOH:AcOH:H_2O$)	48 hours	0.07; 0.12; 0.24; 0.39; 0.56; 0.69; 0.98.

* 0.125 M and 0.25 M oxalic acid fragments hydrolysates were found to contain the same oligosaccharides.

** Taken to dryness and put to run again.

The oligosaccharide mixture in the trifluoracetic acid hydrolysate failed to separate on paper chromatograms.

Attempted separation of the oxalic acid hydrolysates on H.P.L.C. (Exp. 4.5) was unsuccessful. Two broad bands were obtained, presumably one for monosaccharides and the other containing the oligosaccharides.

The small molecules in the hydrolysates were separated from larger polymeric material by dialysis.

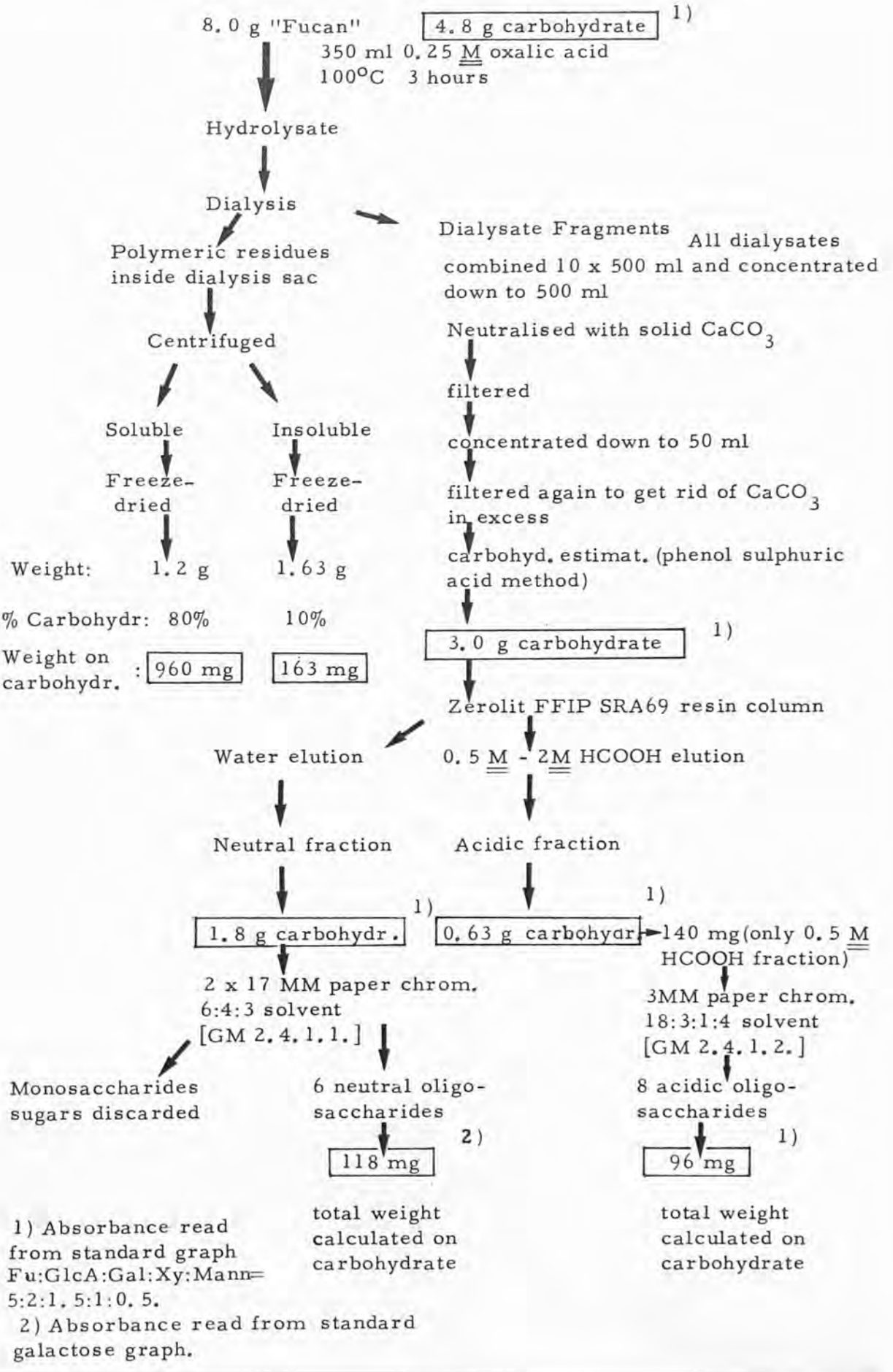
Insoluble material inside the dialysis sac, about 18% of the starting material,contained only 10% of carbohydrate, and soluble material inside the sac was freeze-dried to a white solid, 10% of the starting material,which contained 77% of carbohydrate.

The fragments which passed through the dialysis sac were separated on a resin column into neutral carbohydrate (ca. 25% of the starting material) and acidic oligouronic acids (ca. 16% of the starting material).

In view of these results a large scale hydrolysis on 8 g "fucan" (4.8 g carbohydrate) was carried out with 0.25 M oxalic acid (see Exp. 4.6 and Flow chart VI).

Flow chart VI

Large scale partial hydrolysis of "Fucan" hot aqueous, 2% $CaCl_2$, extract



1) Absorbance read from standard graph Fu:GlcA:Gal:Xy:Mann= 5:2:1.5:1:0.5.

2) Absorbance read from standard galactose graph.

After separation by dialysis, 1.63 g of insoluble material containing 10% of carbohydrate was separated, freeze drying the solution from inside the sac gave 1.2 g of polymeric material containing 80% of carbohydrate (ca. 20% of the starting material) as a white solid.

The solution which passed through the dialysis sac was neutralised and a solution containing approximately 3 g of carbohydrate (ca. 62.5% of the starting material) was obtained. This was separated on resin into 1.8g of carbohydrate as a neutral fraction and 0.63 g of acidic oligouronic acids (81% recovery from the column).

Attempted separation of the neutral fraction into mono-, di-, tri-, etc. saccharides on Bio-Gel P2 resin (see Expt. 4.8) was unsuccessful.

4.2.1. Characterisation of the neutral oligosaccharides.

From the above neutral fraction however six oligosaccharides* (118 mg, see Table 4.2) were separated from the monosaccharides on No. 17 Whatman chromatography paper (Expt. 4.9). The monosaccharides which consisted of fucose, xylose, galactose and mannose were discarded. A photograph of the chromatograph of the separated oligosaccharides can be seen in Fig. 4.1 p. 151.

The weight based on carbohydrate content and the mobility of each of these oligosaccharides in different solvents is given in Table 4.2. p. 152

* some oligosaccharides were mixtures (see later).

Figure 4.1 Paper chromatogram photograph of the separated neutral oligosaccharides in solvent 6:4:3 = nBuOH:Py:H_2O

TABLE 4.2

Mobilities and weights of the carbohydrate in neutral oligosaccharides.

Oligo-saccharide	R_{Glc} 18:3:1:4	R_{Glc} 6:4:3	R_{Glc} 40:11:19	Weight (mg)*
1	0.04	0.04	0.05	15.3
2	0.09	0.10	0.10	14.8
3	0.12	0.18	0.15	17.8
4	0.27	0.24	0.29	27.2
5	0.40	0.43	0.42	14.7
6	0.76	0.77	0.82	27.7

* Absorbance read from a standard graph of galactose.

The specific rotation and degree of polymerization[1] for each of these neutral oligosaccharides can be seen in Table 4.3.

TABLE 4.3

Specific rotation and degree of polymerization of neutral oligosaccharides.

