POLYSACCHARIDES OF PHAEOPHYCEAE FROM THE ANTARCTIC AND CALIFORNIA

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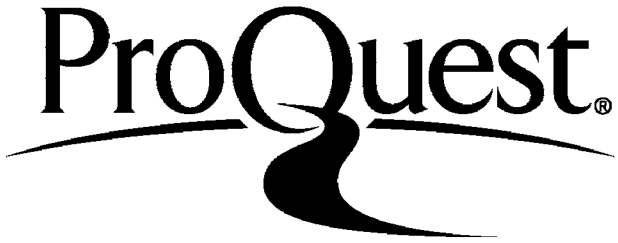

ABSTRACT

1.1. The polysaccharides of two Antarctic brown algae have been studied.

2.1. Ascoseira has been chemically investigated for the first time.

2.2. After extraction of traces of mannitol and some monosaccharides, sequential extraction with aqueous $CaCl_2$, dilute acid and dilute alkali gave mixtures of laminaran, "fucan" and alginate in unusually low total recovery.

2.3. Laminaran fractions from the aqueous and alkali extracts contained different proportions of β-(1→3)- and β-(1→6)- linked units, with evidence of branching in both fractions.

2.4. The "fucans" contained varying proportions of fucose, galactose and glucuronic acid, small amounts of xylose, mannose and glucose, and half-ester sulphate and protein.

2.5. The alginate in the aqueous extract contained about 75% mannuronate residues, that of the alkali extract about 80% guluronate residues. Direct alkaline extraction gave a low molecular weight alginate with a mannuronate to guluronate ratio of about 0.45 and only a small proportion of sequences of alternating residues.

3.1 The mucilaginous exudate, and the "fucan" therein, from the kelp Macrocystis pyrifera have been found to be physically and chemically similar to the same materials from a Californian sample of M.pyrifera.

3.2. Sequential extraction of the Antarctic sample gave "fucan", alginate and a trace of laminaran.

3.3. The "fucan" in the aqueous $CaCl_2$ extract was shown by partial hydrolysis to be composed of subunits containing about 70% fucose, varying small proportions of xylose, mannose, glucose, galactose and glucuronic acid, and half-ester sulphate and protein. Sulphate was linked only to fucose, and predominantly to carbon atom 4. Further partial hydrolysis gave complex fragments with evidence of mutual and various linkages of most of the monosaccharide residues. Tentative evidence of covalent linkage of protein and polysaccharide was found.

3.4. Direct alkaline extraction of Antarctic M.pyrifera gave a high molecular weight alginate with a mannuronate to guluronate ratio of about 0.95 and a relatively high proportion of sequences of alternating residues.

TO MY MOTHER

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CHAPTER I

INTRODUCTION

1.1. Background to the Research

The signing of the Antarctic Treaty in 1959 marked the start of a rare case of international cooperation in basic scientific research. Without prejudicing prior claims to territorial and mineral rights the treaty preserved the Continent for non-military scientific research for a period of thirty years. Since then the area has been subjected to extensive study in a number of fields. Geological and climatological studies have contributed to a better understanding of the total world environment. Large mineral deposits and oil fields have been located and the abundant marine life represents a potentially vast source of food, though the commercial exploitation of these resources is restricted by the large transportation costs.

The British Antarctic Survey is one of the most active organisations in the Antarctic; from its base in Cambridge it directs research in several fields and maintains a number of bases on the Continent. One area of interest is in ecologically orientated studies of the adaptation of plant life to the polar environment. Some 800 terrestrial plants and at least 550 species of algae

have been identified in the Antarctic polar region (generally defined as below 60^{o} South). The terrestrial plants are predominantly lichens, mosses and hardy grasses, with only three native flowering plants. Both marine and non-marine algae are found, including some species which are endemic to the Antarctic.[1]

The synthesis of mono-, oligo- and polysaccharides and their subsequent interconversion and degradation are fundamental processes in plants and hence it seems logical to suggest that concepts of adaptation must be based on a knowledge of the detailed structure of these carbohydrate materials as well as their interrelation. The present knowledge of carbohydrates of Antarctic plants is limited to the seasonal variation of abundance of a few low molecular weight compounds. From an ecological viewpoint aquatic and terrestrial plants are of similar importance but the ready availability of data on the carbohydrates of algae from temperate zones suggested that this project should concentrate on algae.

Algae are primitive plant-like organisms with a great diversity of form, ranging from microscopic unicellular species to the giant kelp Macrocystis pyrifera which grows up to sixty metres in length. They are photosynthetic organisms but lack true leaves,stems, roots and vascular systems.

The need for light restricts their habitat; the free-floating types are found in many parts of the seas but most of the larger species require a firm anchorage and are therefore found only in continental shelf areas. Species in intertidal regions are periodically exposed and must have a wide osmotic tolerance as their saline environment is either diluted by rain or concentrated by evaporation. They must also tolerate changes in air temperature, the high light intensities and the mechanical stresses of wave action. Polysaccharides play a large part in most of these processes.[2] In polar environments the low temperatures and the presence of ice demand modification of the processes to prevent freezing and counter the low photosynthetic rate.

The classification of algae is based primarily on their morphology, particularly in respect of the reproductive systems, but the nature of pigments and food reserves present are crucial to the segregation into groups.[3] Four major divisions are well-established; Phaeophyceae (brown algae), Rhodophyceae (red algae), Chlorophyceae (green algae) and Cyanophyceae (blue-green algae). All contain chlorophyll a, but the characteristic colours are derived from masking pigments. These classes include the economically important algae and most of the

species that have been examined chemically. The sub-divisions of these groups are generally agreed upon but are revised periodically as new species are discovered and older ones are reclassified.

There is a noticeable, but by no means rigid or universal, zonation of algae by species. An important factor in this is the change in spectral distribution of light at different depths of water; the utilisation of light is dependent on pigmentation and hence green, brown and red algae tend to occur at increasing depths.[3] However the exceptions to this trend indicate that other factors are involved in the ecology of algae.[4]

Examples from all these groups have been found in the Antarctic region and the surrounding islands, including some of the commercially important species. The species chosen for investigation here are examples of the Phaeophyceae. Around the Antarctic continent the bulk of the brown algal biomass is provided by species of the order Desmarestiales,[5] but in the sub-antarctic region there are vast stands of species of the order Laminariales.[6] One of these is Macrocystis pyrifera (family Lessoniaceae) which is also found on the Pacific coasts of the United States and South America, in South Africa and Australia. In California it is harvested commercially as a source of alginic acid. It grows in the middle

sublittoral zone at depths of up to 30m and is anchored to the rocky seabed by a padlike holdfast. Arising from this are long, flexible stipes up to several metres in length. Secondary stipes branch off these; at their base they carry a single air bladder and then open out into fronds with serrated edges. The air bladders help to extend the plant and hold the fronds close to the surface where light is most abundant. The extended nature of the plant allows maximum access to available nutrients in the water but the consequent strain on the holdfast generated by wave action can be large, requiring a particularly flexible stipe to dampen the strain.[7] Its position close to or above the surface make the extended weed particularly susceptible to erosion by ice, possibly accounting for its absence from more polar regions. To counter the exposure it exudes a sticky liquid which appears to retain water tenaciously. Ascoseira was formerly assigned to the order Fucales[1,8] but has subsequently been given its own monotypic order, the Ascoseirales.[9] It is essentially endemic to the Antarctic though it has migrated to other locations. The stipes arising from the holdfast are short and divide to produce long narrow blades. The whole plant is less than a metre in length and would appear to be rarely exposed; the sample studied here was collected at a depth of

four metres.

Both algae were collected by members of the British Antarctic Survey from Signy Island and South Georgia. These islands are considered sub-antarctic by virtue of being south of the tree line. They are covered by snow for most of the year and support only hardy grasses and other tundra plants. The Macrocystis was collected in late October (i.e. spring) 1978 and the Ascoseira in early February (late summer) 1979.

No chemical study has been made of Ascoseira. Published work on the carbohydrates of Macrocystis pyrifera is limited to the extraction and composition of the alginic acid[10-14] and the composition of the polysaccharide in the sticky exudate, this latter work being on weed from Southern California.[15]

The carbohydrates found in the brown algae, and those of the other classes of algae, can be roughly divided into three groups; low molecular weight carbohydrates, water-soluble polysaccharides and cell-wall or structural polysaccharides. The second group may be sub-divided into food reserve polysaccharides and sulphated polysaccharides. Few of the Phaeophyceae have been investigated chemically but in those that have considerable information has been obtained on the structure and location, and hence the function, of the various

polysaccharides. The low molecular weight carbohydrates have attracted little attention.

1.2 Carbohydrates of the Phaeophyta

1.2.1. Low molecular weight carbohydrates

D-mannitol appears to be ubiquitous in the brown algae; its reported absence in two cases[16,17] is probably due to loss during collection of the alga. It is a storage material and substrate for respiration,[18] its proportion of the dry weight depending on season, species, location and state of development.[19-25]

D-volemitol, a seven carbon sugar alcohol, and some β-glycosides thereof, have been found in Pelvetia canaliculata[26,27]

1-O-D-mannitol-β-D-glucopyranoside and 1,6-O-D-mannitol-di-(β-D-glucopyranoside) have been found in several algae,[28] and are accompanied by 1-D-mannitol acetate in Fucus virsoides.[29]

Traces of sucrose, galactose and mannose have been reported in species of Cladostephus;[30] sucrose has also been reported in Fucus virsoides,[29] though this may be due to associated Chlorophyceae.

A monouronic acid and its lactone, with chromatographic and electrophoretic mobilities identical to mannuronic acid, was observed in Desmarestia firma.[31]

A number of oligouronic acids containing fucose, xylose, mannose, galactose and glucuronic

acid have been isolated from Lessonia nigrescens,[32] though these may be derived from degradation of polymeric species.

1.2.2. Water soluble polysaccharides

1.2.2.1. - Food reserve material

The reserve polysaccharide of the Phaeophyta is laminaran, and its abundance, seasonal variation and relative disposition in the plant have been extensively studied. It appears to be absent during the rapid growth period of spring[33] but increases to a maximum value during autumn and winter, when it may comprise up to 35% of the dry weight of the frond.[34] It then declines again, largely through erosion or breakage of fronds.[35,36] In extensive studies of 19 species of algae, Powell and Meeuse[37] found laminaran contents ranging from 2 to 34% of the algal dry weight and reported that laminaran increased in quantity in going from the stipes to the tips of the fronds, confirming earlier results.[21,38]

Laminaran analyses have also been reported for Saccorhiza,[39] Fucus spp.,[40-42] Dictyopteris spp.,[40,41] Cystoseira spp.,[33,40,41,43,44] Laminaria spp.,[45-47] Arthrothamnus,[46] Cymathaera spp.,[46] Sargassum,[33] Chordaria,[47] Punctuaria[47] and Desmarestia.[47] In general these studies support the growth pattern outlined above.

Early studies[48-50] indicated that laminaran is essentially a linear (1 → 3)-β-D-glucopyranan of degree of polymerisation 20 to 25.[51-55] The presence of 2 - 3% of mannitol linked through one primary alcohol group to some of the reducing, terminal glucose residues was established by Hough and others;[56-61] a suggestion [62] that the mannitol residues were disubstituted was later excluded.[63] The mannitol-terminated and glucose-terminated chains were separated by oxidation of glucose terminal residues to gluconic acid and fractionation on an anion-exchange resin.[62]

Laminaran is commonly reported as occurring in "soluble" and "insoluble" forms distinguished by their solubility in cold water,[64] but it is clear that a range of structures exist. Attempts to distinguish the two forms revealed little consistency in the proportion of mannitol-terminated chains in the two,[65] but established that the "soluble" form tends to have more highly branched chains[65-67] and a higher proportion of glucose units linked through positions 1 and 6 only.

Early work produced contradictory views on the presence of the latter feature. Results derived from analysis of hydrolysis products, periodate oxidation and methylation analysis have been advanced in favour of (1 → 6)-intra-chain linkages,[68-70] but these have been questioned after

Barry and Smith Degradation studies and careful analysis of the products. The failure to produce small fragments through cleavage of the chain precluded the presence of (1→ 6)-linked units in the main chain[65,66,71] and the occurrence of 2,3,4-tri-O-methylglucose in the methylation products has been either rejected as insignificant or as due to demethylation of tetramethylglucose.[66]

More convincing evidence of (1 → 6)-intrachain linkages has been presented from research on laminaran extracted from Eisenia bicyclis. On the basis of methylation analysis and partial hydrolysis experiments Handa and Nisizawa postulated a linear sequence of (1→ 3)- and (1→ 6)-linkages in a ratio of 2:1 with some of each type of unit occurring in sequences of at least three.[72] Further work led to isolation of a laminaran with the linkages in the ratio 3:1.[73] More recently evidence of branching has been found, the development of ^{13}C n.m.r. spectroscopy allowing Usui and co-workers to confirm units linked through 1,3 and 6 as well as units linked through 1 and 3 and 1 and 6 only.[74] Similar features have been found in laminaran from Ishige okamurai,[75,76] Ecklonia radiata[77] and Cystophora scalaris;[77] in the latter two cases the intrachain (1→ 3)-linked and (1→ 6)-linked units occur in a ratio of 1:1.

1.2.2.2. - Sulphated polysaccharides

The water-soluble sulphated polysaccharide that may be isolated from brown algae has generally been referred to as "fucoidan" and consists mainly of L-fucose units. However even the "purest" fractions contain other sugar residues and since extraction of the alga with acid or alkali often removes large amounts of sulphated fucose-containing polymers, it is best to consider "fucoidan" as just one fraction of a family of polydisperse hetero-molecules. Varying proportions of galactose, mannose, xylose and glucuronic acid are found with fucose and, in the native state, protein and possibly alginic acid are often associated with the polysaccharide. For simplicity these polysaccharides will be referred to as "fucans" on the understanding that the term is strictly inaccurate.

A fucose-containing material was reported in the cell membrane of *Fucus* in 1890,[78] but the first recorded extraction was by Kylin,[79,80] who isolated a mucilaginous polysaccharide from *Fucus vesiculosus*, *Laminaria digitata* and *Ascophyllum nodosum* and termed it fucoidin. Subsequently it was identified in *Macrocystis pyrifera*.[81] Combined ester sulphate was confirmed by several groups.[82-84] Attempts to isolate a pure "fucan" sulphate from *Fucus spp.*,[85-87] *Laminaria hyperborea*,[85] *Himanthalia lorea*,[85] *Macrocystis pyrifera*[15] and *Ascophyllum nodosum*[88,89]

failed; galactose and xylose were the most common of the other residues present.

Early structural studies indicated that the fucose units are predominantly linked α-(1 → 2)- and carry sulphate at position 4, with evidence of α-linkage and sulphation through all possible positions.[90-92] Later work has generally supported this variety of fucose units.[93-95]

That "fucoidan" represented but a small proportion of a large family was shown by the isolation of at least three electrophoretically distinct fucose-containing polysaccharides from Ascophyllum nodosum;[96] subsequently the "fucans" of A.nodosum have been subjected to extensive studies by the Norwegian workers. The predominant "fucan", termed "ascophyllan" by Larsen and Haug was electrophoretically homogeneous between pH2 and 10 and contained 12% protein, 12% SO_3Na, 25% L-fucose, 26% D-xylose, 19% D-glucuronic acid and traces of mannuronic acid.[97] Mild acid hydrolysis split "ascophyllan" into an insoluble polypeptide, a complex mixture of free and sulphated mono- and oligo-saccharides among which 3-O-β-D-xylopyranosyl-L-fucose was identified, and a sulphate-free glycuronan containing nearly all of the original glucuronic acid (74%) and residual fucose (26%).[98] The authors concluded that the molecule had a backbone of β-(1 → 4)-linked glucuronic acid units to

which sulphated side chains of mutually linked fucose and xylose residues were attached; the polypeptide appeared to be linked to the latter.

Two other glucuronoxylofucans were present in the same extract, both structurally similar to "ascophyllan" but with a much higher proportion of fucose.[97,98]

Further extraction of the alga under more vigorous conditions gave another "fucan" fraction with 49% fucose, 10% xylose, 12% glucuronic acid, 21% SO_3Na and 4% protein. The disaccharide isolated earlier, 4-O-α-L-fucopyranosyl-D-xylose and 3-O-(β-D-glucopyranosyluronic acid)-L-fucose were isolated after partial acid hydrolysis. Methylation data indicated that the glucuronic acid and xylose residues were linked through positions 1 and 4 and were also present as non-reducing end-groups, and that the fucose residues were linked as in "fucoidan".[99,100]

All these fractions were isolated after acid treatments that would cause substantial cleavage of glycosidic linkages. Later detailed studies showed that under very mild extraction conditions "ascophyllan" and "fucoidan-like" material were extracted as a single unit, only breaking up under acid hydrolysis. The breakdown products included "ascophyllan" and "fucoidan" as described above, as well as a fraction in which

residues of mannuronic and guluronic acid appeared largely to replace the glucuronic acid in the "ascophyllan-type" sub-unit, indicating covalent linkage of "fucoidan" and alginic acid.[101,102] Another small fraction comprised a galactofucan, with single fucose residues branching off a galactose backbone; glucuronic acid was also present as end-groups.[103]

Similar studies in Fucus vesiculosus revealed the same type of macromolecular complexes.[101,102] Systematic fractionations and structural investigations of the "fucans" from Padina pavonia, Bifurcaria bifurcata and Himanthalia lorea showed a very broad composition distribution, ranging from "fucoidan-like" to "ascophyllan-like".[94,104] Analogous experiments on Desmarestia spp. indicated similar structural features with the addition of non-sulphated end-group and (1 → 3)-linked galactose residues; D.ligulata also contained D-mannose residues.[95,105] After partial acid hydrolysis of the "fucan" from Lessonia nigrescens, Venegas was able to isolate neutral oligosaccharides of up to six galactose residues with a variety of linkages, complex acidic oligosaccharides including 3-O-(β-D-glucopyranosyluronic acid)-L-fucose and 3-O-(β-D-glucopyranosyluronic acid)-D-glucuronic acid, and a backbone of about 44 alternating residues of glucuronic acid and mannose.[32]

A glucuronomannan backbone was also found in a detailed study of a fucan fraction of Sargassum linifolium; 12-13 glucuronic acid and 3-4 mannose residues were uniformly (1 → 4)-linked. From the partial acid hydrolysis fragments neutral tri-, tetra-, and penta-saccharides of mutually linked fucose, xylose and galactose were isolated. The sulphated fragments included fucose-4-sulphate, a fucobiose, a galactobiose, a galactotriose and a galactotetraose and were interpreted as indicating a predominance of 6-sulphated, (1 → 4)-linked β-D-galactose residues and 4-sulphated, (1 → 2)-linked α-L-fucose residues in the side chains.[106] An independent report was in general agreement and similar features were reported in a Pelvetia species.[107,108] These remain the only recorded instances of sulphated galactose in the Phaeophyta.