Oligosaccharides	Specific rotation $[\alpha]_D^{25^\circ}$	Approximate degree of polymerization
1	+ 68.8 ($c = 2.76 \times 10^{-2}$ g/100 ml H_2O)	Heptasaccharide
2	+ 37.2 ($c = 1.21 \times 10^{-2}$)	Pentasaccharide
3	+ 126.7 ($c = 1.46 \times 10^{-2}$)	Tetrasaccharide
4	+ 53.9 ($c = 2.04 \times 10^{-2}$)	Trisaccharide
5	+ 65.9 ($c = 1.22 \times 10^{-2}$)	Trisaccharide
6	+ 45.3 ($c = 3.2 \times 10^{-2}$)	Disaccharide

It can be deduced from the facts presented in the last two tables that these oligosaccharides are not a homologous series.

That with the highest mobility (neutral oligosaccharide 6) corresponds to a disaccharide and that with the lowest mobility (neutral oligosaccharide 1) approximately to a heptasaccharide. The latter was analysed on a Sepharose 4B column (Expt. 4.15) and its molecular weight estimated (Figure 4.2). A value of ca 15×10^2 calculated from the Selectivity curve for Sepharose 4B on Figure 3.7. p. 98 was obtained. This corresponds to an octasaccharide which is in reasonable agreement with the figure obtained by the Timell method[1].

Hydrolysates of these neutral oligosaccharides run on paper chromatograms (Expt. 4.11) revealed from each a single spot with $R_{Glc} = 0.86$ and 0.88 in the different solvents; identical with that of galactose run as standard. This was confirmed when the alditol acetate derivatives of the hydrolysates were analysed by g.l.c. A single large peak with the retention time of that of galactitol acetate was obtained. Insignificant peaks for the alditols of the other sugars present in the "fucan" could also be seen. These can only be regarded as trace contaminants particularly since the amount was too small to be detected on paper chromatograms with aniline oxalate reagent.

Each of the neutral oligosaccharides was methylated (see Expt. 4.12) and the derived partly methylated alditol acetates were analysed on g.l.c. and g.l.c.-m.s. The partly methylated sugars in the hydrolysates are detailed in Table 4.4.

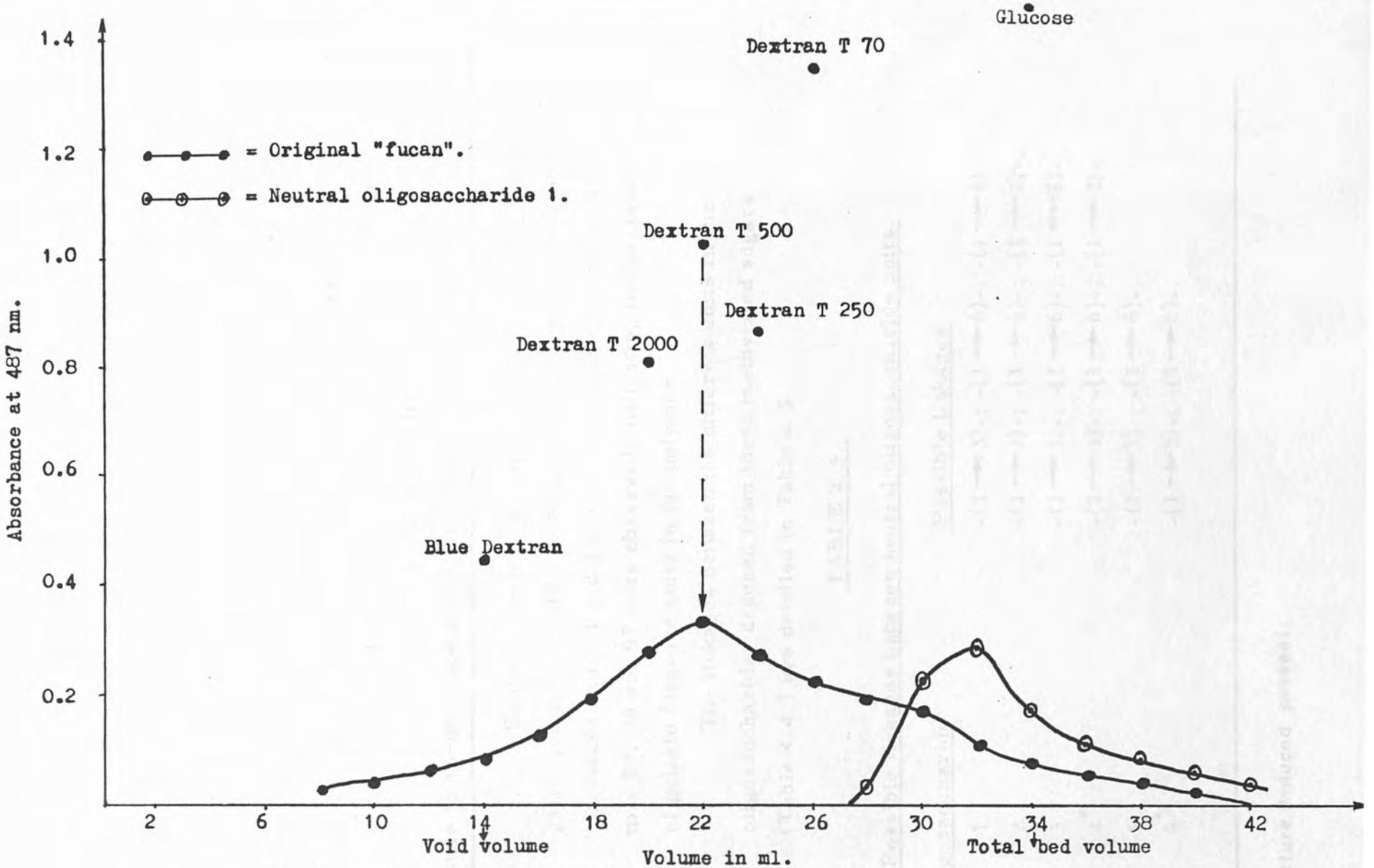


Fig. 4.2 Elution profiles of "fucan" and neutral oligosaccharide 1, obtained after partial hydrolysis of this "fucan". Column Sepharose 4B.

TABLE 4.4.

Methylated sugars derived from the methylated neutral oligosaccharides.

Oligosaccharides	Methylated sugars	G.l.c. retent. time**
1	2, 3, 4, 6-Tetra-O-methyl galactose	1.18
2	2, 4, 6-Tri-O- methyl galactose	2.01
3	2, 3, 4-Tri-O- methyl galactose	2.85
4*	3, 4, 6-Tri-O-methyl galactose	2.12
5	2, 3, 4, 6-Tetra-O-methyl galactose	
6*	2, 4, 6-Tri-O-methyl galactose	
	2, 3, 4-Tri-O-methyl galactose	

**Relative to 1,5-Di-O-acetyl-2,3,4,6-tetra-O-methyl-glucitol.

Evidence for the presence of 2, 3, 5, 6 tetra-O-methyl galactitol-1, 4-acetate was obtained in the hydrolysates of oligosaccharides 1 and 4 when in the mass spectra the peaks m/e 89, 59 and 97 were observed, indicating the presence of galacto furanose units in the polymer.

The linkages between the different units in the oligosaccharides deduced from these methylated sugars (Table 4.4.) are detailed in Table 4.5.

TABLE 4.5.

Possible linkages between neutral oligosaccharide units.

Oligosaccharide	Possible linkages
1	-(1 → 3)-; -(1 → 6)-; -(1 → 2)-.
2	-(1 → 3)-; -(1 → 6)-; -(1 → 2)-.
3	-(1 → 3)-; -(1 → 6)-; -(1 → 2)-.
4*	-(1 → 3)-; -(1 → 6)-; -(1 → 2)-.
5	-(1 → 3)-; -(1 → 6)-.
6*	-(1 → 3)-; -(1 → 6)-.

*mixture deduced present.

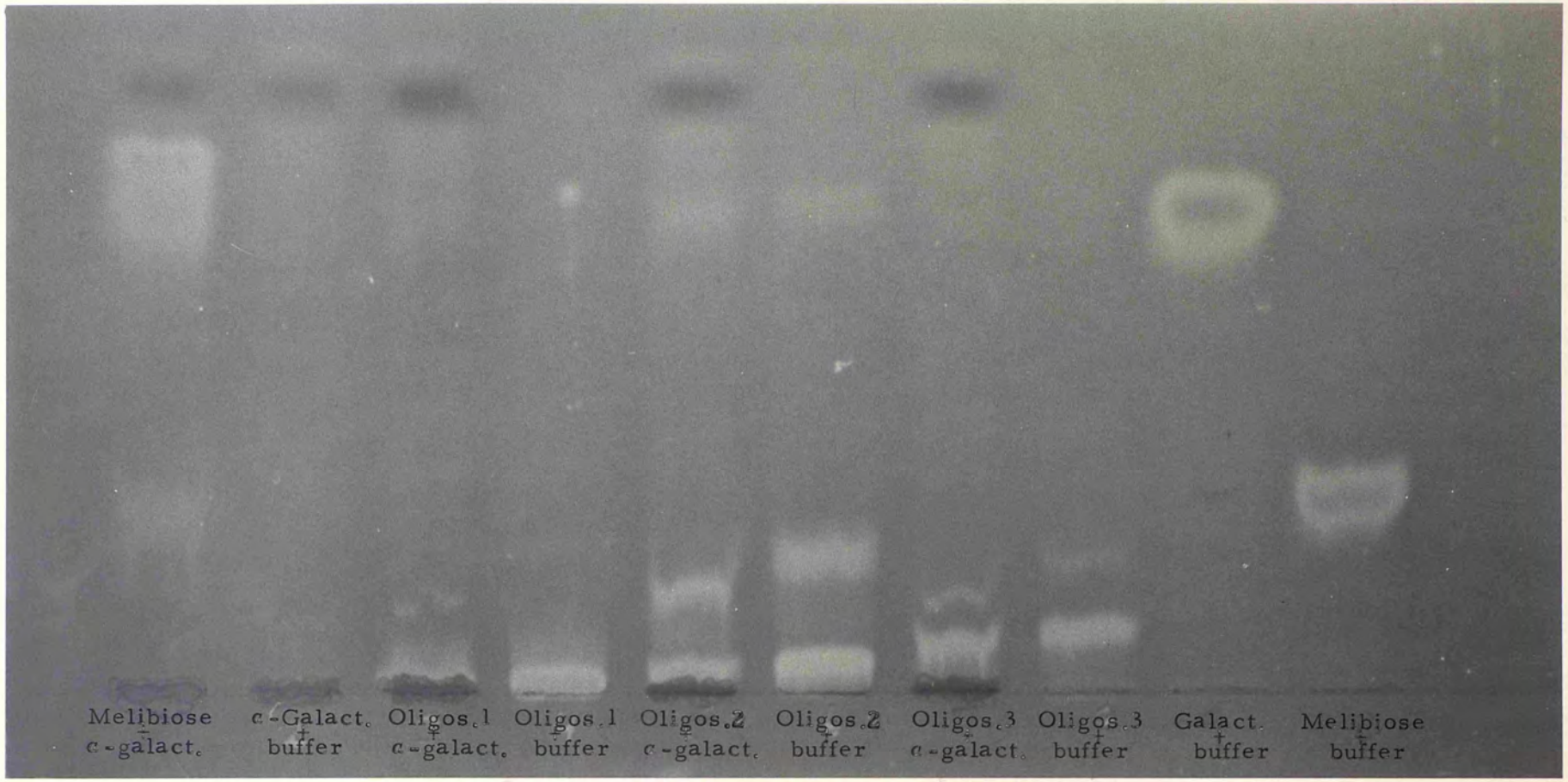


Figure 4.3 Enzymic reaction of neutral oligosaccharides with α galactosidase. Photograph taken under U.V. lamp.

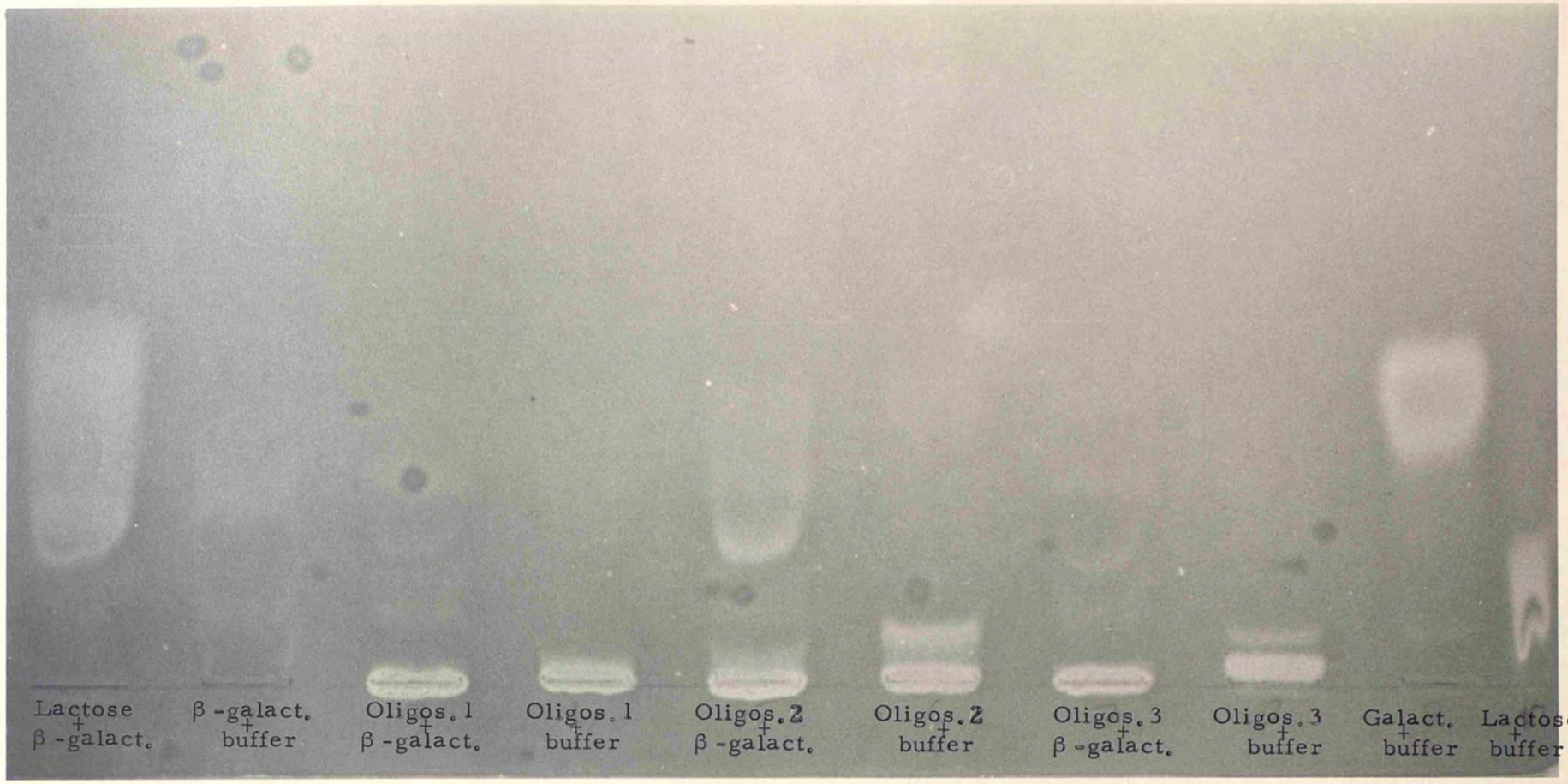


Figure 4.4. Enzymic reaction of neutral oligosaccharides with β galactosidase. Photograph taken under U.V. lamp.