Less detailed studies, generally only involving the proportions of the different sugars, have been carried out on fucans from Fucus virsoides,[109] Fucus spiralis,[86] Pelvetia wrightii,[110-114] Pelvetia canaliculata,[114,115] Sargassum pallidum,[116,117] Undaria pinnatifida,[118,119] Nemacystus discipiens,[120] Lessonia flavicans,[121] Dictyota dichotoma,[122,123] Dictyopteris plagiogramma and Macrocystis integrifolia.[125] The "fucans" have been reviewed on several occasions.[64,126-128]

The range and complexity of the "fucans"

suggests that they are multifunctional molecules; indeed they may be synthesised or structurally modified during growth in response to changes in requirements. Three specific functions have commonly been cited; prevention of desiccation during prolonged exposure of the plant by retention of water,[64,129,130] selective control of cation movement by their ion-exchange capability,[64,129,131] and preservation of the structural integrity of the plant in the face of tidal action by formation of gels or highly viscous fluids.[64,129,132]

In support of the inhibitory effect on desiccation, Lestang and Quillet have shown that "fucan" from Pelvetia canaliculata has a very high affinity for magnesium ions, which are highly hydrated and therefore retain water in the fronds when associated with the ester sulphate groups.[131] Further weight is added to this theory by the general observation that species that are frequently exposed have high "fucan" content whereas those which grow below low tide mark contain little or no "fucan".[20,105,133] In a recent review Kloareg suggests that this effect is more appropriately described as a tolerance to abrupt changes in surrounding salinity.[134]

In some algae a certain proportion of the "fucan" exudes from the weed as a mucilage,[15,21,84,88] but most of it is located in the intercellular region

and in the cell wall.[135] That it occurs in the cell wall is supported by the fact that vigorous conditions are required to remove the final traces of "fucan".[99]

The synthesis and subsequent modification of the "fucans" has been studied in several species. In ^{14}C studies on Fucus vesiculosus it appeared that a xylogalactofucoglucuronan is synthesised first and this is transformed into xyloglucanono-galactofucan, and finally into a "fucan" containing 90% of fucose units.[136] Quatrano and co-workers have studied the development of zygotes of species of Fucus. Four hours after fertilisation a "fucan" comprised about 20% of the cell wall polysaccharides; its structure appeared similar to that of ascophyllan described earlier.[137] This "fucan" is subsequently sulphated[138-140] and accumulates in the rhizoid region .[141-148] Electrophoretic experiments suggested at least three different fractions of the latter, heavily sulphated "fucan".[149]

The Leeds group have shown the Golgi complex to be the site of polysaccharide sulphation in Laminaria and Fucus serratus.[150-152] They have also identified galactosyl-[153] and fucosyltrans-ferases,[154] and implicated fucosyl- and mannosyl-containing species in the fertilization process.[155,156]

The sulphate is subsequently exchanged rapidly with free sulphate in the seawater;[157-159]

Lestang and Quillet postulated that this formed part of a mechanism for removing toxic concentrations of sodium ions from the plant tissue.[131] This is an enzyme-mediated, energy driven process.

1.2.3. Structural Polysaccharides

1.2.3.1. - Alginic acid.

Quantitatively the major polysaccharide of the brown algae is alginic acid. It occurs in all species of Phaeophyta and has also been found in some bacteria. Because of its commercial importance it has been extensively investigated.

As with the other polysaccharides of algae its proportion of the dry solids, and its absolute amount vary according to species, season and location of the plant.[20,160] In general it forms a smaller proportion during the periods of rapid growth than during the colder months when little growth takes place.[21]

Nelson and Cretcher were the first to show that mannuronic acid was a constituent and that all the residues were of uronic acid.[10,11] Methylation analysis and periodate oxidation studies established that a high proportion of the residues were β-(1 → 4)-linked.[12,161,162] These two techniques later led to the confirmation of varying amounts of (1 → 4)-linked L-guluronic acid residues.[163-167] A finding that some of the units were resistant to periodate seemed to indicate

(1 → 3)-linkages,[168,169] but the discrepancy was later traced to the spontaneous formation, during oxidation, of stable, six-membered hemiacetal rings between the aldehyde groups of oxidised units and the closest hydroxyl groups on adjacent unoxidised residues, preventing further oxidation.[171-175] This observation also precluded (1→ 5)-linkages which would require seven-membered rings. Further evidence for a completely (1 → 4)-linked polymer was given by the observed depolymerisation by β-elimination, both at high pH[176] and with lyase enzymes.[177] The low specific rotations of alginates suggested that the D-mannuronosidic linkages are all in the β-configuration and the L-guluronosidic linkages all in the α-configuration, a view supported by fibre X-ray diffraction experiments[178] and subsequently confirmed by n.m.r. spectroscopy.[179-182] The same methods all indicate that the mannuronic acid residues are in the $^{4}C_{1}$ conformation and the guluronic acid residues in the $^{1}C_{4}$ conformation.

None of these results makes it clear whether alginic acid is a true co-polymer rather than a physical mixture of D-mannuronan and L-guluronan. Early attempts to fractionate alginic acid into two forms gave fractions approaching, but never achieving, pure homopolymers.[183-186] Vincent separated a number of oligouronic acids

containing both acids from a partial hydrolysate, but was unable to prove that any fraction constituted a single entity.[187] The first proof of the mutual linkage of the two acids came when mannosylgulose was isolated from a partial hydrolysate of reduced alginic acid.[188]

A major advance in the knowledge of the sequence of monomer units in the chain came with the partial acid hydrolysis experiments of Haug and collaborators. Working on alginate from Laminaria digitata they proposed that it is a block copolymer in which long homopolymeric sequences of both types are joined through sequences of mixed composition.[189-193] Similar results were obtained for alginates from Ascophyllum nodosum and Laminaria hyperborea.[190,194] A more random arrangement was suggested for Cystoseira barbata alginate,[195] a view that was supported by enzymic degradation studies on Ishige okamurai and several commercial alginates, where it was clear that the "alternating" sequences were not purely alternating but contained irregularities of pairs of identical residues.[196,197] However the "three block" concept remains essentially valid.[198,199]

The development of high resolution n.m.r. spectroscopy has greatly assisted the characterisation of monomer sequence in alginate samples. Early p.m.r. work on slightly depolymerised samples

facilitated measurement of the mole fractions of the four possible disaccharide sequences("diads").[181] In ^{13}C n.m.r. spectra the carbon atoms showed a sequence-dependent splitting of resonances, allowing computation of the eight trisaccharide ("triad") as well as the four "diad" frequencies. The results obtained earlier for Laminaria digitata alginate were confirmed, blocks of more than eight units being evident.[180,182,200]

C.D. spectroscopy has also been used for characterisation of sequence.[201-209] It entails comparison of a whole alginate with a "synthetic" spectrum derived by computer from individual contributions of the three types of block, but its speed, the milligram sample size, and the avoidance of preliminary depolymerisation have made it useful for some studies.

More recently a polarimetric method has been devised but it is hindered by the need for samples free of contaminants.[210]

The relative amounts of the two acids in an alginate sample vary with species, location, season and also in different parts of the plant. Tables of mannuronic acid to guluronic acid (M/G) ratios have been published with values for whole algae ranging from 0.25 to 2.25.[64] Extraction of different parts of the plant gives a greater range; an essentially pure mannuronan has been

found in receptacles of Ascophyllum nodosum.[211,212] In general, the older and tougher the tissue the higher the guluronic acid content. Meaningful comparisons between species are difficult since the proportion of different tissues in an algal population depends on its average age and the season.

The ion-binding properties of alginates have received considerable attention and have been investigated by several techniques.[132,213-234] The general conclusion is that the affinity of alginates for divalent metal ions, particularly calcium, increases with increasing content of L-guluronate residues. The mechanism of this calcium binding, and the formation of rigid gels that results therefrom, is related to the solution conformations of the different types of alginate blocks. In the solid state polymannuronic acid adopts a flat ribbon-like twofold conformation,[178] which in the salt form may be modified to a threefold conformation which is still extended and ribbon-like.[235] In polyguluronic acid by virtue of the nature of the linkages, rotation about the glycosidic linkages in more restricted and a buckled twofold conformation is adopted which is maintained in all the salt forms studied.[236]

The enhanced binding of the calcium shown by polyguluronate segments when the chain length

exceeds a critical length of about 20 residues strongly suggested a cooperative mechanism[228,234] and led Rees to postulate the "egg-box model",[201] based on the assumption that the fibre conformation is maintained in solution. The array of coordination sites is formed by the alignment of two polyguluronate chains; the buckled shapes creating cavities, lined with carboxylate and other electronegative oxgen atoms, which are of the right size to accommodate calcium ions.[237] This model is in line with theoretical calculations[238-245] and is confirmed by c.d. spectroscopy[201-209] and competitive inhibition studies which also indicate that the association stops at dimerisation rather than continuing to form layered structures of several chains.

All the above studies show that polymannuronate and alternating sequences are not involved in binding but serve as flexible regions between the junction zones, thereby sustaining the alginate as a gel rather than a solid.

Hence the extent of gelation is controlled by the number and length of polyguluronate sequences. Following the isolation of D-mannuronan C-5 epimerase from bacteria[246-248] and brown algae,[249-250] studies of the sequence generating process were possible. Though UDP-L-guluronic acid

has been isolated from a brown alga,[251] it is generally considered that alginate is first synthesised as a pure mannuronan. Units are then selectively epimerised to generate the polyguluronate and alternating sequence blocks; the two types are probably generated by different epimerases.[252,253] The epimerase activities are strongly dependent on calcium ion concentration. The reverse epimerisation has been observed and it seems that the alga synthesises alginate in response to the prevailing calcium ion concentration; when it is low an alginate is synthesised that binds it strongly to ensure adequate gel rigidity, and when it is too high an alginate is synthesised to bind it weakly preventing a too brittle gel from forming.

1.2.3.2. - Cellulose

The presence of cellulose in brown seaweed was postulated by several early workers[80,254,255] but Dillon and O'Tauma were the first to separate, from Laminaria digitata.[256] Structural studies on samples from several algae indicated close similarity to cotton cellulose, differences probably being due to degradation during extraction.[257,258] Subsequent studies indicate that it is present in small quantity in all brown algae.[104,259]

CHAPTER 1. REFERENCES

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CHAPTER 2

CARBOHYDRATES OF ASCOSEIRA

2.1. Preliminary Investigation of Ascoseira

2.1.1. Extraction

The algae are generally of more primitive morphology than terrestial plants and show little differentiation into tissues with specialised functions. Where differences in tissue are marked, for example, stipe and frond of some species of Laminaria, separate analyses reveal variations in the quantities rather than the nature of the constituents.[1,2] The differentiation into photosynthetic and storage tissues of land plants does not appear to occur in the algae. On this basis the use of whole algae for the study of constituents seems justified.

Experience has shown that some separation of the carbohydrates is often possible by differential extraction, the ease of extraction depending on the location and chemical nature of the materials.[3] Refluxing the alga in 80% aqueous ethanol extracts all the low molecular weight carbohydrates without extracting any polysaccharide. The extraction of the polysaccharides is more problematical; most methods yield mixtures, often contaminated with protein and colouring material, and in all but the mildest conditions considerable modification and

degradation of macromolecular structure may occur. This is not so important where only one polysaccharide is sought, such as in the commercial extraction of alginic acid, but may seriously hamper study of the function and biosynthesis of the various polysaccharides where the structure "in vivo" must be known. No standard method has been formulated, the procedure usually being established by trial and error. The stages are generally selected from the sequence: cold water, hot water, cold dilute acid, hot dilute acid, cold dilute alkali and hot dilute alkali. The residual material will often still contain cellulose and uronic acid-containing polysaccharides but the latter are usually too highly degraded to be worth investigating.

The sequence hot 80% aqueous ethanol, cold water, hot water, hot acid and hot alkali was chosen to keep the number of extracts small.

To facilitate the extractions the whole alga was first ground to a fine powder under liquid nitrogen (Expt. 5.1). (This powder will be referred to as the dried alga and concentrations of the various carbohydrates will be quoted as percentages of the dry weight of this material). Treatment of this powder with formaldehyde solution polymerised phenolic compounds in the alga, rendering them insoluble in most of the extractants and giving

clean extracts. The residual, adhering solution was allowed to evaporate in a stream of air rather than drained off, to avoid loss of carbohydrate. The treated material was dried again to a powder. The full extraction procedure is shown in FLOW CHART 1. 2% of calcium chloride was present in the aqueous extractions in order to retain alginic acid in the alga as its insoluble calcium salt; in the hot acid extraction the free acid form should remain insoluble (Expts. 5.2-5.6).

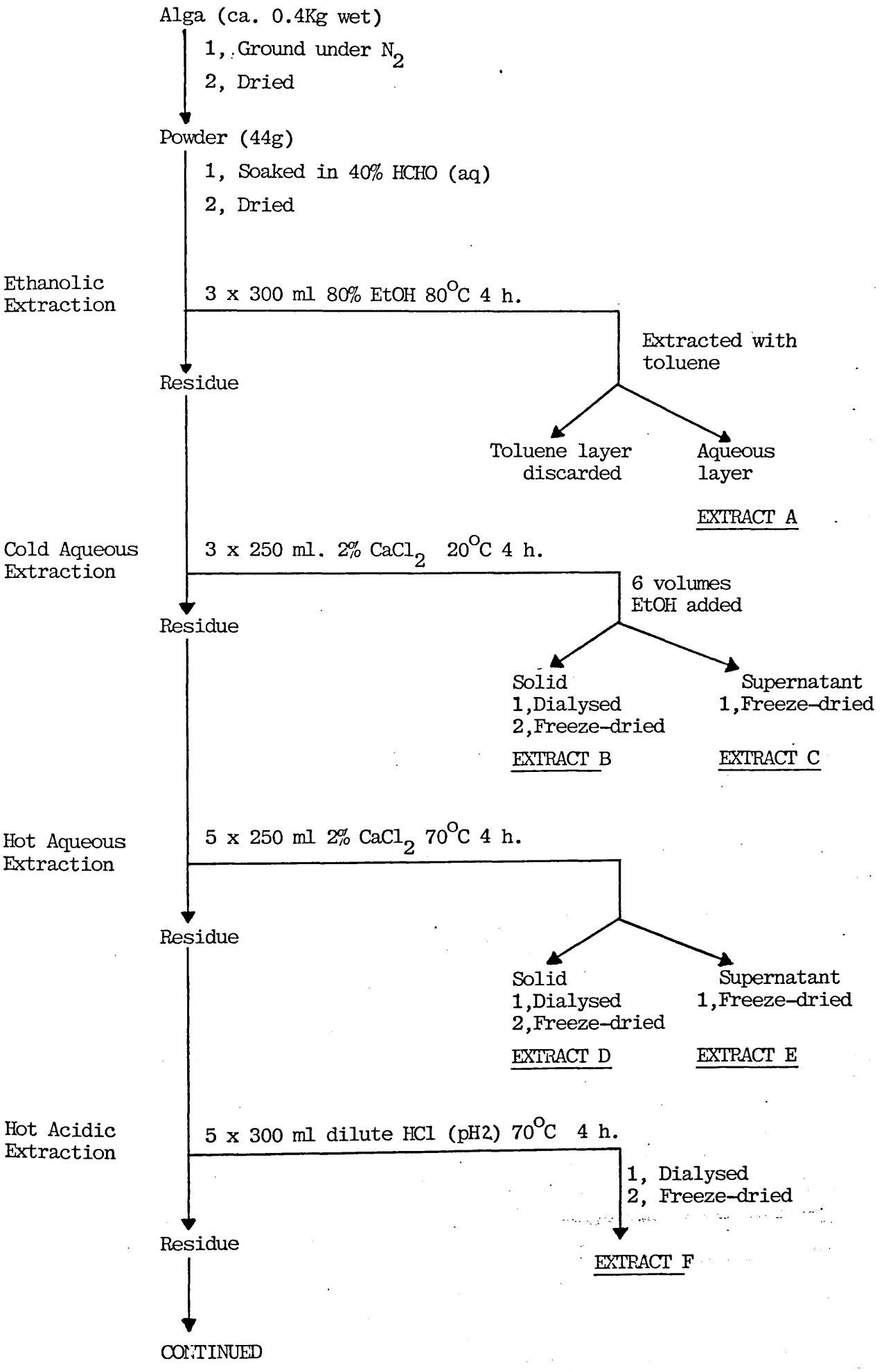
The ethanolic extract was not isolated as a solid. The yields of the remaining extracts are listed in TABLE 2.1.

TABLE 2.1. RECOVERY OF POLYMERIC EXTRACTS FROM ASCOSEIRA (44 g)

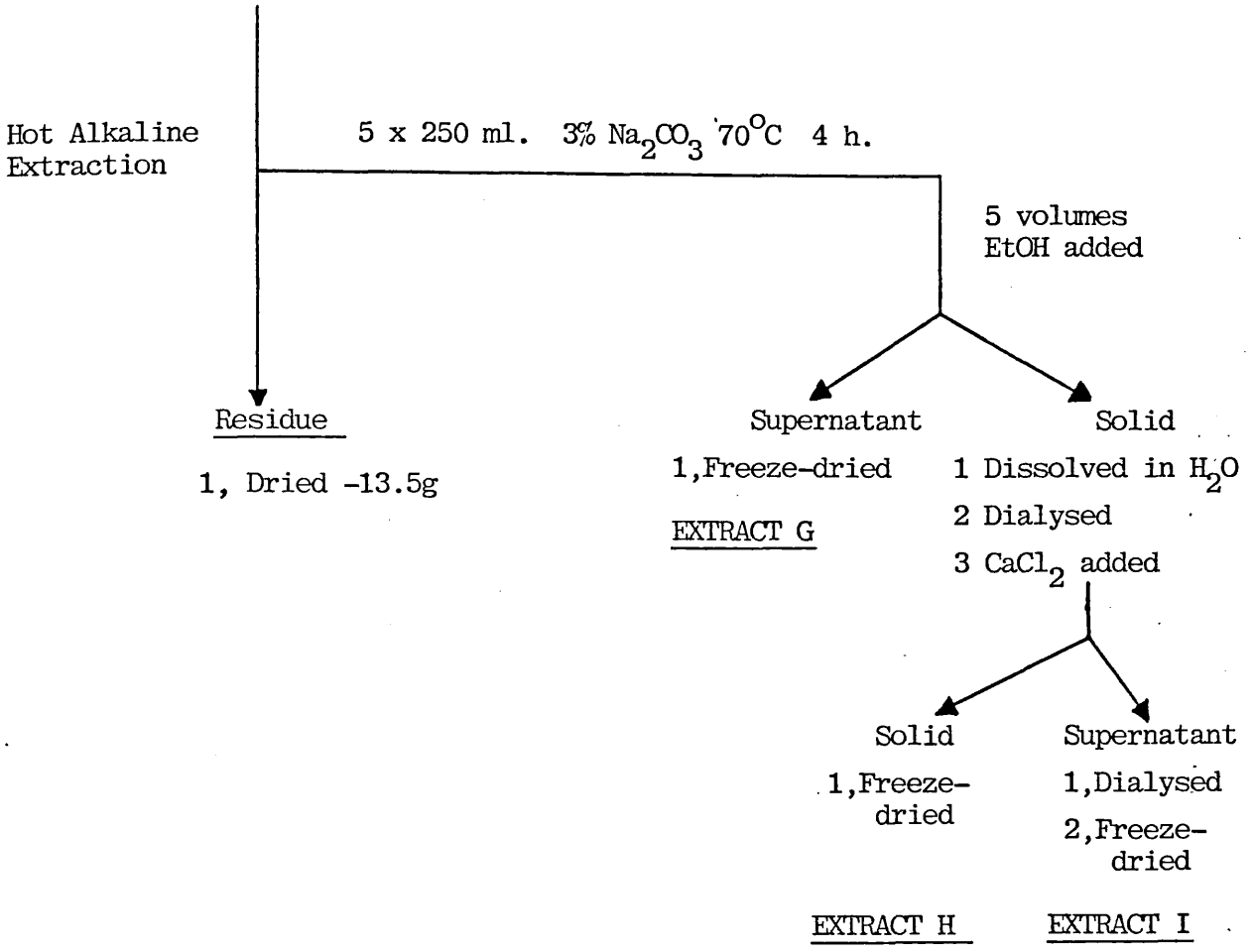
Extractant	Extract	Weight(mg.)	% of Dry Weight
Cold water	B	590	1.3
Cold water	C	50	0.1
Hot water	D	826	1.9
Hot water	E	35	0.1
Hot acid	F	790	1.8
Hot alkali	G	20	0.1
Hot alkali	H	1770	4.0
Hot alkali	I	236	0.5
	Total	4317	9.8

FLOW CHART 2.1.