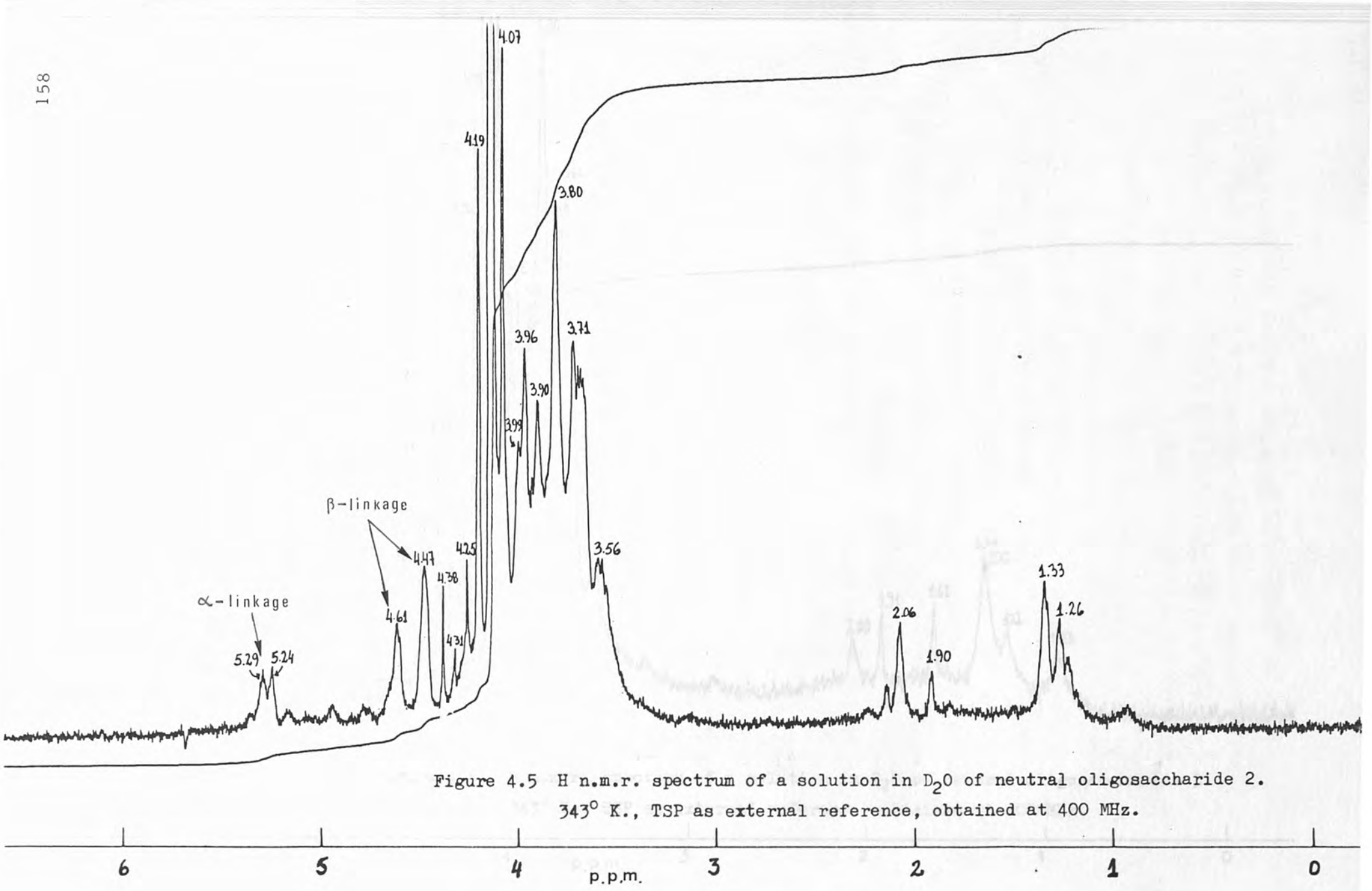


Figure 4.5 H n.m.r. spectrum of a solution in D_2O of neutral oligosaccharide 2. 343° K., TSP as external reference, obtained at 400 MHz.

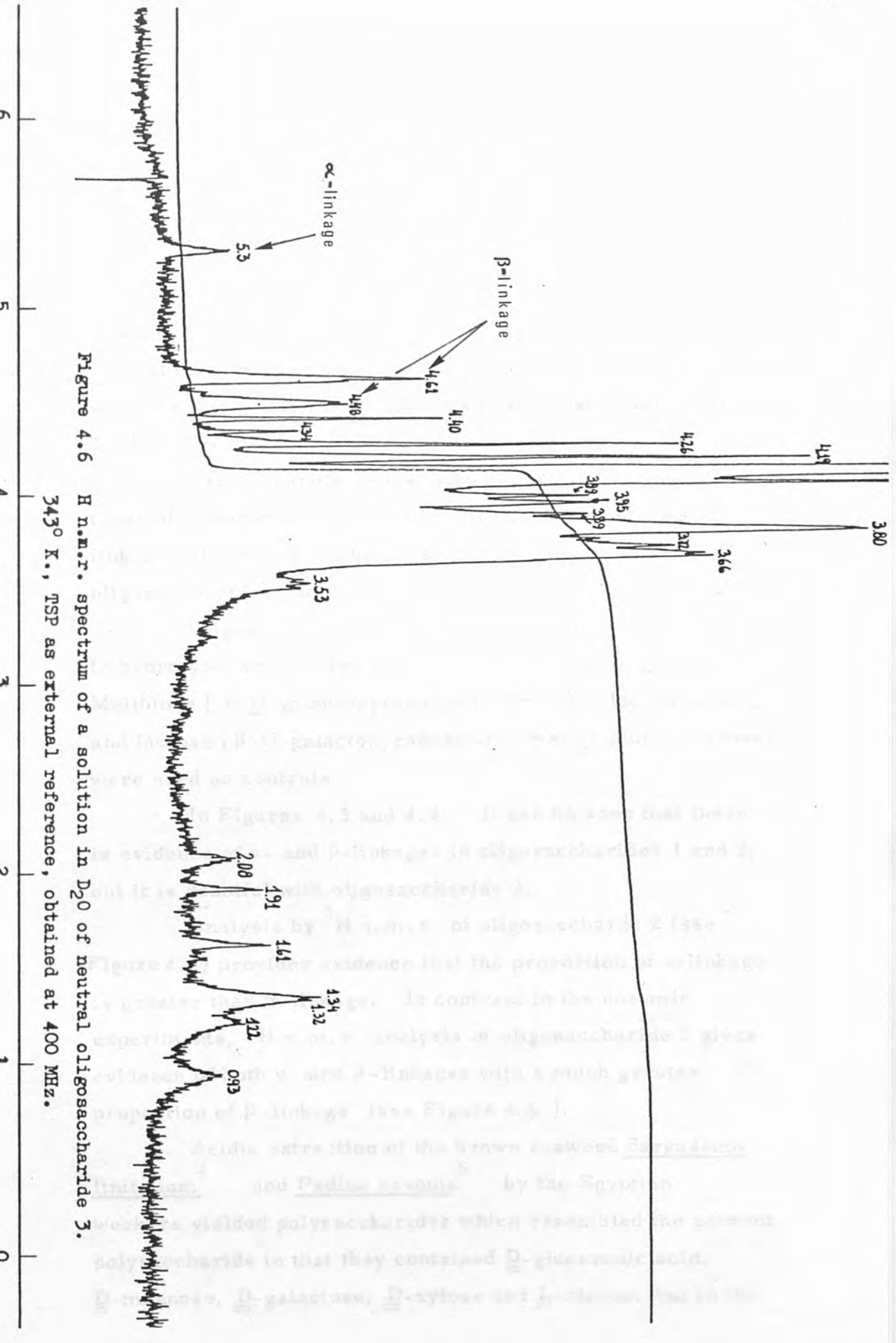


Figure 4.6 H n.m.r. spectrum of a solution in D_2O of neutral oligosaccharide 3. 343° K., TSP as external reference, obtained at 400 MHz.