SEQUENTIAL EXTRACTION OF ASCOSEIRA



FLOW CHART 2.1. (continued)



2.1.2. Low Molecular Weight Carbohydrates in the Ethanolic Extract.

After removal of the inorganic salts (Expt.5.7), mannitol was recovered and recrystallised. Its melting point, mixed melting point and the retention time of its peracetylated derivative on g.l.c. all confirmed its authenticity. Its yield (500 mg, 1.4% of the dry weight) is low, values of 3% or more being common in the brown algae. Some loss may have occurred during collection of the alga, though the low yield may be due to the seasonal variation in the amount of mannitol. The alga was collected during late summer, when the mannitol might be expected to be at a high level,[4] but may be temporarily depleted by conversion to laminaran during a period of slow growth.

The residual syrup contained 160 mg of carbohydrate measured by the phenol/sulphuric acid method, and based on a glucose standard graph. It should be pointed out here that only a few standard graphs have been used for calculating carbohydrate contents in this work and will be quoted where appropriate. This introduces some inaccuracy but it is impractical to prepare standard graphs for all the polysaccharides encountered, and the graphs would be of doubtful value given the problems in accurate determination of monosaccharide compositions.

Generally the carbohydrate content was read from either a glucose or a fucose standard graph and then "corrected" using a more appropriate graph when the approximate monosaccharide composition had been determined.

Several sugars were found in the syrup. After paper chromatography sucrose and fructose were identified by ketose spray, glucose by glucose oxidase, and fucose and xylose tentatively by aniline oxalate. The latter three sugars were confirmed by g.l.c.,which also revealed glycerol; these four components appeared to form the bulk of the syrup. A minor slow moving component ($R_{MANNITOL}$ ~ 0.25 in solvent 18:3:1:4) was separated on paper and hydrolysed, giving mannitol, glucose and xylose on paper chromatograms and g.l.c. The proportions suggested a mixture of oligomers rather than a single species. There was insufficient material for structural study.

Sucrose, glycerol and oligomers of glucose and mannitol have been reported previously in the brown algae and would appear to have some metabolic function. Fucose and xylose may be derived from degradation of polymeric species.

2.1.3. Composition of the Aqueous, Acidic and Alkaline Extracts (Expts. 5.8, 5.13).

The ethanol-soluble components (Extracts C,E and G)

of the various extracts all had carbohydrate contents of about 20% (glucose graph) and in view of the small amounts were not investigated further.

The insoluble calcium salt (Extract H) was converted to the sodium salt (Expt. 5.13); at the free acid stage a small amount of carbohydrate, approximately 5% of the extract, was solubilised. The sodium salt gave uronic acids but no neutral sugar on paper chromatograms. On g.l.c. glucitol hexaacetate and mannitol hexaacetate were identified; they are probably derived from reduction of guluronic acid and mannuronic acid. These results are consistent with the assumption that this extract is comprised of alginic acid, the 5% of soluble material being probably "fucan", indicating that the addition of calcium ions does not achieve a sharp separation of alginic acid from the remainder of the alkali-extracted material. No further attempt was made to confirm these inferences at this stage. The compositions of the remaining extracts are summarised in TABLE 2.2. Extracts B, D and F were white solids, Extract I was buff-coloured.

TABLE 2.2. COMPOSITION OF MAJOR EXTRACTS FROM ASCOSEIRA

EXTRACT	CARBOHYDRATE	URONIC ACID	SULPHATE	PROTEIN
	%(a)	%(b)	%(c)	%
B	62	10	10	0
D	62	15	8	0
F	50	49	3	<1
I	70	19	5	16

(a) Based on a glucose standard graph.

(b) Based on a glucuronic acid standard graph and expressed as a percentage of the carbohydrate.

(c) Expressed as a percentage of the carbohydrate.

Portions of each extract were acid-hydrolysed by two methods (GM 5.3.1,5.3.3.) and the hydrolysates examined by paper chromatography and g.l.c. Fucose, xylose, mannose, glucose, galactose and uronic acids appeared on paper chromatograms from all the extracts, the glucose being confirmed in each case with glucose oxidase. The five neutral sugars were identified as their alditol acetates on g.l.c. This may be taken as confirmation of fucose and xylose, but the hexitol acetates may be partially derived from the respective uronic acids.

The enantiomeric identities of the component sugars were not established; it is assumed throughout this thesis that they do not differ from those previously identified, namely L-fucose, D-xylose,

D-mannose, D-glucose, D-galatose, D-glucuronic acid, D-mannuronic acid and L-guluronic acid. The alternative enantiomers have not been found in brown algae; study of several algae failed to find evidence of D- fucose, [5] which occurs in other organisms.

During hydrolysis of the extracts the acid conditions induce extensive lactonisation of uronic acids, the extent being dependent on the uronic acid and the hydrolysis conditions.[6] The lactones are subsequently reduced, along with the neutral sugars, to the alditols, thereby confusing chromatographic results. Uronic acids not present in lactone form are reduced to aldonic acids which give rise to variable amounts of 1,4- and 1,5-lactones. These may appear on g.l.c. after acetylation but do not interfere with the other alditol derivatives.[7]

Several methods of avoiding this problem have been suggested. Those dependent on conversion of the lactones back to the free acids or salts with alkali face the likely alkaline degradation of the reducing sugars or the uronic acid lactones. Blake and Richards[6] found that titration with potassium hydroxide, maintaining the pH at 7.5 - 9.0, produced minimal degradation of lactones. The neutral sugars and uronic acids could then be separated on ion-exchange resins. Complete reduction of the uronic

acids to lactones (and hence to the alditols) by repeated borohydride treatment has been claimed[8], allowing determination of the total sugar, though the neutral sugars still have to be quantified separately. A similar extra analysis is necessary when the uronic acids are reduced prior to hydrolysis - in itself a difficult step.

Perry and Hulyalkar[9] claimed successful analytical trimethylsilylation of lactonised aldonic acids but were unable to achieve quantitative conversion of uronic to aldonic acid during hydrolysis. Jones and Albersheim[10] avoided the problem by using a mild acid hydrolysis followed by enzymic degradation to the aldoses and uronic acids. However this method is hardly convenient for routine analysis.

Under the conditions used here mannuronic acid was converted to mannitol to an extent of about 50%; no other products were detectable in appreciable amounts on g.l.c. under any of the acid conditions used. Glucuronic acid did not give reproducible results, though up to 10% conversion to glucitol occurred together with products tentatively identified as derived from lactonised gluconic acid. Whether these results are representative of what would happen when the acids are linked in a polymer is not clear but analysis of an alginic acid sample confirmed partial conversion of mannuronic acid and a small but significant conversion of guluronic acid to glucitol.

The consequences of this ambiguity in the g.l.c. results will be discussed later when the extracts have been fractionated. For the moment there is only tentative evidence for mannose in these extracts.

In addition to the above, the calculation of the proportions of the monosaccharides by g.l.c. proved difficult. For all four extracts the two hydrolysis methods gave different figures though in all cases glucose was the major sugar (TABLE 2.3). Repeat experiments showed the figures to be reproducible, implying that the two acids must give different patterns of hydrolysis. In view of the complexity of the extracts and in particular the high uronic acid contents this was not too surprising and hence the extracts were fractionated before any further attempt at monosaccharide quantification was made.

TABLE 2.3. RELATIVE PROPORTIONS OF NEUTRAL SUGARS IN ASCOSEIRA EXTRACTS.

EXTRACT	ACID[a]	NEUTRAL SUGAR DISTRIBUTION (%)[b]				
		FUC	XYL	MAN	GLU	GAL
B	1	9	5	72	12	3
	2	8	3	24	59	7
D	1	14	5	26	50	5
	2	5	4	12	72	8
F	1	7	8	30	48	7
	2	5	2	23	57	14
I	1	25	8	6	49	30
	2	7	8	6	49	30

(a) 1: 90% formic acid (GM 5.3.1).

2: 2M trifluoroacetic acid (GM 5.3.3).

(b) Calculated from g.l.c. considering total peak area of five peaks at 100%.

2.1.4. Fractionation of the Polymeric Extracts.

A variety of fractionation methods for polysaccharides has been developed on an analytical scale, but for preparative work the choice is somewhat limited. Fractional precipitation with organic solvents (usually ethanol) has been widely practised but tends to give poor resolution, particularly where there are only slight differences in solubility. It has however proved useful here to effect a rapid separation of polymeric material from small fragments and contaminants. Selective precipitation with

metallic ions is more successful; as already described alginic acid can be precipitated from solutions of algal polysaccharides by addition of calcium ions, and may be further fractionated into guluronate- and mannuronate-rich fractions by addition of manganous salts to solutions of the sodium salt. Barium and copper salts have also been used.[11-13]

Quaternary ammonium salts such as cetylpyridinium chloride form insoluble salts with anionic polysaccharides, giving a sharp separation of neutral and charged species.[14] However, problems may be experienced in resolubilising the polysaccharide.

The technique used here has been found to be successful before for algal polysaccharide extracts, Large quantities of polymeric material may be fractionated on columns of diethylaminoethyl-cellulose (DEAE cellulose). Anionic polymers are readily adsorbed on the column at near neutral pH, allowing neutral polymers to be eluted with water. The anionic polymers are then desorbed either by lowering the pH or by increasing the ionic strength of the eluant. Species of different adsorption affinities may be eluted selectively by applying a gradient of increasing ionic strength to the column.

Portions of Extracts B,D,F and I were fractionated on DE52 cellulose columns (Expt.5.9). Neutral carbohydrate material was eluted from all four

extracts and isolated by freeze-drying (Fractions BN,DN,FN and IN respectively). Previous fractionations of brown algal extracts have been carried out with steps of increasing potassium chloride concentration. In general polysaccharide eluted over a broad range of ionic strength (up to 1$\underline{M}$ KCl), the eluate of low ionic strength containing material with a high proportion of glucuronic acid and a low proportion of fucose and sulphate; as the ionic strength was increased the proportion of uronic acid decreased and the fucose and sulphate increased.[15-17] This stepwise elution may mask a partial resolution of fractions, so a linear gradient of KCl was used in this work to elute the anionic material from the column.

In fact, in contrast to the results described above, the anionic material from Extracts B,D and F (from the Cold Water, Hot Water and Acid Extracts respectively) eluted as a single band, in each case at the ionic strength given by 0.1 $\underline{M}$ KCl. In view of this the acidic components were isolated as single polysaccharide Fractions (BA, DA, FA respectively).

The elution profile (based on carbohydrate content of column fractions) of Extract I (FIGURE 2.1) showed three distinct peaks, which were isolated separately (Fractions IA1, IA2 and IA3 respectively in order of elution).

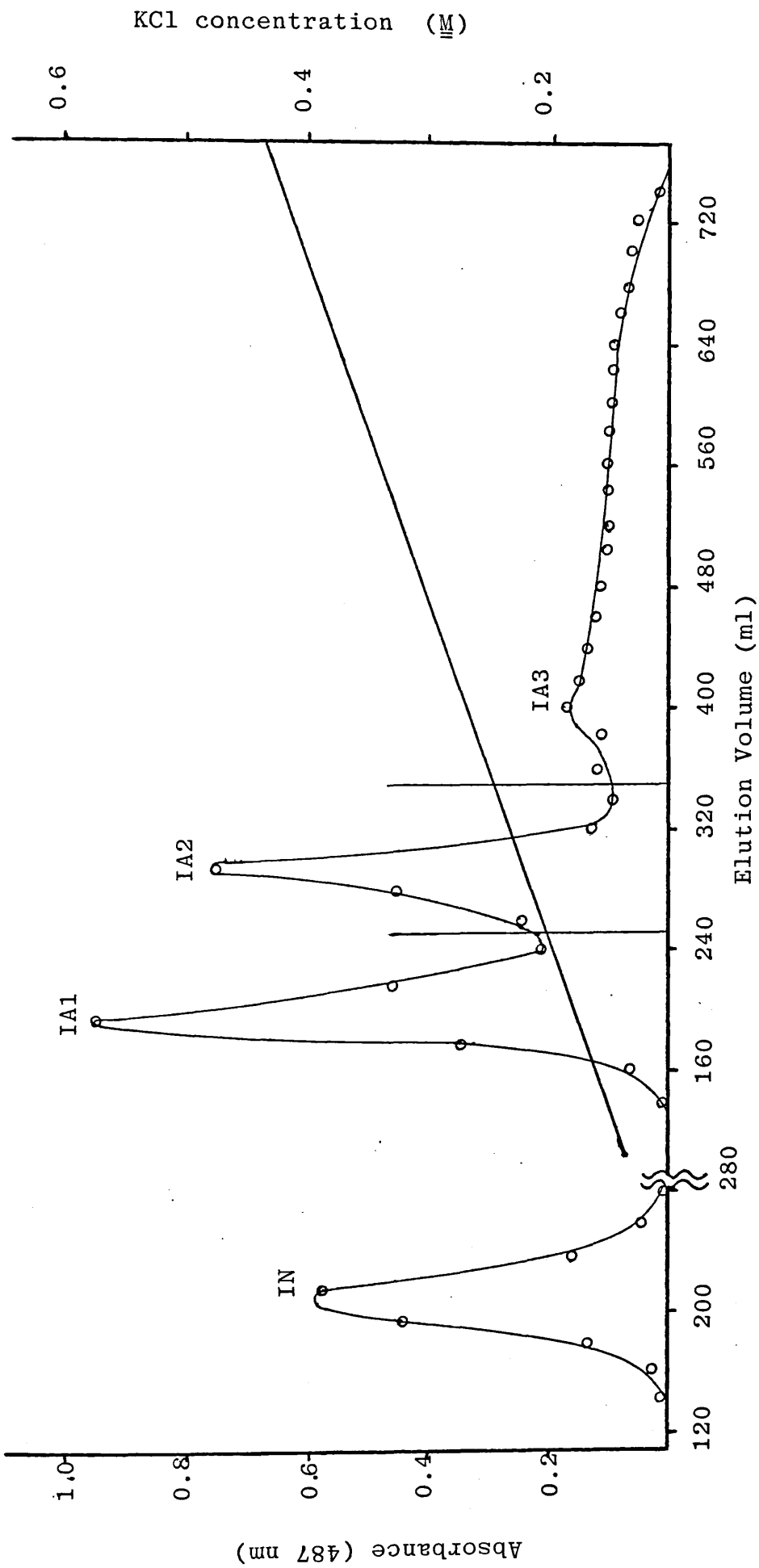


FIGURE 2.1 ELUTION PROFILE OF ASCOSEIRA EXTRACT I ON A DE52 COLUMN

2.1.4.1. Examination of Neutral Fractions (Expt. 5.10)

Fractions BN and DN (from the Cold Water and Hot Water Extracts) dried to amorphous white solids; the yields represented 21% and 31% respectively of the original extracts. The carbohydrate contents (glucose graph) were 88% and 60% respectively. Dialysis of the latter reduced the yield to about 20% and increased the carbohydrate content to 85%, indicating the presence of inorganic material, possibly resulting from incomplete washing of the column during regeneration. After hydrolysis glucose was the only reducing sugar detectable in both. Mannitol was identified with silver nitrate and confirmed as about 3% of the carbohydrate in both cases. These results are consistent with the expected presence of laminaran in the aqueous extracts.

The neutral fractions FN and IN (from the Acid and Alkali Extracts respectively) freeze-dried to syrups, containing glucose as the only reducing sugar after hydrolysis. The recoveries as a percentage of the extracts could not be calculated but on the basis of carbohydrate content the neutral fractions accounted for respectively 10% and 18% of the carbohydrate in the Acid and Alkali Extracts. The mannitol observed on g.l.c. amounted to only 1%

of the glucose in both cases, too small to be considered as confirmation of its presence.

These two fractions would appear to be laminaran which has not been extracted by the aqueous extractants, though this is not a common observation.

2.1.4.2. Examination of acidic fractions

The yields and compositions of the six acidic fractions are summarised in TABLE 2.4 (Expt. 5.11).

TABLE 2.4. ACIDIC FRACTIONS FROM MAJOR ASCOSEIRA EXTRACTS

FRACTION	RECOVERY (a) %	CARBOHYDRATE (b) %	URONIC ACID (c) %	SULPHATE (d) %	PROTEIN (e) %
BA	63	41	20	15	0
DA	56	53	11	10	0
FA	57	45	37	5	2
IA1	18	69	4	0	0
IA2	30	42	11	10	15
IA3	31	43	13	10	20

(a) Percentage of the original extract.

(b) Fuc:Gal:GlcA (1:1:1) standard graph

(c) GlcA standard graph and expressed as percentage of carbohydrate.

(d) Expressed as percentage of the carbohydrate

(e) Expressed as percentage of the weight.

Samples of each were hydrolysed with 2M trifluoroacetic acid and examined by paper chromatography and g.l.c. The approximate relative proportions of the monosaccharides found are shown in TABLE 2.5. Mannose was estimated visually from paper chromatograms and the uronic acid content was based on the previously calculated value; otherwise the proportions were determined by g.l.c. The glucitol hexaacetate was assumed to arise entirely from glucose rather than glucuronic or guluronic acid. The errors introduced by these assumptions are unavoidable without a great deal of time-consuming separation of uronic acids and neutral sugars with the inherent difficulties described earlier (Section 2.1.3.).