It can be seen that oligosaccharide 6, the disaccharide, must be a mixture of galactosyl-(1 → 3)- galactose and galactosyl-(1 → 6)-galactose, probably derived by cleavage of the higher galactose oligosaccharides.

The specific rotation of the former in the β-form is + 65°[2] and of the latter in the β-form is + 31°[3]. Assuming equal proportions of the two disaccharides in neutral oligosaccharide 6, the calculated specific rotation would be + 48° which is in agreement with that reported in Table 4.3. p. 152.

Trisaccharide 4 must also be a mixture since three types of linkages are indicated. Although three types of linkages also appear to be present in four of these neutral oligosaccharides, no evidence for branching was obtained.

Oligosaccharides 1, 2 and 3 were subjected separately to hydrolysis with α- and β- galactosidase (see Expt. 4.13). Melibiose [α-D-galactopyranosyl-(1 → 6)-D-glucopyranose] and lactose [β-D-galactopyranosyl-(1 → 4)-D-glucopyranose] were used as controls.

In Figures 4.3 and 4.4 it can be seen that there is evidence of α- and β-linkages in oligosaccharides 1 and 2, but it is doubtful with oligosaccharide 3.

Analysis by ^{1}H n.m.r. of oligosaccharde 2 (see Figure 4.5) provides evidence that the proportion of α-linkage is greater than β-linkage. In contrast to the enzymic experiments, ^{1}H n.m.r. analysis of oligosaccharide 3 gives evidence of both α- and β-linkages with a much greater proportion of β-linkage (see Figure 4.6).

Acidic extraction of the brown seaweed Sargassum linifolium[4] and Padina pavonia[5] by the Egyptian workers yielded polysaccharides which resembled the present polysaccharide in that they contained D-glucuronic acid, D-mannose, D-galactose, D-xylose and L-fucose, but in the

case of the latter D-glucose as well. However the molar ratios of the constituents differed considerably from those of the extracts from Lessonia nigrescens particularly in the higher proportion of fucose and lower proportion of mannose in all extracts of the latter. In contrast to the present results, similar partial hydrolysis of the Sargassum[4] polysaccharide led to the isolation not of neutral galactose oligosaccharides with a positive specific rotation but of sulphated galactose oligosaccharide with negative specific rotations. Further partial hydrolyses experiments on Sargassum[4] and on Padina[5] polysaccharides gave neutral oligosaccharides again with negative specific rotations, but consisting of varying proportions of galactose, xylose and fucose together with glucose from Padina.

4.2.2. Characterisation of the acidic oligosaccharides.

Three fractionsof different formic acid concentration 0.5 M, 1 M and 2 M formic acid, containing the acidic oligosaccharides were eluted separately from the column after all the neutral oligosaccharides and monosaccharides had been eluted with water from the same column (Expt. 4.16). These fractions were separately estimated for carbohydrate and together gave 0.63 g. See flow chart VI.p. 149.

On electrophoresis analysis at pH 6.8 (Expt. 4.17), three spots with mobilities $M_{GlcA.}$ = 0.4; 0.7 and 0.9 were developed, the same for each fraction with only slight differences in the intensities of the spots.

Each of these formic acid fractions was analysed by paper chromatography (see Expt. 4.18), and by this method the 0.5M formic acid fraction was found to give the best separation in solvent 18:3:1:4 = Ethyl Acetate: Acetic Acid: Formic Acid: Water, with very defined spots whose mobilities were $R_{GlcA.}$ = 0.05; 0.19; 0.35; 0.52; 0.80 and 1.00; the one at 0.8 being the largest and fairly elongated probably containing

more than a single oligosaccharide. This was found to be true when a aliquot (1/10 of the total volume) of the 0.5M formic acid sample containing the acidic oligosaccharides was spotted over a strip of 3 MM paper and eight oligosaccharides were clearly separated. (See Expt. 4.19). Figure 4.7. p.163 shows this separation on a photograph of the developed strip.

The rest of the 0.5M formic acid fraction, ca. 130 mg, was treated similarly and the separated oligosaccharides were analysed by paper chromatography to test the purity of each. The following spots with mobilities R_{Glc} = 0.03; 0.18; 0.27; 0.37 0.54; 0.67; 0.84 and 1.0 were developed. The mobilities, carbohydrate content and specific rotations of these acidic oligosaccharides are shown in Table 4.6.

TABLE 4.6

Mobilities, carbohydrate content and specific rotations of the acidic oligosaccharides.

Oligosaccharide	R Glc *	Weight (mg of carbohydrate) **	Specific rotation $[\alpha]_D^{25^\circ}$
1	0.03	14.60	+ 6.8 (c= 1.46×10^{-1} g/100ml H_2O
2	0.18	5.60	+ 12.5 (c= 5.6×10^{-2})
3	0.27	4.80	± 0 (c= 4.8×10^{-2})
4	0.37	21.00	- 26.2 (c= 4.2×10^{-2})
5	0.54	30.25	+34.7 (c= 6.05×10^{-2})
6	0.67	13.50	- 16.3 (c= 1.35×10^{-2})
7	0.84	3.30	-9.1 (c= 3.3×10^{-2})
8	1.0	2.90	-44.8 (c= 2.9×10^{-2})

* Solvent 18:3:1:4 = EtOAc : AcOH:HCOOH:H_2O. Running 2 x 20 hours

** Read from standard graph Fu:GlcA : Gal: Xyl : Mann = 5:2:1.5:1:0.5.

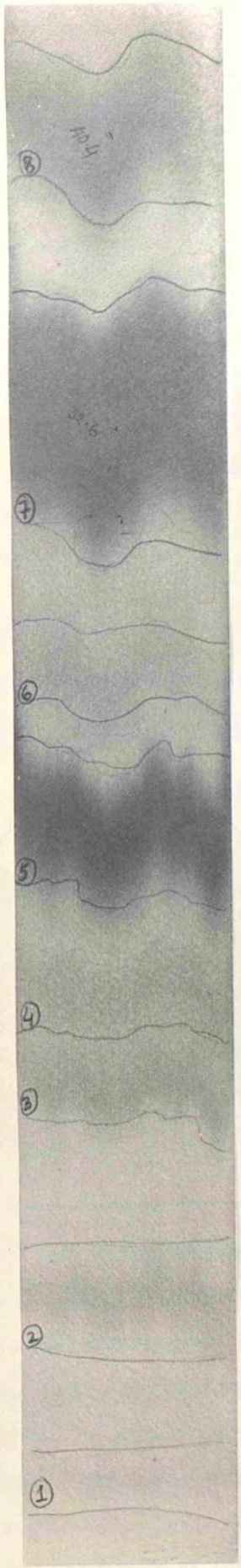


Figure 4.7 Paper chromatogram photograph of the separated acidic oligosaccharides.
Solvent 18:3:1:4 = EtOAc:AcOH:HCOOH:H_2O

The degrees of polymerization estimated by the Timell method[1] appear to be less accurate with acidic samples than with the neutral ones and it was impossible to obtain repeatable results by this method.

The acidic oligosaccharides 2, 3, 7 and 8, isolated in small yield, see Table 4.6 were esterified with methanolic hydrogen chloride, reduced, hydrolysed, reduced again and finally converted to the alditol acetates (See Expt. 4.22). Analysis of these derivatives on g.l.c. gave the molar proportions shown in Table 4.7 for the sugar constituents.

TABLE 4.7

Relative molar proportions of monosaccharides in the acidic oligosaccharides after esterification, reduction and hydrolysis.

Oligosaccharide	Fucose	Xylose	Mannose	Galactose	Glucose
1	1.0	0.5	4.0	9.0	5.0
2	1.0	0.5	4.0	2.0	4.0
3	1.0	0.5	1.0	1.5	2.0
4	1.0	0.4	7.0	-	11.0
5	1.0	-	-	-	2.0
6	1.0	-	-	-	4.6
7	1.0	0.6	3.3	1.3	1.1
8	1.0	2.4	0.4	0.6	1.2

In this Table 4.7 the reported molar proportions of glucose are double those found. This follows from the results of Expt. 4.25 where a standard mixture 1:1 = Glucuronic acid: xylose was esterified, reduced and hydrolysed. Half of this hydrolysate analysed by paper chromatography and the developed paper showed a strong spot corresponding to xylose and a weaker spot for glucose, derived from the reduced glucuronic acid. The other half of this hydrolysate was converted to the alditol acetate derivative and peak areas

from the g.l.c. spectra gave a proportion of 1:2 for glucitol:xylitol, showing that half of the uronic acid was lost during esterification and reduction. A more accurate estimation was carried out to confirm this loss when a weighed sample of 7.8 mg of glucuronic acid was esterified, reduced and hydrolysed and the hydrolysate was found to contain only ca. 4 mg of carbohydrate, proving again the loss of half of the uronic acid. These results were checked and confirmed when samples of acidic oligosaccharides 4 and 6 (R_{Glc} = 0.37 and 0.67 respectively, see Table 4.6) were estimated for carbohydrate after esterification, reduction and hydrolysis. Table 4.8 shows the weight of initial carbohydrate before esterifying, reducing and hydrolysing the sample and the final carbohydrate content after these procedures were performed. It can be seen again that nearly half of the carbohydrate was lost.

TABLE 4.8

Carbohydrate estimation for acidic oligosaccharides 4 and 6 once esterified, reduced and hydrolysed.*

Oligosaccharide	Initial weight of carbohydrate (mg)	Wt. of carbohydr. after esterific. reduct. and hydrol.	
4	14	8.4	ca. 50% loss
6	13.5	6.2	

*Absorbance compared with standard graph Fu:GlcA:Gal:Xy:Mann = 5:2:1.5:1.05.

In spite of the fact that oligouronic acids 7 and 8 gave single discrete spots on paper chromatograms with the mobilities of di- or tri-saccharides (see Table 4.6) it is clear from the mixture of sugars in different proportions found in their hydrolysates (see Table 4.7) that they must each comprise a mixture of oligouronic acids. Although oligouronic acids

2 and 3 with mobilities R_{Glc} = 0.18 and 0.27 respectively must be larger entities it is impossible to build up single oligouronic acids with the monosaccharide compositions found and they too must be regarded as mixtures.

The acidic oligosaccharides isolated in higher yield, 1, 4, 5 and 6 (R_{Glc} = 0.03; 0.37; 0.54 and 0.67 respectively. See Table 4.6) were esterified with diazomethane instead of methanolic hydrogen chloride (see Expt. 4.23) in the hope that the loss of glucuronic acid could be avoided or at least reduced. However this was not the case for 4 and 6 as shown in Table 4.8 (see Expt. 4.26) where it can be seen that the same phenomenon occurred with this method of esterification. Possibly reasons for the loss of uronic acid were the result of incomplete esterification, incomplete reduction or destruction during hydrolysis. That the glucuronic acid was completely esterified was shown by the fact that when samples already esterified, reduced and hydrolysed were analysed through paper chromatography in solvent 6:4:3 = n-BuOH:Py:H_2O, no uronic acid could be recognised when the paper was developed.

Half of these four acidic oligosaccharides already esterified with diazomethane and reduced were hydrolysed and analysed by paper chromatography. Glucose derived from the reduced glucuronic acid was present in all of them as well as the other sugars already found in the "fucan"; xylose, galactose, mannose and fucose in acidic oligosaccharide 1; fucose, xylose and mannose in acidic oligosaccharide 4 and only fucose in acidic oligosaccharides 5 and 6. These results were confirmed when the other half of these hydrolysates were reduced and converted to the alditol acetate derivatives which were analysed by g.l.c. Table 4.7 shows the molar proportion of the

constituents sugars in which the glucose estimation has been doubled. (See p. 164).

The rest of the acidic oligosaccharides, 1, 4, 5 and 6, already esterified with diazomethane and reduced, were methylated (see Expt. 4.24), then hydrolysed, reduced and finally converted to the alditol acetate derivatives and analysed by g.l.c. and g.l.c.- m.s. both with E.I and C.I. method.

Spectra of g.l.c.- m.s. analysis of the methylated oligosaccharides were difficult to interpret because of the complex mixtures of sugars present in oligosaccharides 1 and 4 and the conclusions can only be regarded as tentative.

Acidic oligosaccharide 1 seems to have a very complex structure and probably is a very large molecule. The presence of the following acetylated and methylated alditols was detected:

1, 5-Di-O-acetyl-2, 3, 4, 6-tetra-O-methyl glucitol
1, 5-Di-O-acetyl-2, 3, 4, 6-tetra-O-methyl mannitol
1, 5-Di-O-acetyl-2, 3, 4-tri-O-methyl fucitol
1, 5-Di -O-acetyl-2, 3, 4-tri-O-methyl xylitol
1, 3, 5- Tri-O-acetyl-2, 4, 6-tri-O-methyl glucitol
1, 3, 5- Tri-O-acetyl-2, 4, 6-tri-O-methyl mannitol
1, 2, 5- Tri-O-acetyl-3, 4, 6-tri-O-methyl glucitol
1, 2, 5- Tri-O-acetyl-3, 4, 6-tri-O-methyl mannitol
1, 2, 5- Tri-O-acetyl-3, 4, 6-tri-O-methyl galactitol
1, 4, 5-Tri-O-acetyl-2, 3, 6-Tri-O-methyl glucitol
1, 3, 4, 5-Tetra-O-acetyl-2, 6, Di-O-methyl galactitol
1, 2, 4, 5-Tetra-O-acetyl-3, 6, Di-O-methyl mannitol
1, 4, 5, 6-Tetra-O-acetyl-2, 3-Di-O-methyl mannitol
1, 2, 5, 6-Tetra-O-acetyl-3, 4, Di-O-methyl glucitol
1, 4, 5, 6-Tetra-O-acetyl-2, 3, Di-O-methyl galactitol

The presence of tetra-O-methyl hexitols and tri-O-methyl fucitol and xylitol together with di-O-methyl galactitol, mannitol and glucitol from this oligosaccharide, assuming

complete methylation, indicates the presence of a highly branched macromolecule. In acidic oligosaccharide 4 g.l.c. - m.s. spectrum shows the presence of the following methylated alditol acetates:

1, 5-Di-O-acetyl-2, 3, 4, 6-tetra-O-methyl-glucitol

1, 5-Di-O-acetyl-2, 3, 4, 6-tetra-O-methyl-mannitol

1, 5-Di-O-acetyl- 2, 3, 4-tri-O-methyl- fucitol

1, 3, 5-Tri-O-acetyl-2, 4, 6-tri-O-methyl- glucitol

1, 2, 5-Tri-O-acetyl-3, 4, 6-tri-O-methyl-mannitol

1, 4, 5-Tri-O-acetyl-2, 3, 6-tri-O-methyl-glucitol

1, 5, 6-Tri-O-acetyl-2, 3, 4-tri-O-methyl-mannitol

1, 3, 5-Tri-O-acetyl-2, 4-di-O-methyl-fucitol

It is difficult to draw any other firm conclusions from the methylation results of these two oligosaccharides 1 and 4 apart from the fact that the original polymer contains (1 → 3)- and (1 → 2)-linked glucuronic acid and mannose; (1 → 4)-linked glucuronic acid and (1 → 2)-linked galactose. They do however provide conclusive evidence that all the sugars are present in a single polysaccharide and are not present as chains of single polysaccharides held in a single macromolecule by linkage to protein as in some of the animal proteo glycans[6]

Similar methylation of acidic oligosaccharide 5 gave the following alditol acetates.