TABLE 2.5.

RELATIVE PROPORTIONS OF SUGARS IN ASCOSEIRA ACIDIC FRACTIONS

FRACTION	SUGAR DISTRIBUTION					
	FUC	XYL	MAN	GLU	GAL	URONIC ACID
BA	x	x	xx	xx	x	xxx
DA	xx	x	xx	x	xx	xx
FA	x	x	xx	x	x	xxxx
IA1				xxxx		x
IA2	xx	x	x	xxx	xx	xx
IA3	xx	x	x	x	xxxx	xx

Fraction IA is apparently a glucan. The probable explanation is that it is laminaran which has been degraded by the alkali,[18] giving meta-saccharinic acids which would be adsorbed to the column. The remaining fractions all appear to be complex polysaccharides.

The paper chromatograms suggested that there may be more than one uronic acid present in all of these fractions. The uronic acid contents were initially determined by the metahydroxydiphenyl method, which cannot distinguish different uronic acids, so the fractions were examined by the carbazole method. This method allows approximate proportions of different acids to be determined. Glucuronic acid is commonly found in algal polysaccharides, and mannuronic and guluronic acids are found in alginic acid; the latter two have been reported both separately and together in extracts containing the neutral monosaccharides.[19] Mannuronic acid oligomers are commonly found in material extracted under acidic conditions.[20]

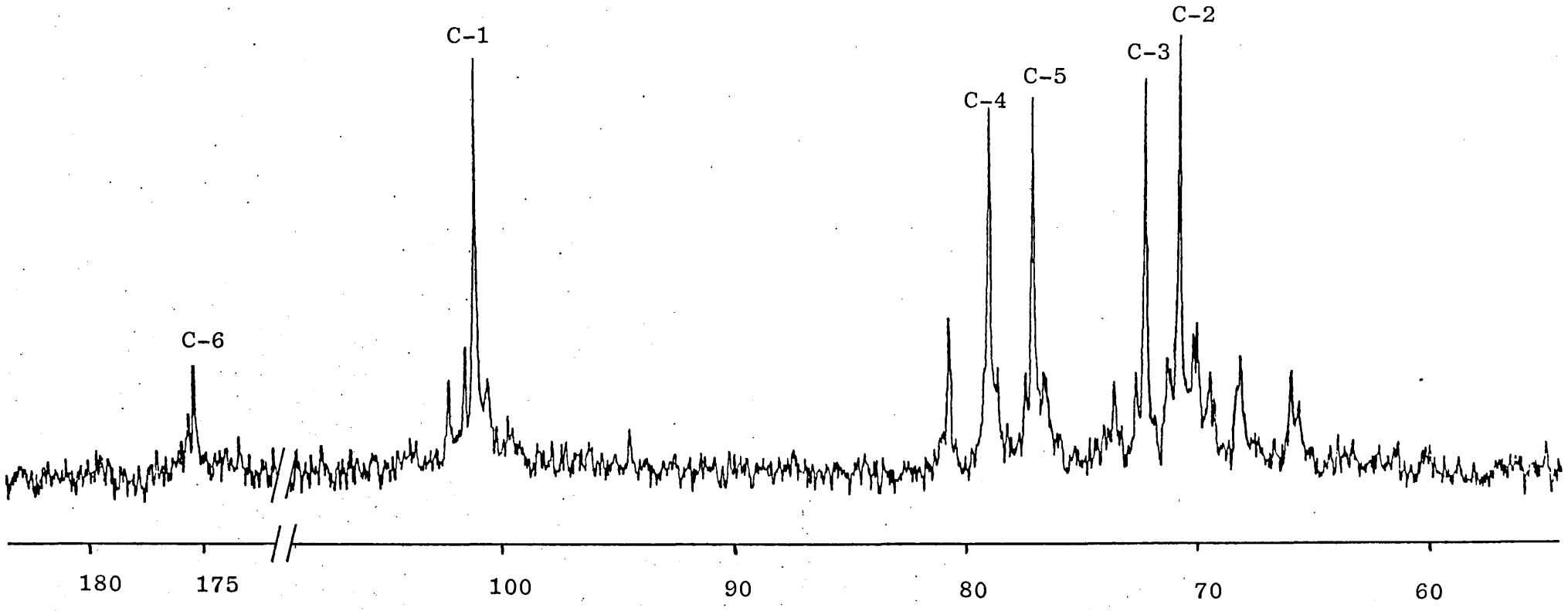
The carbazole analysis gives only approximate proportions of the three possible acids, but for all the fractions it was clear that glucuronic acid comprised only a small part of the acids and the remainder was a mixture of mannuronic and guluronic acids. These results suggest that all the fractions are contaminated with alginic acid, despite the

stringent attempts to render it insoluble in the aqueous extractants and to separate it from the Alkali Extract. As stated above, the presence of mannuronic acid in acid extracts is not unusual. On the basis of the high "mannose" values found on g.l.c. for all the fractions it seems reasonable to assume that there is substantially more mannuronic acid than guluronic acid. The conclusion that most of the uronic acid is mannuronic alters the uronic acid contents of the fractions which were calculated from a glucuronic standard graph. Recalculation using a mannuronic acid graph gives uronic acid contents which are approximately doubled, thus the values in TABLE 2.4 may be more accurately expressed as:- BA 40%, DA 20%, FA 67%, IA2 20% and IA3 24%.

It is now apparent that all the fractions contain a substantial proportion of alginic acid, the remainder in each case being "fucan"-like material (see Introduction). The fact that only one acidic band eluted from the columns for Extracts B,D and F, and two bands of roughly similar composition from Extract I, suggests that the alginic acid and "fucan" may be linked.

A clearer picture of the composition of Fraction BA was obtained from the ^{13}C n.m.r. spectrum (FIGURE 2.2). Comparison with published spectra[21]

FIGURE 2.2 ^{13}C N.M.R. SPECTRUM OF ASCOSEIRA FRACTION BA



shows the six principal peaks to correspond to the six-carbon repeating unit of polymannuronic acid (TABLE 2.6).

TABLE 2.6.

ASSIGNMENT OF PRINCIPAL PEAKS IN ^{13}C N.M.R. SPECTRUM OF FRACTION BA.

	CHEMICAL SHIFT (p.p.m.)					
	C-1	C-2	C-3	C-4	C-5	C-6
Fraction BA[a]	100.9	70.8	72.3	78.9	77.1	175.3
Polymannuronate[b]	102.8	72.2	74.1	80.7	78.9	177.1

(a) External TMS reference.

(b) Internal TSP reference. The correction for TMS is ca - 1.8 p.p.m. [22]

The predominance of these peaks suggests that the fraction is mainly polymannuronic acid; indeed the uronic acid content is apparently higher than suggested by colorimetric determination.

Further discussion of n.m.r. spectra and of the composition of all the acidic fractions will follow a second series of extractions.

2.1.5. Examination of the residual alga.

After the final alkali extraction the residual alga was rinsed with water and dried, giving 13.5 g (31% of the dry weight). A portion was hydrolysed (Expt. 5.12) and the carbohydrate content determined

to be only about 1%. Uronic acid, glucose and traces of fucose, xylose, galactose and mannose were identified in the hydrolysate by paper chromatography.

In view of the small amount of carbohydrate and the vigorous conditions required for extraction the residue was not investigated further.

2.1.6. Summary

The five-step sequential extraction has given four polymeric extracts, all of which appear to contain glucan, "fucan" and alginic acid. This would suggest that the procedure used is not the most convenient or effective way of extracting the various polymers but it is difficult to see how it can be varied without compromising the aim of obtaining relatively undegraded polymers. The Cold Water and Hot Water Extracts seem to be of similar composition and may be rationalised into one step.

The total recovery of polysaccharides (4.3 g. 9.8% of the dry weight) is unusually low; all three major polysaccharides are commonly found as a greater proportion of the dry weight.

Further study of the polysaccharide structures requires a fresh extraction on a larger scale and a method of separating the "fucan" and alginic acid, if indeed they can be separated.

2.2. Large Scale Extraction of Ascoseira

2.2.1. Extraction

The extraction procedure was modified slightly, (Expts. 5.14-5.18); the Cold Water Extraction was omitted and the attempted fractionation of extracts by pouring into ethanol was only carried out with the Acid Extract.

The full procedure is outlined in FLOW CHART 2.2. During the concentration of the Hot Water Extract some material precipitated. This was removed by centrifugation and freeze-dried; on attempted dissolution about 65% dissolved again.

The recoveries and composition of the polymeric extracts are shown in TABLE 2.7 (Expt. 5.19-5.21, 5.25-5.27, 5.30).

TABLE 2.7

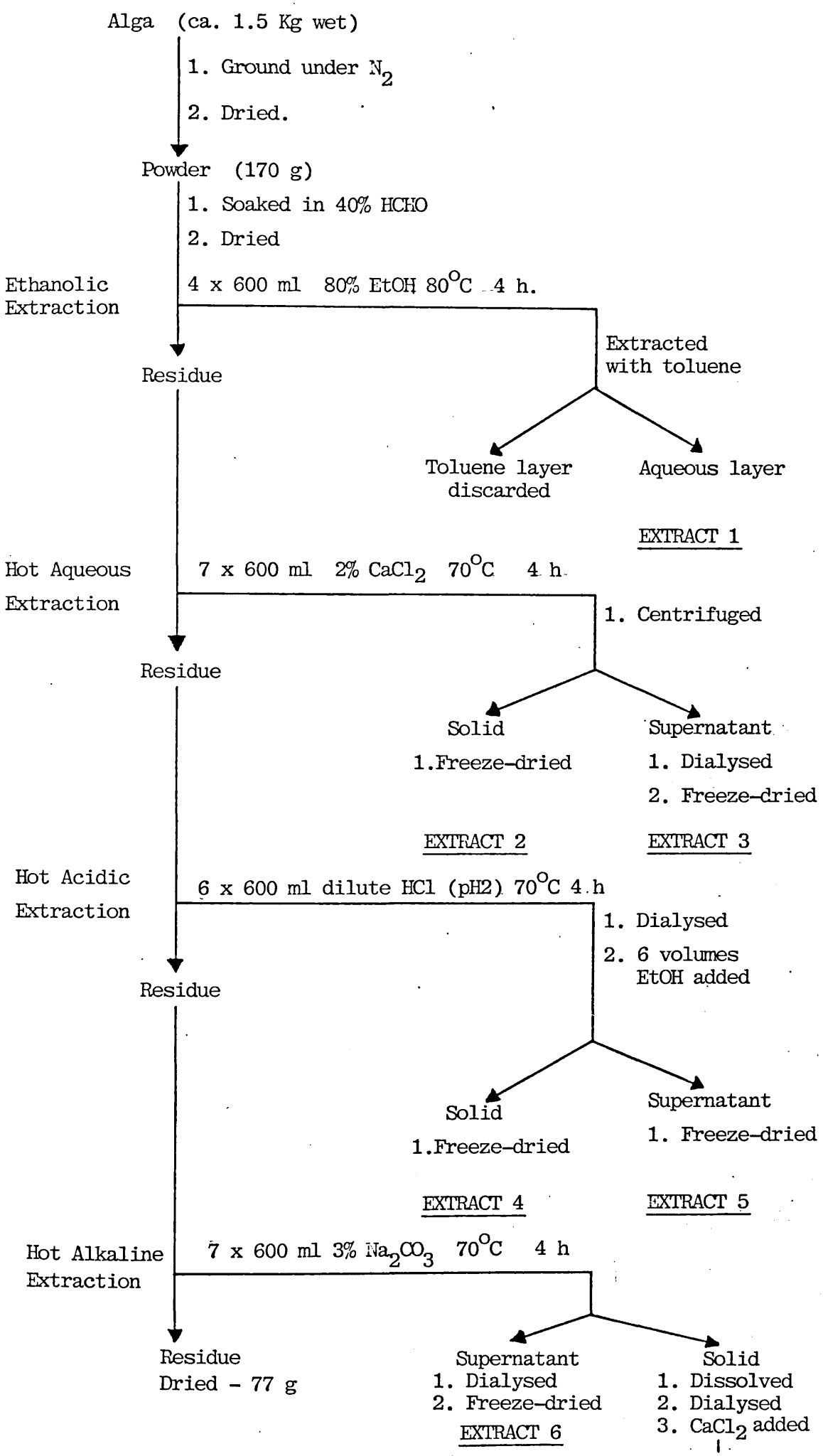
RECOVERY AND COMPOSITION OF POLYMERIC EXTRACTS FROM ASCOSEIRA

Extractant	Extract	Recovery	Carbohydrate	Uronic Acid	Protein
		g.	%(a)	%(b)	%(c)	%(d)
Hot aq.$CaCl_2$	2	0.77	0.5	35	40	0
Hot aq.$CaCl_2$	3	3.81	2.2	60	45	0
Hot Acid	4	2.26	1.3	50	55	6
Hot Acid	5	0.45	0.3	55	35	1
Hot Alkali	6	0.05	-	50	(e)	(e)
Hot Alkali	7	13.83	8.1	(e)	(e)	(e)
Hot Alkali	8	2.42	1.4	60	40	16
	TOTAL	23.59	13.9			

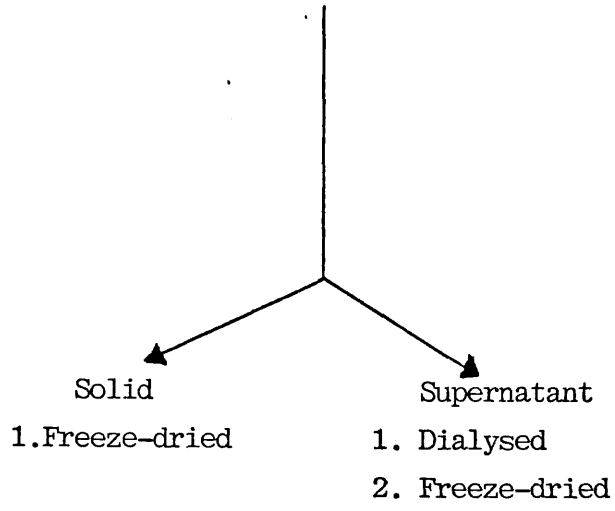
(a) Percentage of the dry weight.
(b) ManA:GlcA (1:1) standard graph.
(c) ManA standard graph and expressed as percentage of the carbohydrate.
(d) Percentage of the extract.
(e) Not determined.

FLOW CHART 2.2

SECOND SEQUENTIAL EXTRACTION OF ASCOSEIRA



FLOW CHART 2.2 (continued)



Comparison with TABLE 2.1 shows that the proportions of each extract are rather different from the first extraction. In particular there is a substantially larger recovery of alginic acid, Extract 7, though the total recovery of the other polysaccharides is roughly the same. The reasons for this are not clear. In both cases each extraction step was repeated exhaustively i.e. until negligible carbohydrate was detectable in the extract. The alga was from a single batch, though no attempt was made to ensure a similar distribution of fronds, stipes and holdfast. The two samples were ground to a similar consistency and hence the efficiency of each extraction step should be similar in both. Both were dried at room temperature and hence the final dry material may still contain a variable amount of water; however this should not affect the relative proportions of each polysaccharide.

The principal feature of the first extraction - the low percentage of carbohydrate - is confirmed.

2.2.2. Investigation of the Ethanolic Extract (Expt.5.19)

The same procedure was followed as for the previous extraction. 1.46 g (0.9% of dry weight) of mannitol was separated and characterised. The proportion (640 mg, 0.4% DW.) and composition of the residual carbohydrate were the same as

previously. The amount of oligomeric material was again small.

2.2.3. Composition of the polymeric extracts.

(Expts. 5.20, 5.21, 5.25-5.27, 5.30).

Portions of each extract were hydrolysed with 2M trifluoroacetic acid and the hydrolysates examined by paper chromatography and g.l.c. The approximate proportions of the monosaccharides were determined in the same manner as for the acidic fractions in the first extraction (TABLE 2.8).

TABLE 2.8

SUGAR DISTRIBUTION IN POLYMERIC EXTRACTS FROM ASCOSEIRA

EXTRACT	SUGAR DISTRIBUTION					
	FUC	XYL	MAN	GLU	GAL	URONIC ACID
2	xx	x	x	xxx	x	xxxx
3	xx	x	x	xxx	x	xxxx
4	x	x	x	xxx	x	xxxx
5	x	x	x	xxx	x	xxxx
6	xx	x	x	xxx	xxx	xxx
8	xx	x	x	xxx	xxx	xxx

The similarity in composition of Extracts 2 and 3 and Extracts 4 and 5 suggests that no fractionation was occurring when Extracts 2 and 4 precipitated and thus the four extracts will be considered as two (3 and 5).

With the proviso that the glucan has not been

separated, the compositions are similar to those found previously for the corresponding acidic fractions (TABLE 2.5).

2.2.4. Fractionation of the Polymeric Extracts (Expts. 5.22, 5.30).

In view of the poor recovery from the column and the high mannuronic acid content of the previous Acid Extract, no fractionation was attempted on DEAE cellulose.

The Hot Water and Akali Extracts were fractionated on large-scale DEAE cellulose columns. Neutral fractions were eluted with water. The acidic fractions were recovered by exhaustive elution with 0.5M KCl for the HWE, and by elution with 0.1M KCl (1 l) followed by 0.5M KCl (exhaustive) for the Alkali Extract.

The recoveries and composition of the various fractions are listed in TABLE 2.9. The neutral fraction from the Alkali Extract was successfully dried to a solid (Expts. 5.23, 5.24, 5.31-5.33).

TABLE 2.9

COMPOSITION OF DE52 COLUMN FRACTIONS FROM ASCOSEIRA EXTRACTS

EXTRACT	ELUANT	FRACTION	RECOVERY % (a)	CARBOHYDRATE %	SUGAR DISTRIBUTION (b)							
					FUC	XYL	MAN	GLU	GAL	MANA	GULA	GLCA
3	Water	3N	12	86 (c)				100				
3	0.5M KCl	3A	60	60 (d)	13	5	4	5	8	55	5	5
8	Water	8N	28	85 (c)				100				
8	0.1M KCl	8A1	2	65 (c)				100				
8	0.5M KCl	8A2	40	60 (e)	20	11	5	3	24	20	5	10

(a) Percentage of the extract.

(b) Neutral sugars determined by g.l.c.; mannose determined visually from paper chromatograms. Uronic acids estimated by carbazole method.

(c) Glucose standard graph.

(d) Fucose: Xylose: Mannose: Glucose: Galactose: Mannuronic acid (1:1:1:1:1:10).

(e) Fucose: Galactose: Mannuronic Acid (1:1:1) standard graph.