1, 5-Di-O-acetyl-2, 3, 4, 6-tetra-O-methyl-glucitol

1, 3, 5-Tri-O-acetyl-2, 4-di-O-methyl-fucitol

1, 3, 5-Tri-O-acetyl-2, 4, 6-tri-O-methyl-glucitol

1, 5, 6-Tri-O-acetyl-2, 3, 4-tri-O-methyl-glucitol

Considering that this molecule is probably a trisaccharide (See R_{Glc} in Table 4.6), and that the molar proportion of the constituents is 2:1 = glucuronic acid:fucose (see Table 4.7) the following structures are possible for this acidic oligosaccharide:

1) Gluc. Acid - (1 → 3)-Fucose-(1 → 3)-Glucuronic acid.

2) Gluc. Acid- (1 → 3)-Gluc. Acid - (1 → 3)-Fucose

Acidic oligosaccharide 6 which from its mobility (see Table 4.6) is probably a disaccharide gave rise to the following methylated alditol acetates.

1, 5-di-O-acetyl-2, 3, 4-tri-O-methyl fucitol

1, 3, 5-tri-O-acetyl-2, 4-di-O-methyl fucitol

1, 5, di, O-acetyl-2, 3, 4, 6-tetra-O-methyl glucitol

1, 3, 5-tri-O-acetyl-2, 4, 6-tri-O-methyl glucitol

This material contains at least 4 molecules of glucuronic acid to one of fucose (see Table 4.7) and is therefore probably a mixture of two aldobiouronic acids of which the possible structures could be:

Gluc.Acid - (1 → 3) - Fucose

Gluc. Acid - (1 → 3)- Gluc. Acid

with a higher proportion of the latter.

The Egyptian workers[4] also isolated complex oligouronic acids (each containing at least four monosaccharides) from their partial acid hydrolysates and they too found complete characterisation of these fragments impossible. From none of their hydrolysates did they succeed in separating fragments containing only fucose and glucuronic acid.

4.2.3. Characterisation of the water soluble and water insoluble polymeric material residues after partial acid hydrolysis with 0.25 M oxalic acid.

The small molecules in the hydrolysate were separated from the larger polymeric material by dialysis. The larger polymeric material comprised a water soluble and a water insoluble residue which after freeze-drying weighed 1.2 g. (15% of starting material) and 1.63 g. (20.4% of starting material) respectively. See Flow chart VI on page 149 In Table 4.9 below, the properties of these residues are shown.

TABLE 4.9

Constituents of water soluble and water insoluble residues recovered from the partial hydrolysis with 0.25M oxalic acid.*

	Carbohydrate content	Uronic Acid content	Protein content	Ash content
Soluble	70 - 80	25 (30% on carbohydr)	12	5.3
Insoluble	10	3 (30% on carbohydr)	10	23.6

*All the figures are given in percentages.

Water insoluble residue

Because of the low carbohydrate content, the high ash content and the presence of Ca^{+2} (indicated by a strong red colour on the flame with Pt wire), it is reasonable to conclude that the insoluble polymeric residue is mainly inorganic, but with glucuronic acid, mannose and glucose in small proportion. The presence of these carbohydrates was revealed by paper chromatographic analysis of a hydrolysate of this material (Expt. 4.28). The presence of the above neutral sugars as well as traces of fucose, xylose and galactose was confirmed by g.l.c. analysis of the alditol

acetate derivatives of the hydrolysate. Approximately 45% of glucitol and 38% of mannitol were found (see Table 4.10).

The relatively high percentage of glucose found in this insoluble material could be another indication of the presence of some cell wall material as previously found in the original extract. The percentage of carbohydrate and protein content (see Table 4.9) could indicate also that this insoluble material comprises a major protein moiety linked to the polysaccharide.

A sample of this insoluble polymeric material (100 mg.) was dispersed in water (50 ml) and treated as stated in Experiment 4.30 in an attempt to remove the maximum of carbohydrate and protein initially insoluble in water. Freeze-dried soluble material, 16 mg (16% of the starting material) was recovered and 61 mg were found to remain insoluble. This is a 77% recovery of the initial sample. The 23% loss can be explained by the difficulty of complete separation of insoluble material from the glass fibre millipore filter. From the 16 mg of soluble product, 4.3 mg were found to be protein and 5.7 mg carbohydrate.

TABLE 4.10

Molar relative proportions of sugars constituents in water soluble and water insoluble residues.

	Fucose	Xylose	Mannose	Galactose	Glucose
Soluble	1	1	14.7	1.6	3
Insoluble	1.5	1	15	4.2	17.5

Water soluble residue

The soluble polymeric residue from the partial acid hydrolysis recovered from inside the dialysis sac has a high carbohydrate content (ca. 80%, as shown in Table 4.9) which represents about 20% of the initial polysaccharide.

A hydrolysate of this soluble material analysed by paper chromatography (see Expt. 4.28) showed the presence

of glucuronic acid and mannose with some traces of glucose. This was confirmed for mannose and glucose when the acetate derivative of the hydrolysate was analysed by g.l.c. and a 70% of mannose was found and only 7% of glucose.

Molecular weight of this soluble residue was estimated by elution of a sample through a Sepharose 4B column (see Expt. 4.29).

Figures calculated from the "Selectivity" curve for Sepharose 4B (Fig. 3.7 p. 98) gave a molecular weight of ca. 8,000 confirming that this is a comparatively large polymeric material with approximately 44 units of monosaccharide residues.

These results together with the high glucuronic acid content of this residual material provides evidence that the interior of the "fucan" macromolecule consists of some 44 units of a glucuronosyl mannan and that this comprises some 20% of the whole "fucan".

This structure is supported by the ^{1}H and ^{13}C n.m.r. spectra of the residual material.

^{1}H n.m.r. studies were carried out at 400 MHz at room temperature and 250 MHz at 358°K with and without DCl. Data are referred to 400 MHz with external reference DSS.

Some clear features from the spectrum are,

1) 5.38 δ, a very broad peak. Must be an equatorial anomeric proton from shift. So, an α-D-(or β-L) sugar residue must be present.

2) 5.27 δ, a small doublet with a coupling constant J ~2Hz is possibly the reducing version of 1).

3) 4.48 and 4.46, a pair of doublets. The latter of greater intensity and the total intensity, considering both pairs of

doublets is roughly the same as 1). There are clear splittings of ~8Hz in each case. The chemical shift implies an anomeric axial proton and the coupling implies an axial proton on C-2 as in a β-D- gluco unit in the normal conformation.

4) 4.15δ, broad peak with some splitting of ~3Hz.

5) 3.39δ, a quartet. There are clear splittings of 8 and 9 Hz. This must correspond to an axial proton coupled to two different vicinal axial protons, for example protons, 2, 3, 4 of a gluco-unit or proton 4 of a manno-unit in the normal conformation.

The effect of DCl obviously offered some changes, feature at 3.6δ changes losing intensity which possibly reappears at 4.0δ. These δ values are low for a uronic acid H-5.