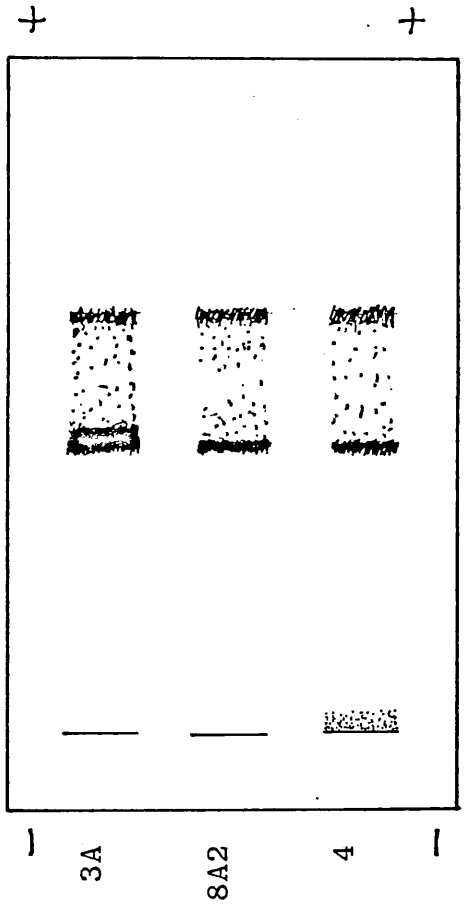
The neutral fractions will be discussed in Section 2.2.7.

As stated earlier, another method is required to separate "fucan" and alginic acid. Gel electrophoresis, cellulose acetate electrophoresis and gel filtration were attempted on the acidic fractions 3A and 8A2. The Acid Extract had freeze-dried as a fine powder and proved only slightly soluble in the buffer systems used; only cellulose acetate electrophoresis was attempted (Expts. 5.20, 5.21, 5.25-5.27, 5.30).

The two acidic fractions and the Acid Extract migrated as single bands of similar mobility on cellulose acetate at pH 8.0; in the case of the Acid Extract a large amount of material remained on the baseline.

At pH 5.1 two bands were evident for all three fractions (FIGURE 2.3). By comparison with known samples the fast band in each case was assigned as "fucan" and the slower band as alginic acid. This is to be expected since at this pH the carboxyl groups of alginic acid are only partially ionised, whereas the sulphate groups in "fucan" remain essentially fully ionised. Unfortunately calculation of the relative distribution of the two polymers depends on quantitative binding of dye molecules, hence the intensity of the bands cannot be taken as

FIGURE 2.3 ELECTROPHORETIC PATTERNS OF ASCOSEIRA ACIDIC FRACTIONS 3A AND 8A2 AND ACIDIC EXTRACT 4 ON CELLULOSE ACETATE AT pH 5.1



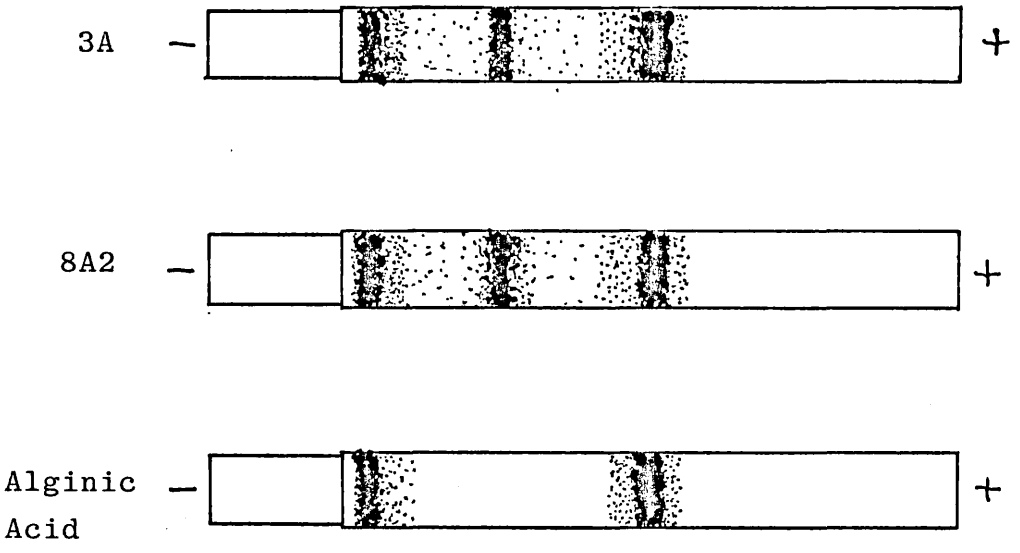
proportional to the amount of polymer. However the predominance of the alginic acid band in Fraction 3A and the roughly similar bands in Fraction 8A2, are consistent with the colorimetrically determined uronic acid contents.

Gel electrophoresis on 7.5% cross-linked polyacrylamide gave rather different results. At pH 8.5 a single migrating band which stained for polysaccharide and protein was observed for 3A and 8A2, tentative evidence of covalent linkage.

A stable, low pH acrylamide gel system could not be cast, but a system at pH 6.5 proved stable. The results however, were anomalous (FIGURE 2.4). In contrast to the cellulose acetate electrophretic patterns, the "fucan" migrated more slowly than the alginic acid in both cases. A possible explanation is that the pH is not low enough to suppress ionisation of the alginic acid, allowing relatively rapid migration, whereas the "fucan" is restrained by the molecular sieving effect of the gel matrix. However this fails to explain the apparently unimpeded migration of the "fucan" at pH 8.5. No explanation can be offered for the two bands for alginic acid.

Staining for protein in Fraction 8A2 did not produce any visible band, though the considerable dispersion of the polysaccharide bands may account for this.

FIGURE 2.4 POLYACRYLAMIDE GEL ELECTROPHORESIS OF ASCOSEIRA FRACTIONS 3A AND 8A2 AT PH 6.5

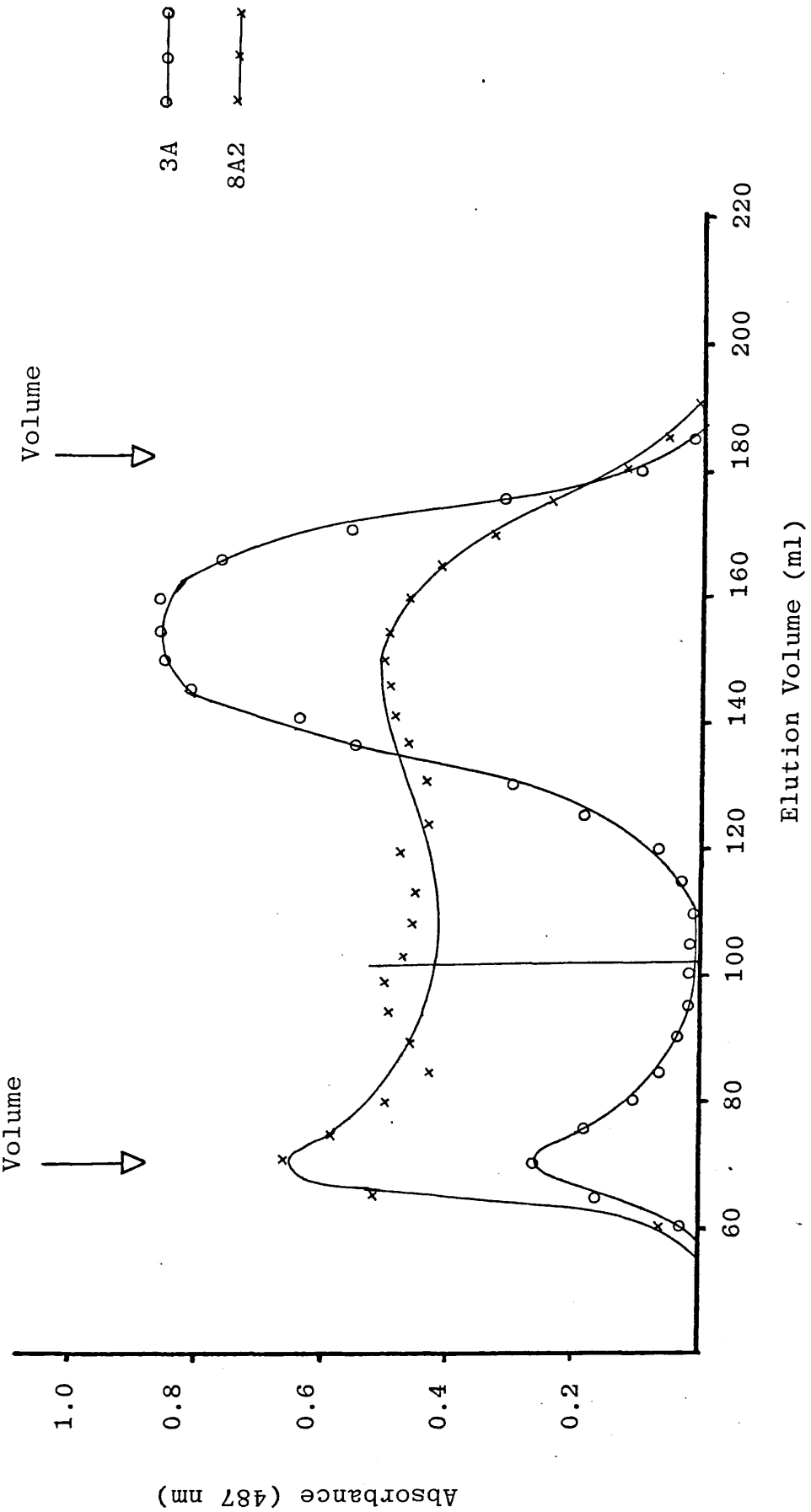


The molecular sieving effect may be reduced by using a more "open" acrylamide gel. However stable gels could not be generated.

The electrophoretic experiments confirm that most of the "fucan" and alginic acid are not mutually linked in any of the extracts. In view of the above, some separation should be effected by gel filtration. Sephadex G100 was chosen in expectation of relatively low molecular weight polymers. The elution profiles of Fractions 3A and 8A2 are shown in FIGURE 2.5; the Acid Extract was insufficiently soluble in the buffer solution.

Two peaks are apparent for Fraction 3A; from the results presented above it is reasonable to assume that the high molecular weight material eluting in the void volume is "fucan" and the later peak is the mannuronate-rich alginic acid. By comparison with a selectivity curve derived from dextrans of known weight the alginic acid was calculated to have an average molecular weight of around 4000. Molecular weights, particularly of charged polymers, calculated in this manner should be treated with caution because of the effects of the charge and the molecular shape, but it is clear that the alginic acid molecule is very small compared with most reported alginic acid extracts. A weight of 4000 indicates chains of 20-25 units. The absence of extended sequences of guluronic acid

FIGURE 2.5 ELUTION PROFILE OF ASCOSEIRA FRACTIONS 3A AND 8A2 ON SEPHADEX G100



units (see n.m.r. data, Section 2.4) may explain the solubility of this material in the presence of calcium ions since formation of insoluble calcium salts depends on the presence of these extended sequences.

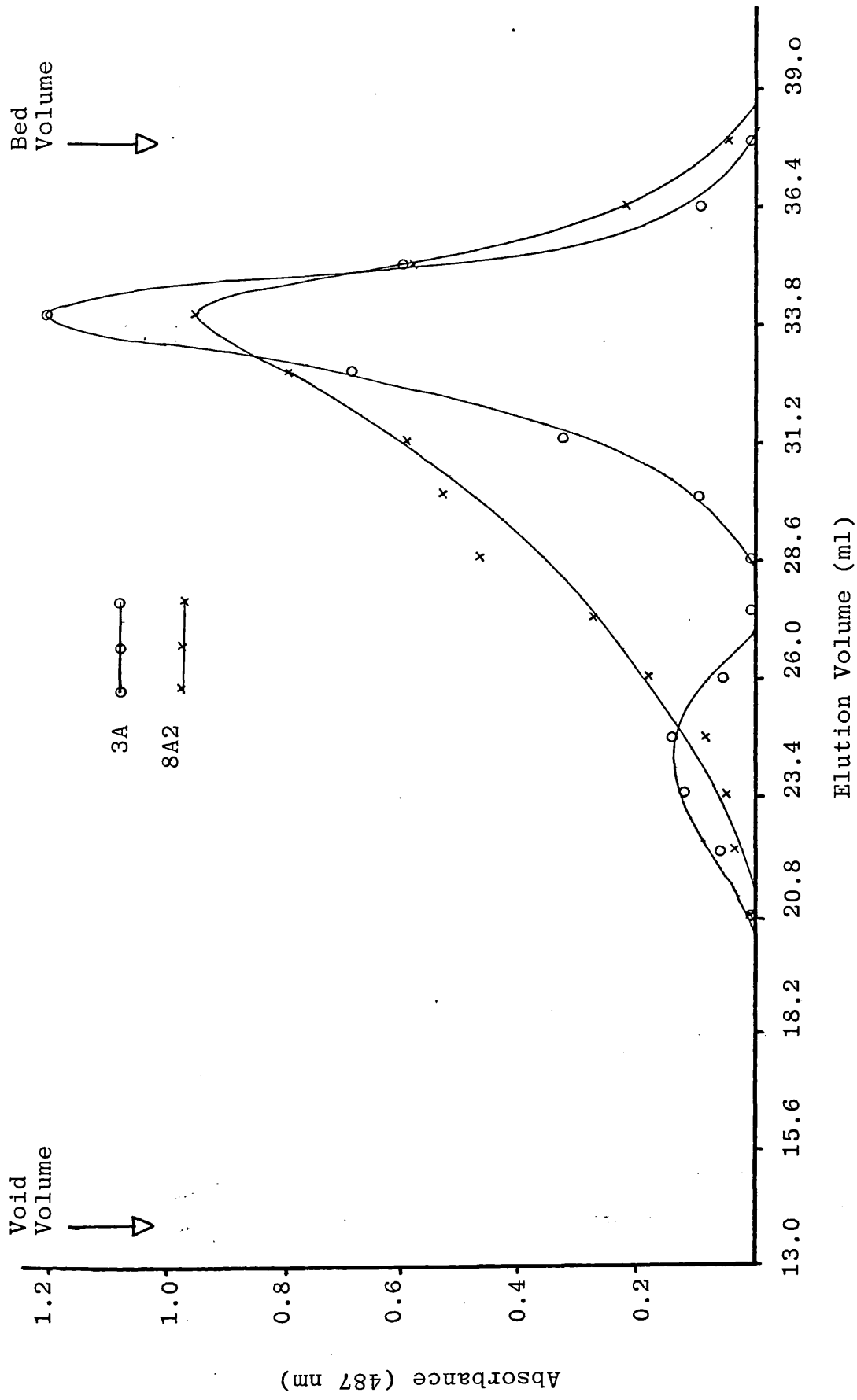
There is no sharp separation of bands for Fraction 8A2, but this is consistent with the expected degradation of the "fucan" giving material that falls within the fractionation range of Sephadex G100.

In an attempt to assess the size of the "fucan", Fractions 3A and 8A2 were fractionated on a column of Sepharose 4B (Fractionation Range 10^4- 5 x 10^6) (FIGURE 2.6). By comparison with the selectivity curve it is apparent that the "fucan" has a molecular weight range extending up to 5 x 10^5 in both cases. The elution pattern for protein in Fraction 8A2 could not be measured directly; the brown pigment interfered with measurement of absorbance at 280 nm., and the buffer interfered with attempts to determine protein by the Lowry Method.

2.2.5. Quantitative Fractionation of Acidic Fractions (Expts. 5.24, 5.33).

The separation achieved on Sephadex G100 offered scope for a quantitative separation of "fucan" and alginic acid. With the column available 600 mg of Fraction 3A were passed through the column in six runs. Each time the two peaks were separated and,

FIGURE 2.6 ELUTION PROFILE OF ASCOSEIRA FRACTIONS 3A AND 8A2 ON SEPHAROSE 4B



finally, after pooling, 200 mg of "fucan" and 390 mg of alginic acid isolated by freeze-drying. Hydrolysis of the alginic acid revealed only traces of neutral monosaccharides. This confirms the earlier assertion that most of this fraction is alginic acid. The composition of the "fucan" will be discussed in the next section.

A similar fractionation of 270 mg of Fraction 8A2 was attempted; with no sharp dividing line the two peaks were arbitrarily separated between Fractions 20 and 21 (see FIGURE 2.5). 83 mg of "fucan" and 181 mg of alginic acid were isolated. However, hydrolysis of the latter showed it to contain substantial amounts of neutral monosaccharide indicating the presence of "fucan" of very low molecular weight. For discussion in the next section this "fucan" and alginic acid will be referred to as "fucans" 8AF and 8AA.

2.2.6. Composition of the Fucans from the Acidic Fractions.

The "fucan" from Fraction 3A (3AF) and "fucans" 8AF and 8AA were analysed for carbohydrate, protein, sulphate and uronic acid, the latter being determined by the carbazole method. Portions of each were hydrolysed with 2M trifluoroacetic acid and the hydrolysates examined by paper chromatography. On the basis of the g.l.c. results the carbohydrate

contents were corrected by comparison with more appropriate standard graphs of mixtures of the monosaccharides. The results are summarised in TABLE 2.10 (Expt. 5.24, 5.33).

TABLE 2.10

COMPOSITION OF "FUCANS" FROM ASCOSEIRA

FRACTION	CARBOHYDRATE	URONIC ACID	SULPHATE	PROTEIN
	%	%(a)	%(b)	%(c)
3AF	62(d)	33	12	0
8AF	68(e)	25	8	15
8AA	62(f)	38	(g)	15

(a) Carbazole analysis.

(b) Expressed as a percentage of the carbohydrate.

(c) Expressed as a percentage of the fraction.

(d) Fucose: Galactose: Glucuronic acid (1:1:1) standard graph.

(e) Fucose: Galactose: Glucuronic acid: Mannose (1:2:1:1) standard graph.

(f) Fucose: Galactose: Mannuronic acid (1:2:3) standard graph.

(g) Not determined.

The monosaccharide compositions are shown in TABLE 2.11. The mannose content was estimated visually. The uronic acid contents were determined on the assumption that there is little guluronic acid present,

and that only mannuronic acid of the uronic acids appears on g.l.c. in significant amount.

TABLE 2.11

SUGAR PROPORTIONS IN ASCOSEIRA "FUCANS"

FUCAN	SUGAR DISTRIBUTION (a)							
	FUC	XYL	MAN	GLU	GAL	GLUA	MANA	GULA
3AF	26	8	7	9	17	23	8	2
8AF	15	8	12	8	27	15	10	2
8AA	10	5	5	7	18	8	28	4

(a) See footnote (b) to TABLE 2.9.

Even with the inherent inaccuracy in quantifying the sugars, the "fucans" all contain a large percentage of galactose, indeed in 8AF and 8AA it is the major sugar. These latter two should perhaps be referred to, more accurately, as fucogalactoglucuromannoxylans. It is possible that they may have been degraded during the extractions, particularly during the acid extraction where fucosyl linkages would be preferentially cleaved. However inspection of the dialysed fragments from the Acid Extract did not reveal enough carbohydrate to suggest any significant change in composition of 8AF and 8AA during extraction.

The approximate monosaccharide composition calculated earlier (Section 2.2.3) for the Acid Extract, with galactose the major sugar in the "fucan"

fraction, suggests a similarity to 8AF and 8AA. With the "fucan" from Fraction 3A representing only a small proportion of the total "fucan" content of the alga it is clear that galactose is the major sugar present. Galactose-rich "fucan" fractions have been reported previously but such a high level of galactose in the total "fucan" has not.

The persistence of glucose in the "fucans" and its confirmation with glucose oxidase, suggest that it is part of the "fucan" molecule despite the fact that it has not previously been confirmed as such. Attempts to separate and characterise mannose as its phenylhydrazone were unsuccessful, because of its low abundance and the difficulty in separating it from glucose and galactose.

The molar ratio of sulphate ester to fucose is about 1.3 for each "fucan" suggesting that each fucose unit carries more than one ester unit, though the possibility of other sugars carrying sulphate is not precluded.

Further structural study of "fucans" was not attempted. Insufficient material was available for partial hydrolysis or detailed periodate oxidation studies and methylation analysis is unsuitable because both the half-ester sulphate groups and the carboxyl functions of the uronic acids hinder complete methylation.[20]

2.2.7. Structure of the Glucans from the Polymeric Extracts. (Expts. 5.23, 5.31).

Two neutral Glucans have been isolated (3N and 8N). A glucan also occurs in the Acid Extract but it has not been isolated.

Past experience has shown that exhaustive extraction with hot water is usually sufficient to remove all the neutral glucan laminaran from brown algae. The other neutral glucan, cellulose, should not be solubilised under the relatively mild alkaline conditions used here.

As described in the Introduction, a range of structures is found for laminaran in different species and within a single plant; an arbitrary division into "soluble" and "insoluble" forms is made on the basis of cold water solubility though there is apparently no sharp structural distinction. Both forms should be contained in Fraction 3N and hence any suggested structure must be an average of the range present. Fraction 8N, if it is laminaran, must differ significantly in its structural form from Fraction 3N.

To establish the structural features the two glucans were studied by periodate oxidation, methylation analysis and n.m.r. spectroscopy.

2.2.7.1. Periodate Oxidation

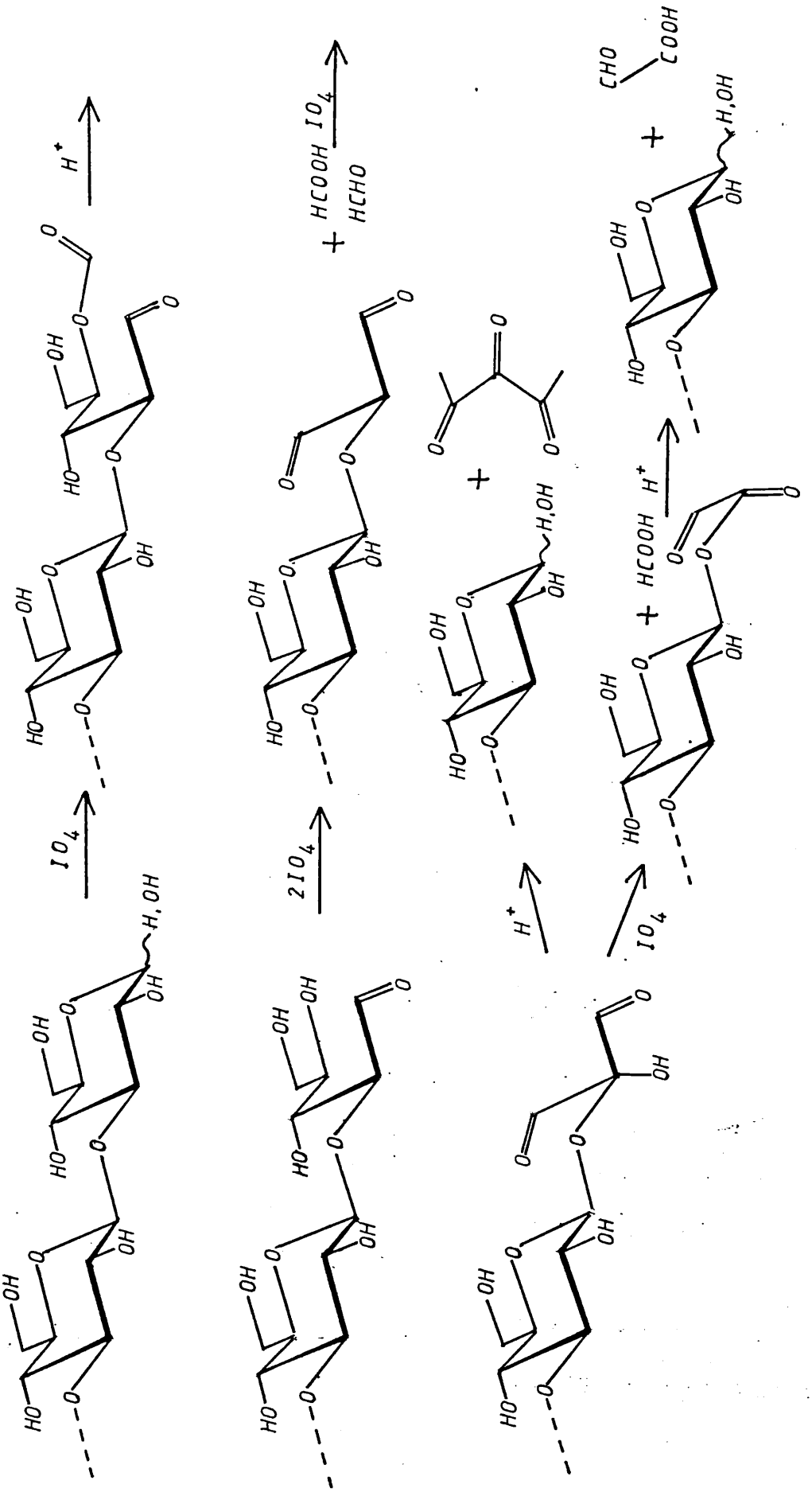
The periodate oxidations were carried out in the dark at 5°C in 0.15M sodium

metaperiodate solution, conditions intended to minimise overoxidation, 1→3-linked units should be unaffected while 1→6-linked units and non-reducing terminal units should consume 2 moles of periodate with the release of 1 mole of formic acid. The reducing end units are most susceptible to over-oxidation; the "normal" oxidation should only consume 1 mole of periodate and give a formyl ester (FIGURE 2.7) but this ester is very labile and easily hydrolysed to a malondialdehyde, with the release of formaldehyde.[23] The malondialdehyde may then be "overoxidised", releasing CO_2 and exposing a further reducing unit. This process is then repeated, eroding the chain away from the reducing end. This effect may be avoided by reducing the end-units prior to oxidation but the conditions used here are generally accepted as sufficient.

The C-1 linked mannitol terminal units found in laminaran should consume 4 moles of periodate and release 3 moles of formic acid and 1 mole of formaldehyde.

Periodate consumption had ceased after 96 h for both 3N and 8N. The reactions were terminated and the oxidised polymers reduced, dialysed and isolated as polyalcohols, which were assayed for carbohydrate to ascertain the percentage of uncleaved glucose units. The dialysis waters were

FIGURE 2.7 "OVEROXIDATION" OF A β-(1 → 3)-LINKED GLUCAN



checked for carbohydrate but none was detected in either case, ruling out the possibility of any hydrolytic breakdown of the chains to oligomeric species.

The periodate consumption, formic acid release, percentage of uncleaved glucose units and the recovery of the polyalcohols are listed in TABLE 2.12.

TABLE 2.12. PERIODATE OXIDATION OF GLUCANS FROM ASCOSEIRA

GLUCAN	PERIODATE CONSUMPTION[a]	FORMIC ACID RELEASE[a]	UNCLEAVED GLUCOSE[b]	POLYALCOHOL RECOVERY[c]
3N	0.96	0.40	60	70
8N	1.80	0.77	39	68

(a) Moles per anhydro unit.

(b) Percentage of the original glucose.

(c) Percentage of the glucan weight.

The results in both cases are consistent with a substantial proportion of 1→6-linked units; the amount of mannitol present in each is too small to account for the high periodate and formic acid figures.

With two possible modes of generation of formaldehyde, from mannitol oxidation and overoxidation of reducing terminal units, knowledge of the formaldehyde generated is of doubtful value.

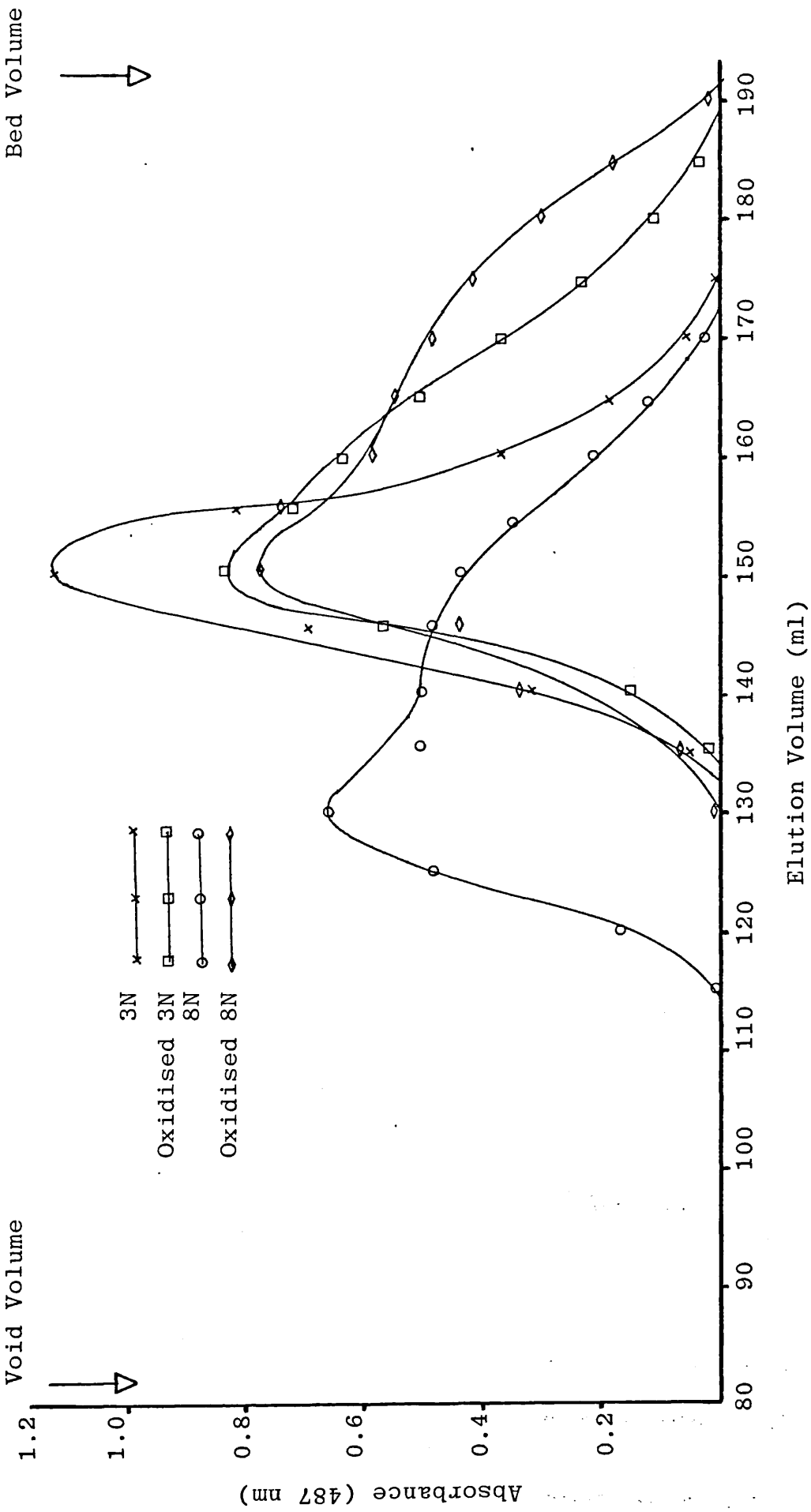
Prior separation of mannitol and glucose terminated chains (M- and G-chains respectively) would allow calculation of the degree of polymerisation from the formaldehyde generation of the former, and of the latter if the end units were first reduced. However the separation was not attempted.

The periodate oxidation results give no information on the relative positions of 1→3- and 1→6-linked units and the number of branch points, if any. Mild acid treatment, cleaving the acetal links in the polyalcohols derived from 1→6-linked units and leaving surviving glycosidic links intact, should lead to oligomeric species indicative of the molecular structure.[24] This procedure (known as Smith Degradation) was applied to both polyalcohols, the conditions being those used previously for degradation of laminaran (0.05M sulphuric acid for 20 h at 20°C.)[25] The reaction solutions were neutralised and dialysed. In both cases about 10% of the carbohydrate content passed through the dialysis membrane, the polymeric contents of the membrane being recovered in about 75% yield. Paper chromatographic examination of the dialysed fragments showed only glucose and smaller fragments rather than glycosides and higher oligomers. These results indicate that some cleavage of glycosidic links has occurred but the bulk of the polyalcohol in both

cases remains undialysable, indicating the absence of 1→6-linked units (other than at branch points) from the middle of the molecular chains.

The gel filtration profiles (FIGURE 2.8) give a reasonable estimate of the sizes of the glucans and the derived polyalcohols; the glucans are uncharged and with a high proportion of (1→6)-linked units should behave in a similar manner to the dextrans used in column calibration. Hence the calculated molecular weights should be close to the actual values. For the original glucans the calibration curves yield molecular weights of 8000 and 11000respectively for 3N and 8N. These correspond to degrees of polymerisation of 50 and 70 respectively. The profiles for the derived polyalcohols are anomalous; for 3N there has been a slight diminution in size consistent with cleavage of peripheral residues, whereas for 8N the apparent decrease in size is greater and there appears to have been partial resolution into two species. A possible explanation of the latter feature may be that the profile of glucan 8N represents an aggregated species, the aggregation breaking down on oxidation or subsequent hydrolysis.

FIGURE 2.8 ELUTION PROFILES OF ASCOSEIRA GLUCANS AND THEIR PERIODATE-OXIDISED POLYALCOHOLS ON SEPHADEX G100



2.2.7.2. Methylation Analysis.

Samples of 3N and 8N were methylated twice by Björndahl and Lindberg's modification of the Hakomori Method. The methylated polymers were hydrolysed with 90% formic acid and the hydrolysates examined by g.l.c. and g.l.c/ms.

Both glucans yielded four O-methylated alditol acetates, which were characterised by retention time and mass spectrum, in significant amounts. The molar ratios are given in TABLE 2.13. Traces of 4,6-di-O-methyl-1,2,3,5-tetraacetylglucitol and fully acetylated glucitol were also identified; these are probably derived from incomplete methylation or partial demethylation during subsequent manipulation.

TABLE 2.13.

METHYLATED SUGARS FROM METHYLATED ASCOSEIRA GLUCANS.

GLUCAN	RETENTION TIME (a)	METHYLATED SUGAR (b)(c)	LINKAGE INDICATED	MOLAR RATIO (d)
3N	0.97	2,3,4,6-Tetra-O-	Glcp-(1→	1.7
	1.82	2,4,6-Tri-O-	→3)-Glcp-(1→	12.3
	2.32	2,3,4-Tri-O	→6)-Glcp-(1→	9.0
	4.18	2,4-Di-O-	→3),→6)-Glcp-(1→	1.0
8N	0.97	2,3,4,6-Tetra-O-	Glcp-(1→	1.3
	1.82	2,4,6-Tri-O-	→3)-Glcp-(1→	7.3
	2.32	2,3,4-Tri-O-	→6)-Glcp-(1→	8.8
	4.24	2,4-Di-O-	→3,→6)-Glcp-(1→	1.0

(a) OV225 column, expressed relative to 2,3,4,6-tetra-O-Me-1,5-di-O-acetylglucitol as 1.00

(b) 2,3,4,6-Tetra-O- = 2,3,4,6-tetra-O-methyl-1,5,-di-O-acetylglucitol, etc.

(c) Confirmed by g.l.c./ms.

(d) Determined from peak areas and corrected for effective carbon response. [26]

TABLE 2.14

^{13}C N.M.R. CHEMICAL SHIFTS OF ASCOSEIRA GLUCANS.(a)

CARBON ATOM	GLUCAN 3N		GLUCAN 8N
	β-D-(1→3)	β-D-(1→6)	β-D-(1→6)
C-1	103.1	103.5	103.8
C-2	73.8	73.8	74.1
C-3	85.3	76.4	76.8
C-4	68.9	70.5	70.9
C-5	76.3	75.6	75.9
C-6	61.5	69.6	69.9

(a) In p.p.m. downfield from external TMS at room temperature.

These results are consistent with those from the periodate oxidation and further indicate approximately one branch point per molecule and a degree of polymerisation of about 25 for 3N and 27 for 8N.