^{13}C n.m.r. studies were carried out at 100 MHz using TSP as external reference.

The spectrum reproduced in Figure 4.8. p.174 shows twelve major peaks at the chemical shift values given below in Table 4.11, together with a number (ca. 17 peaks) of minor peaks.

These twelve peaks are strongly suggestive of a polymer with a disaccharide repeating unit.

Chemical evidence and ^{1}H n.m.r. studies suggest β-D-glucuronic acid and α-D-mannosyl constituents in the ratio 1:1.

The expected ^{13}C chemical shifts for these units in the pyranose forms are given by Barker and Walker[7] and reproduced in Table 4.11.

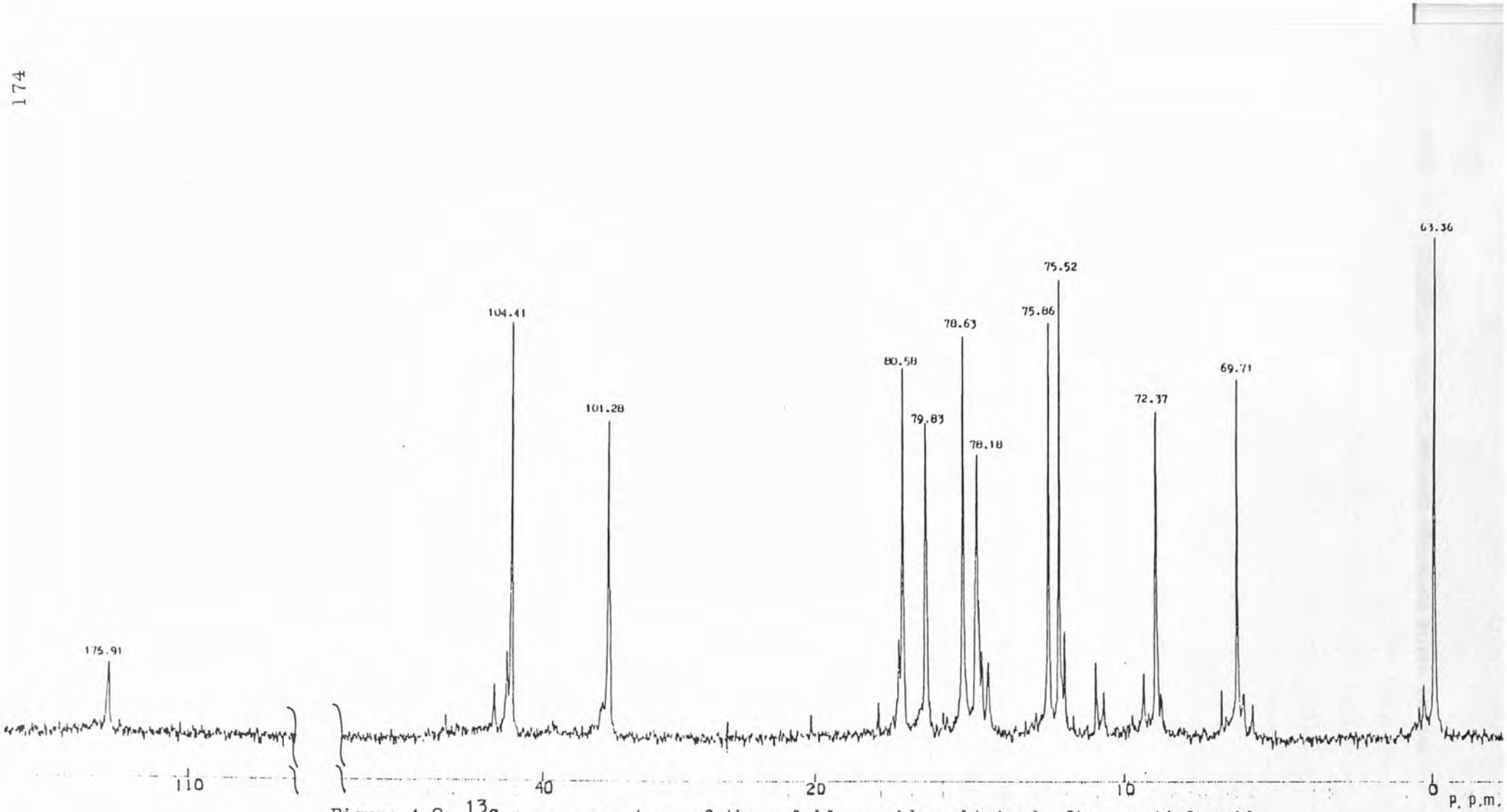


Figure 4.8 ^{13}C n.m.r. spectrum of the soluble residue obtained after partial acid hydrolysis of the "fucan". 343° K., TSP as external reference.

TABLE 4.11

Carbon and Compounds		Chemical shifts from reference (7)	Chem. Shifts observed with tentative assignment
C-6	β-D-Gluc. Ac.		175.41
C-1	β-D-Gluc. Ac.	104.3	104.40
C-1	α-D-Mann.	101.9	101.28
C-3	β-D-Gluc. Ac.	76.5	75.96
C-5	β-D-Gluc Ac.	75.6	75.52
C-2	β-D-Gluc. Ac.	73.8	
C-5	α-D-Mann.	73.7	
C-4	β-D-Gluc. Ac.	72.3	72.37
C-3	α-D-Mann.	71.8	
C-2	α-D-Mann.	71.2	
C-4	α-D-Mann.	68.0	69.71
C-6	α-D-Mann.	62.1	63.36

Four observed peaks at 78.13, 78.63, 79.83 and 80.58 could not be assigned; two of these can be accounted for by linkage positions which always display upfield shifts[8] but this still leaves two peaks unassignable. There are no large additional peaks in the spectrum that might arise from other substituents. Two possibilities could be 3,6-lactone formation by the glucuronic acid units, or the presence of furanose rings. The latter have been reported for galactose, arabinose and fructose containing polysaccharides[9]. A more definite structural assignment in the present case will require further degradative work such as periodate oxidation studies and further ^{13}C n.m.r. reference spectra of model compounds.

Evidence for the presence of a backbone chain of D-glucuronic acid and D-mannose residues for the acid extracted "fucan" of Sargassum linifolium has previously been advanced[4].

At the same time the present partial hydrolysis studies

on Lessonia nigrescens have shown that a proportion of the glucuronic acid residues is linked to fucose, that the majority of the galactose residues are mutually linked in chains, and that on the periphery of the molecules are the majority of the fucose and xylose units.

The fact that in both soluble and insoluble polymeric material, protein and carbohydrate are found, support the indications previously found that protein and polysaccharide are closely associated in the seaweed and difficult to separate in the extracts.

4.3. REFERENCES

CHAPTER IV Results and Discussion

1. Timell E.T., Sven. Papperstidn 63, 668 (1960).
2. Hirst E.L. and Perlin A.S., J. Chem. Soc. 2622 (1954).
3. Freudenberg K., Wolf A., Knopf E. and Zaheer S.H., Ber, 61 1743 (1928).
4. Abdel-Fattah A.F., Hussein M.M-D. and Salem H.M., Carbohydr. Res., 33, 19-24 (1974).
5. Hussein M.M-D., Abdel-Aziz A. and Salem H.M. Phytochem, 19, 2133-2135 (1980).
6. Hardingham T.E., Biochem. Transactions, 9, 489-499 (1981).
7. Barker R. and Walker T.E., Methods in Carb. Chem., 8, 151-171. (1980).
8. T. Ogawa and K. Sasajima, Carbohydr. Res., 97, 205-227 (1981).
9. Beier R.C., Mundy B.P. and Strobel G.A., Can. J. Chem., 58, 2800-2804 (1980).