2.2.7.3. N.m.r. Spectra

^{1}H and ^{13}C n.m.r. spectra were recorded for 3N and 8N (FIGURES 2.9 - 2.12). The ^{13}C spectra were assigned from published spectra[27-30] for β-(1→3) and β-(1→6) linked glucans (TABLE 2.14). The minor signals in the ^{13}C spectrum of 3N are probably due to terminal and branch point residues and are consistent with the degree of polymerisation and branching indicated above, though they do not

FIGURE 2.9 ^{13}C N.M.R. SPECTRUM OF ASCOSEIRA GLUCAN 3N

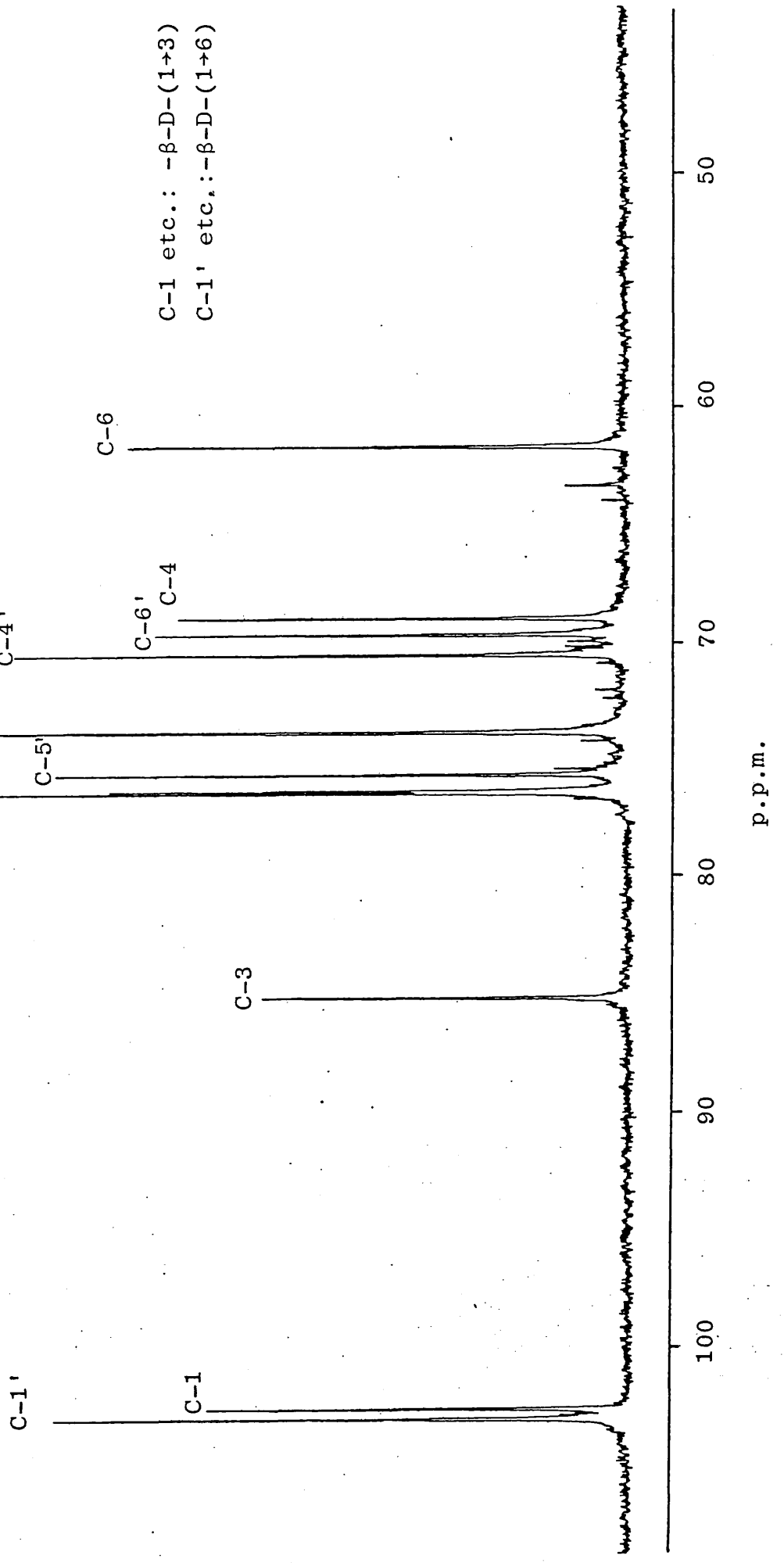


FIGURE 2.10 ^{1}H N.M.R. SPECTRUM OF ASCOSEIRA GLUCAN 3N

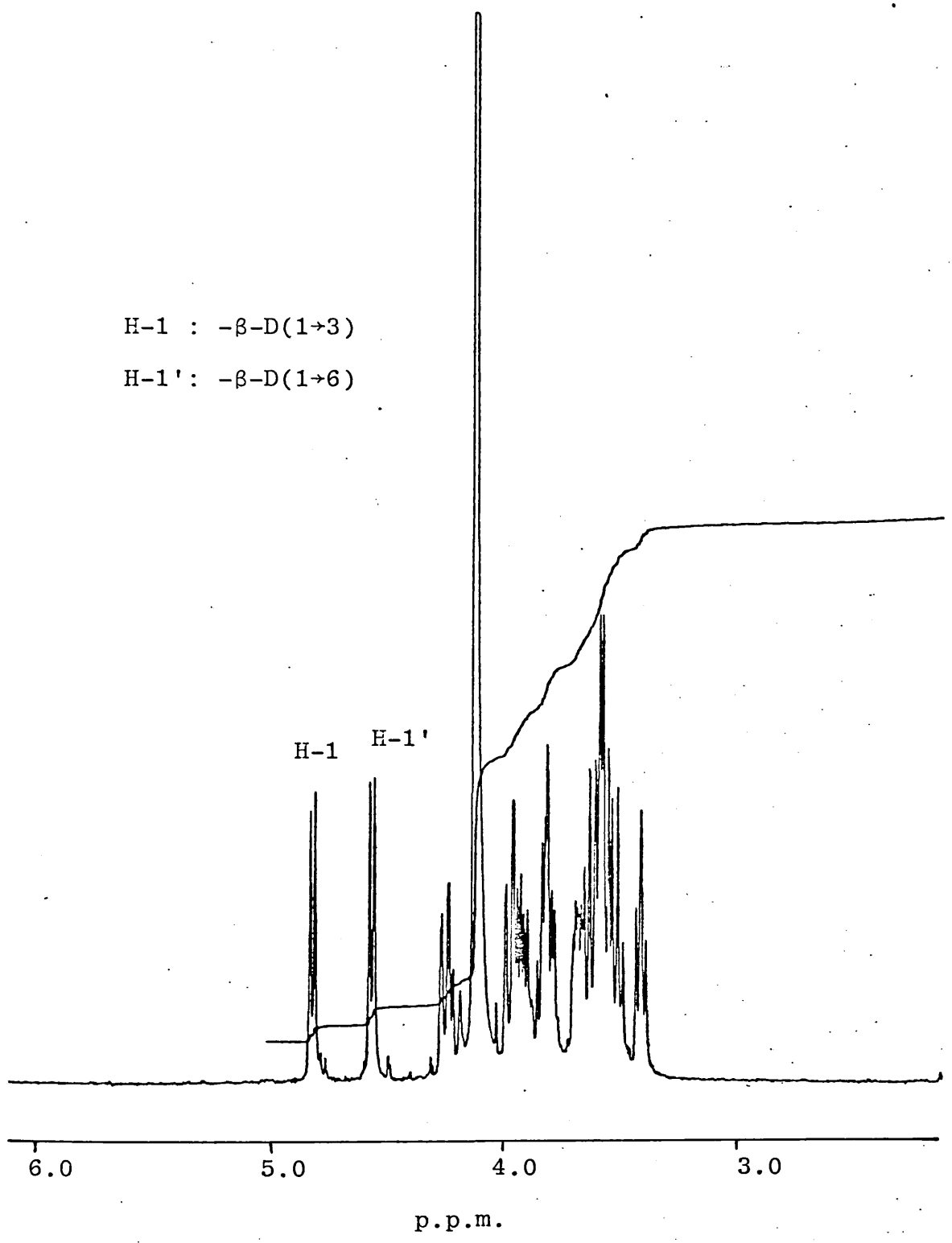


FIGURE 2.11 ^{13}C N.M.R SPECTRUM OF ASCOSEIRA GLUCAN 8N

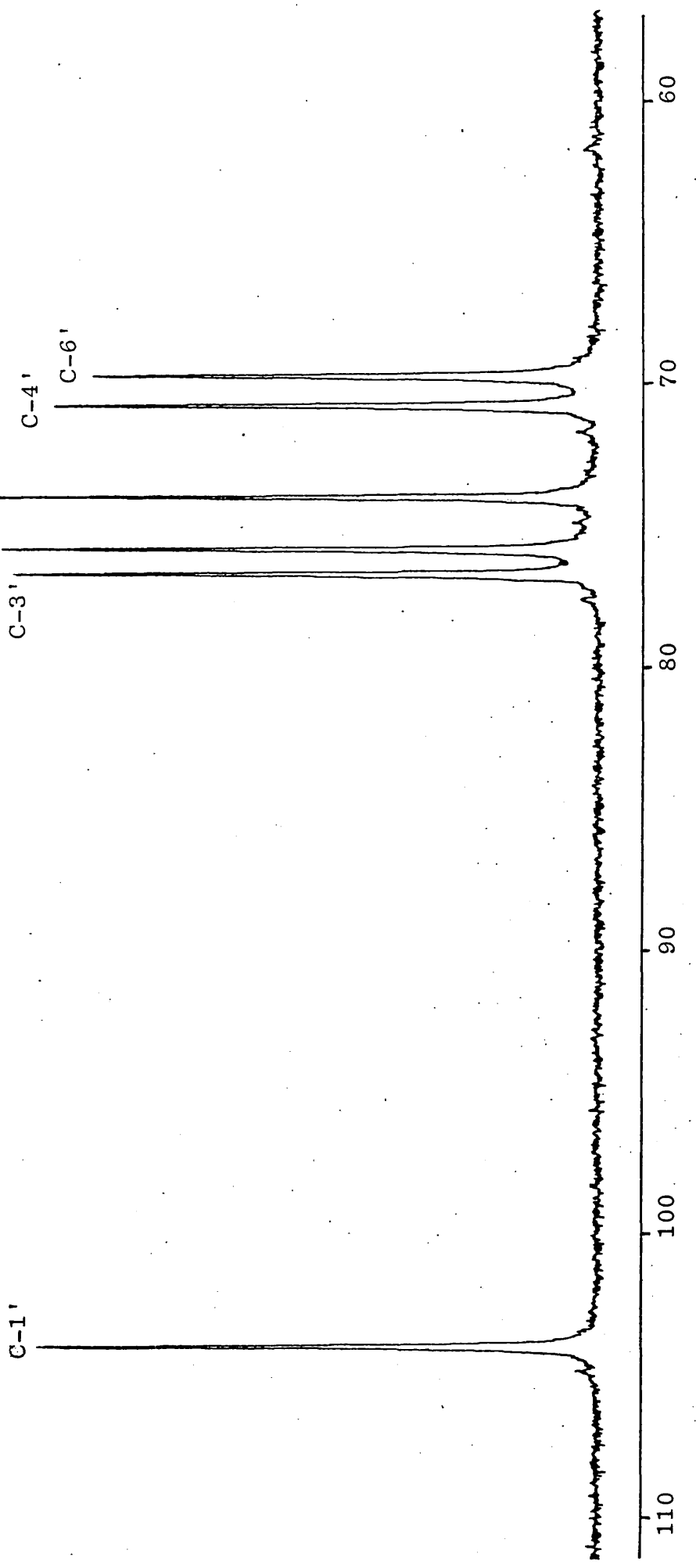
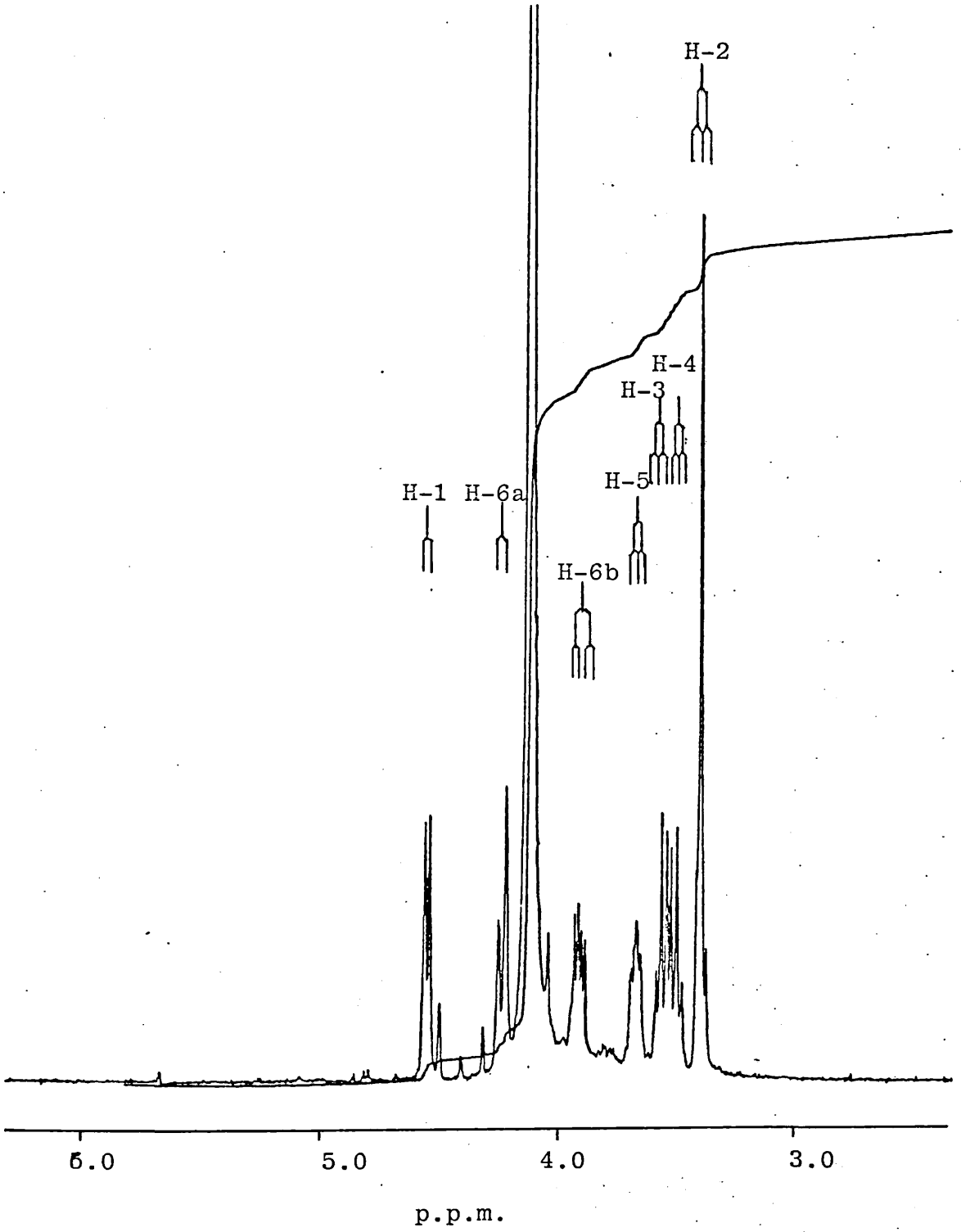


FIGURE 2.12 ^{1}H N.M.R. SPECTRUM OF ASCOSEIRA GLUCAN 8N



confirm the latter since an unequivocal assignment of any minor peaks to branch point carbons is not possible.

The occurrence of only one sharp peak for each carbon atom suggests that the two types of unit occur in separate homopolymeric sequences in 3N. The ratio of (1→3)- and (1→6)- linked units appears to be about 1.0 from comparison both of signals in the ^{13}C spectrum and of the anomeric signals in the ^{1}H spectrum. This is a much lower percentage of (1→3)- linked units than indicated by the methylation results.

Glucose reducing end units should give two sets of signals; in the ^{1}H spectrum the anomeric proton in the α-anomer should give a signal in the downfield region of δ5.0-5.5 ppm. The absence of any observable signal points to a low proportion of G-chains, supporting the calculated mannitol content of about 3%; the degree of polymerisation allows a maximum mannitol content of about 4%.

The spectra of 8N (FIGURES 2.11, 2.12) are somewhat different with only the signals for (1→6)-linked intra-chain units apparent. In the ^{13}C spectrum the very minor signals at 61.9 and 104.7 ppm.are close to the C-6 and C-1 signals for methyl-β-D-glucopyranoside (61.9 and 104.3 ppm. respectively); these may be derived from non-reducing end terminal

units. In the ^{1}H spectrum the doublet at 4.80 (J = 8 Hz) is coincident with the anomeric signal for (1→3)-linked units in 3N; the corresponding signal in the ^{13}C spectrum may be masked by the broad signals,or more probably is too broad to be observed. The remainder of the spectrum is consistent with that published by Bassieux et al.[31] for a β-(1→6)-linked glucan.

The complete loss of the signals from (1→3)-linked units may be accounted for by extreme line broadening consequent upon immobilisation of (1→3)-linked chains by gel formation or microaggregation. The weakness in this hypothesis is that this effect has only been observed in high molecular weight glucans.[30] In addition it does not explain why the same total suppression of signals does not occur for glucan 3N, where the percentage of (1→3)-linked units is higher and hence the tendency to gel might be expected to be higher. However the possible gelation of 8N is consistent with the gel filtration results, which are clearly in conflict with the molecular weight calculated from the methylation analysis.

2.2.8. Examination of Alginic Acid (Expt. 5.28)

Several alginic acid fractions have been obtained from the various extracts; Extracts 3,4 and 8 all contain mannuronate-rich material, in

addition to Extract 7, which was originally anticipated to be the only substantial alginic acid fraction. Past experience has shown that this latter fraction is often considerably degraded during the sequential extraction.[17,32] A crude estimate of the extent of any degradation may be gained by measuring the viscosity of the soluble sodium salt. After conversion of Extract 7 to its sodium salt the viscosity relative to water at 25°C of a 1% solution was found to 1.33, implying considerable degradation giving a low molecular weight polymer.

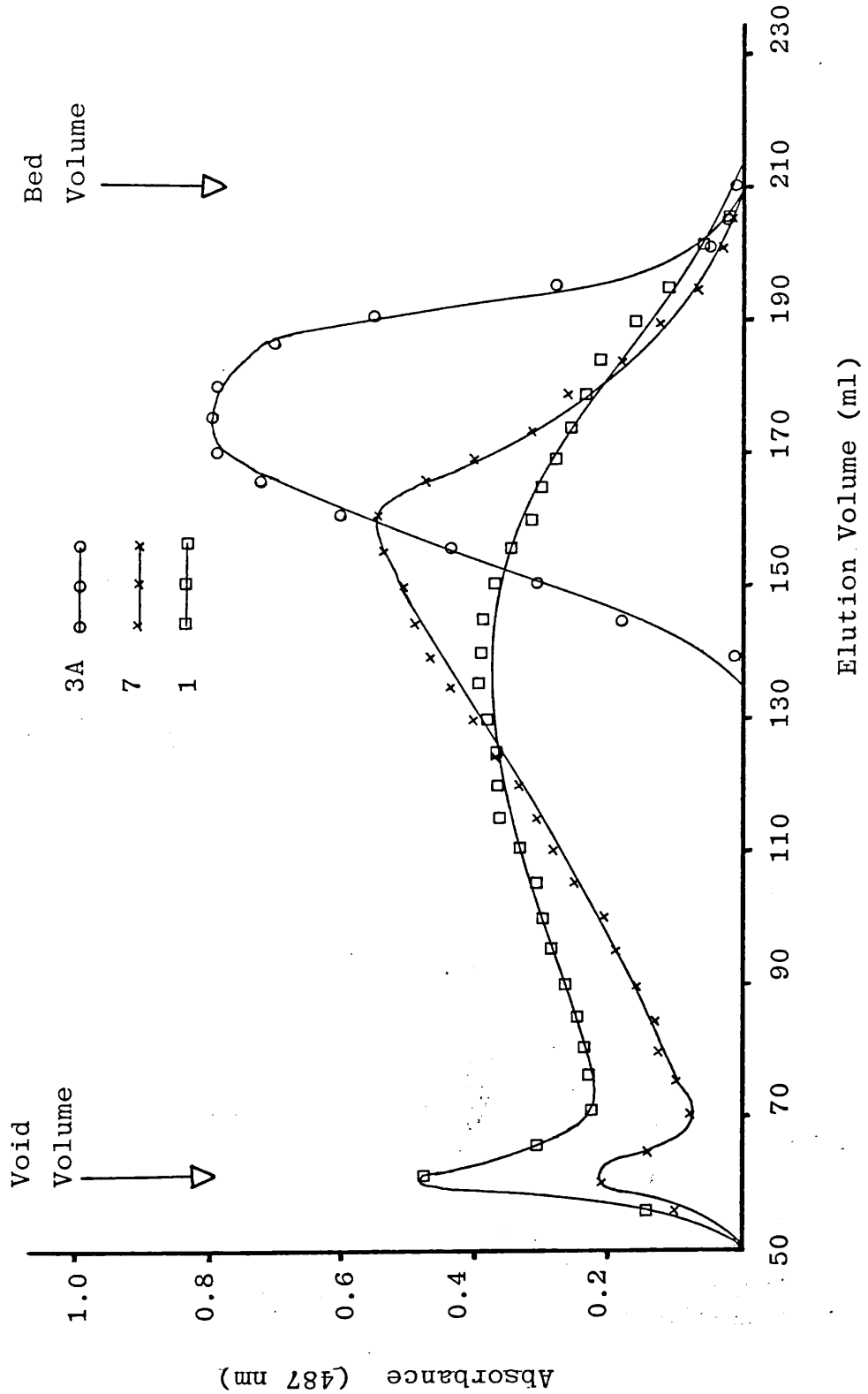
The low molecular weight was confirmed by gel filtration on Sephadex G100 (FIGURE 2.13) with Fraction 3A shown for comparison. The alginate is extremely polydisperse with an average molecular weight, calculated from the selectivity curve, of about 8000, representing a degree of polymerisation of only ca.40. Undegraded alginates are generally accepted as having D.P's of several hundreds.[33]

2.3. Direct Alkali Extraction of Ascoseira (Expt. 5.34).

In order to obtain a relatively undegraded alginic acid more representative of the native polymer a sample of dried ground alga was extracted directly with dilute alkali as outlined in FLOW CHART 2.3.

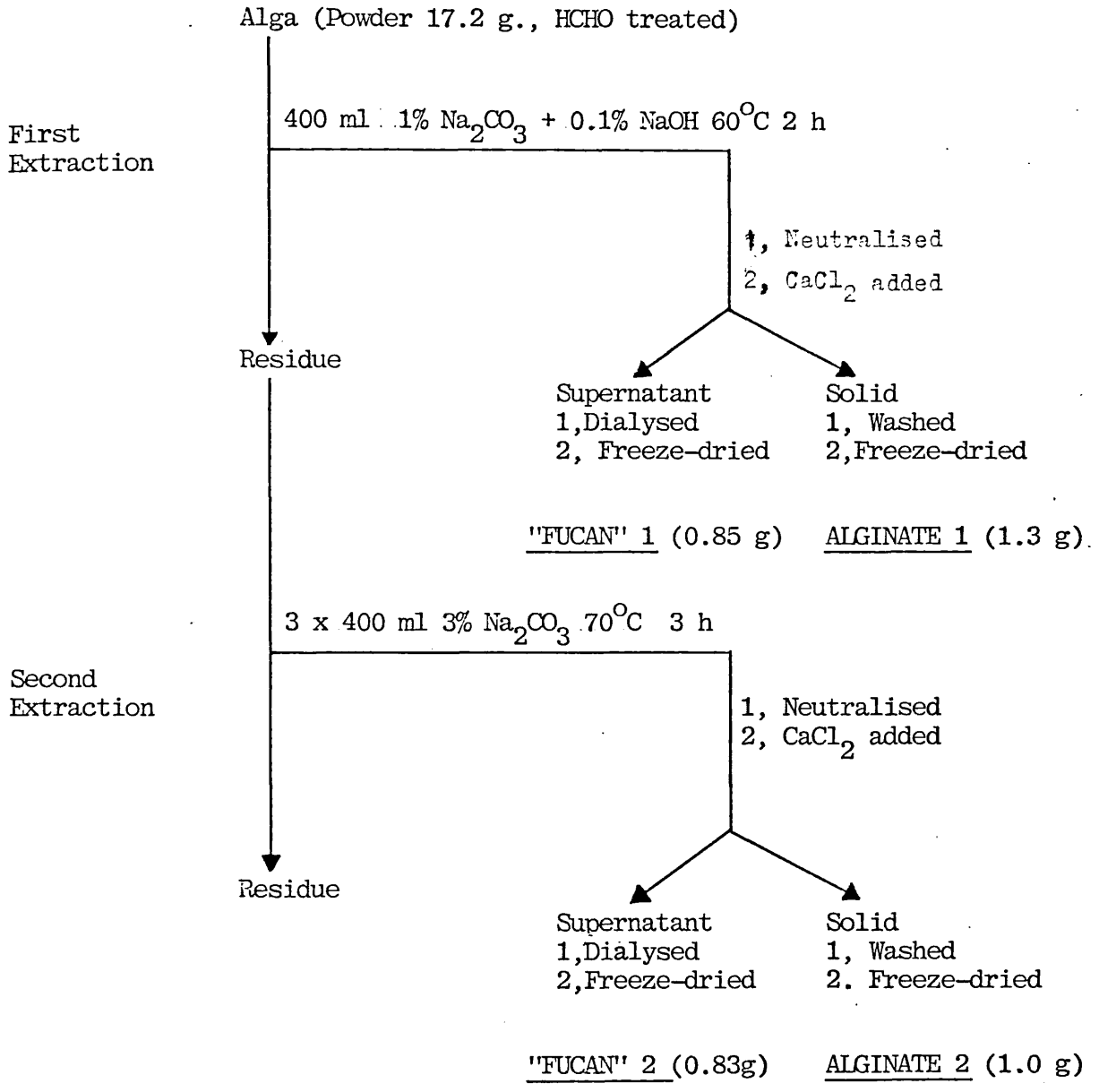
The total recovery of calcium alginate (2.30 g, 13.4% of the dry weight) is higher than for the

FIGURE 2.13 ELUTION PROFILES OF SODIUM ALGINATES FROM ASCOSEIRA ON SEPHADEX G100



FLOW CHART 2.3

DIRECT ALKALINE EXTRACTION OF ASCOSEIRA



sequential extraction, reflecting the absence of the degradative acid extraction and the milder alkaline conditions used. Similarly a higher percentage of "fucan" has been recovered, though hydrolysis and paper chromatography and g.l.c. showed that a considerable amount of mannuronic acid was present, presumably derived from polymannuronate not precipitated by the calcium ions.

Alginate 1 was converted to its sodium salt and the viscosity measured in the same manner as for the sequentially extracted alginate (Expt. 5.35). The relative viscosity was only 2.0, only marginally greater than for the above and much lower than would be expected. The inference of a low degree of polymerisation is supported by the gel filtration pattern (FIGURE 2.13) which indicates a D.P. of about 100.

The limitations of molecular weights determined from dextran selectivity curves has been referred to earlier. Two qualifications are pertinent here; the alginate has a known tendency to aggregate, and the gel filtration support material is known to carry a small residual negative change which would tend to repel the alginate, forcing it through the gel more rapidly. Both these effects would tend to lead to artificially high molecular weights. Hence the calculated weights may be too high; thus

strengthening the idea of low molecular weight alginate.

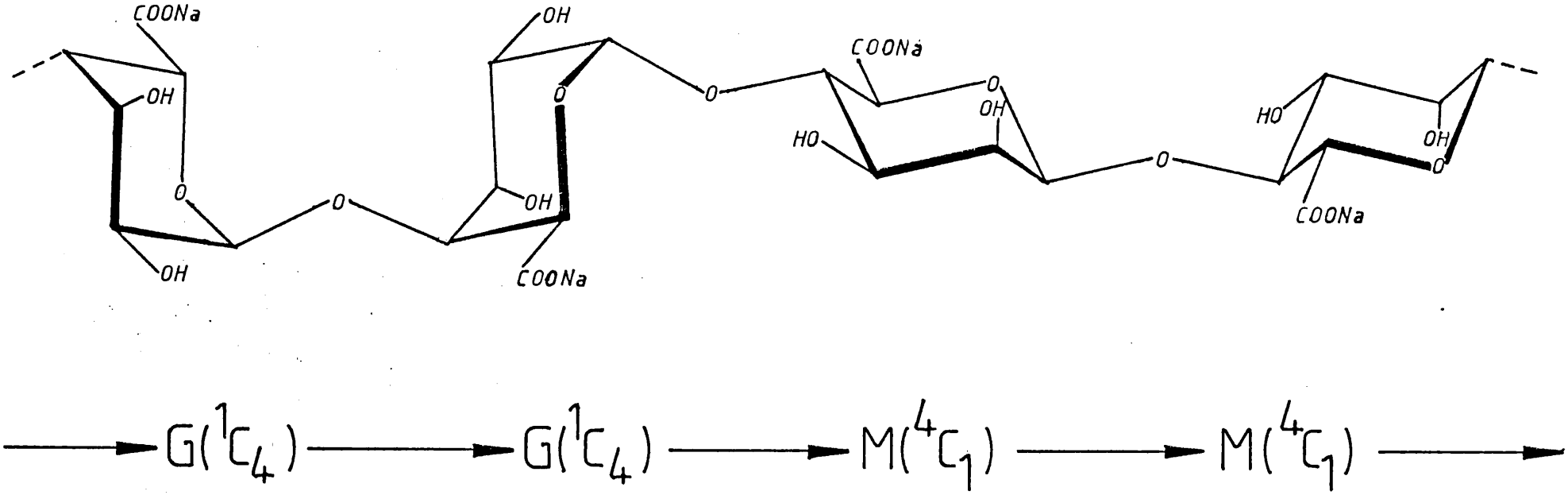
It appears that the alginate is of low molecular weight immediately prior to extraction but it may be that some degradation had taken place during storage or during the grinding and drying. The latter two possibilities may be ruled out on the basis that other workers, using the same conditions, have successfully obtained alginic acid fractions of high viscosity.[17,20,32]

The alga was stored at -20°C from shortly after harvesting (at a temperature below 10°C) until it was ground up, leaving little scope for degradative processes. However, any enzymic degradation would cleave the chain generating 4,5-unsaturated acids at the non-reducing end.[34-40] These may easily be detected with thiobarbituric acid. Tests on both the sequentially extracted alginate and Alginate A failed to reveal any 4,5-unsaturated acid. Further evidence of negligible degradation during storage was provided by the isolation of an alginate of very high viscosity from a sample of the alga Macrocystis pyrifera which had been stored under the same conditions. (see Section 4.2).

2.4. N.m.r. of Alginates (Expts. 5.24, 5.28, 5.35).

Alginic acid is a linear co-polymer of (1→4)-linked β-D-mannuronic acid and α-L-guluronic

FIGURE 2.14 CONFORMATION OF SODIUM ALGINATE



acid residues, arranged in homopolymeric blocks of mannuronic acid (M blocks) and of guluronic acid (G blocks) and blocks of predominantly, but not exclusively, alternating sequence (MG blocks). This structure was first put forward on the basis of partial hydrolysis studies but has subsequently been confirmed by ^{1}H and ^{13}C n.m.r.

Penman and Sanderson[41] split alginates into alternating and homopolymeric fractions by partial acid hydrolysis and then determined the relative proportions of M and G blocks in the latter fraction by ^{1}H n.m.r. They also confirmed that the D-mannuronic acid residues are in the C1 conformation and the L-guluronic acid residues in the IC conformation (FIGURE 2.14).

By recording spectra at low pH, Grasdalen and co-workers[42] established a sequence-dependent deshielding of H-5 of the guluronate residues and hence were able to determine the mole fractions of the four possible doublets of nearest neighbours along the chain. Their alginate samples had to be moderately depolymerised to reduce viscosity but they considered them to be representative of intact alginates.

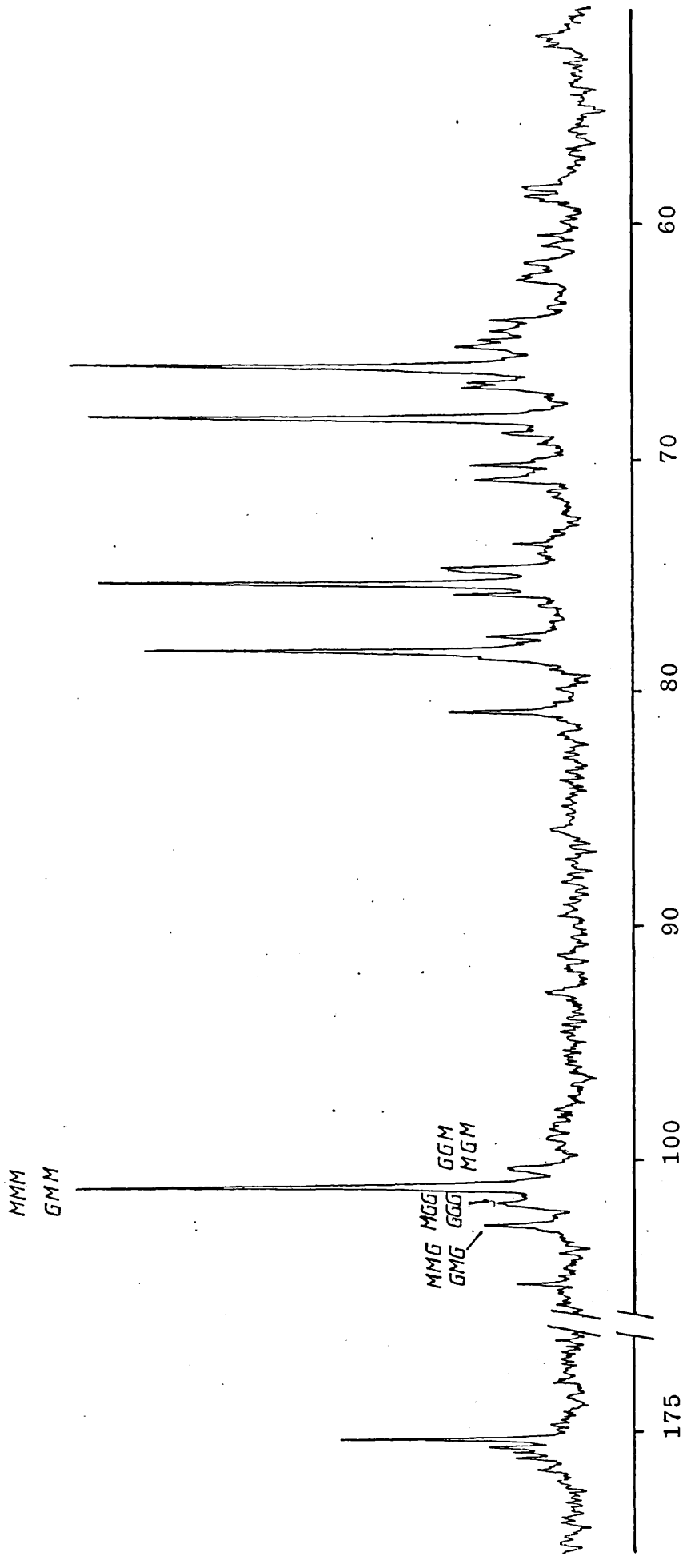
The same authors found that ^{13}C n.m.r. gave a more complete picture of the sequence.[43,44] The greater sensitivity to structural differences caused

resolution of some of the individual carbon resonances into four lines, in evident dependence upon the identities of the units immediately preceding and following them in the chain. Each line corresponded to the resonance of the carbon atom in the central unit of one of the four possible triplets ("triads", e.g. MMM, MMG, GMM, GMG or GGG, GGM, MGG, MGM), Hence it was possible to calculate the relative proportions of the eight possible triads and estimate the lengths of the three types of block. Again moderate depolymerisation to D.P.~ 20-30 was necessary to give good spectral resolution.

For the Ascoseira alginates it has been possible to obtain good spectral resolution without prior depolymerisation by operating at a higher frequency (400 MHz for ^{1}H and 100 MHz for ^{13}C spectra). This is doubtless assisted by the initial low molecular weights, but nevertheless the problems introduced by different rates of hydrolysis of the various linkages are avoided.

The alginate isolated from the aqueous extract (Fraction 3A) contains some "fucan" but in the ^{13}C spectrum (FIGURE 2.15) the signals from the alginate residues clearly dominate. The assignment of peaks was made by reference to the spectra of Grasdalen et al.[44] (TABLE 2.15). The presence of interfering "fucan" signals casts doubt on the assignment of

FIGURE 2.15 ^{13}C N.M.R. SPECTRUM OF ASCOSEIRA FRACTION 3A



some of the smaller signals, but some estimate of the block structure should be possible using the relative peak sizes. The above authors established that for ring carbons in alginate the relative peaks areas represent relative occurrence for the possible triads.

TABLE 2.15

ASSIGNMENTS OF PEAKS IN THE ^{13}C N.M.R. SPECTRUM OF ASCOSEIRA FRACTION 3A[a]

TRIAD SEQUENCE	Carbon atom in central residue of triad					
	C-1	C-2	C-3	C-4	C-5	C-6
MMM	100.8	70.7	72.1	78.8	76.9	175.3
MMG	101.9	(71.3)[b]	(c)	78.8	(76.4)	(c)
GMM	100.8	(c)	(c)	78.4	(77.1)	(c)
GMG	101.9	71.3	72.5	78.4	76.9	175.8
GGG	101.2	65.9	69.9	80.5	68.0	175.5
GGM	100.4	65.6	(c)	80.5	68.0	(c)
MGG	101.2	65.9	(c)	80.5	68.0	(c)
MGM	100.4	65.6	70.1	80.5	68.0	175.8

(a) Shifts in ppm., downfield from external TMS at room temperature.

(b) Values in parenthesis denote tentative assignments.

(c) No assignment made.

TABLE 2.16

DIAD FREQUENCIES AND AVERAGE BLOCK LENGTHS FOR ASCOSEIRA FRACTION 3A AND EXTRACT 7.

SAMPLE	F_M	F_G	Diad F_{MM}	F_{MG}	F_{GM}	F_{GG}	Block Length (a) N_M	N_G
3A	0.75	0.25	0.59	0.13	0.13	0.15	5.8	1.2
7	0.25	0.75	0.21	0.04	0.04	0.71	5.3	18

(a) See text.

In the anomeric region (95-105 ppm.) the eight possible triad resonances described by Grasdalen et al. are incompletely resolved and hence calculation of triad frequencies is not possible. However the resolution is sufficient to allow calculation of diad frequencies from the four major signals. The signal at 101.9 ppm. (from triads MMG and GMG) is the MG diad, the signal at 101.2 ppm. (from triads GGG and MGG) is the GG diad, and so forth for the MM and GM diads. The relative intensities of the four signals give the proportions of the four diads, F_{MM}, F_{MG}, F_{GG} and F_{GM} (TABLE 2.16); as would be expected F_{MG} and F_{GM} are the same.

Grasdalen et al. were also able to calculate triad frequencies from the signals from C-4 and C-5 of mannuronate residues but here the resolution is not adequate. However the mole fractions of M

and G can be calculated by averaging peaks areas for all the ring carbons. From the diad frequencies a number-average block length, $\overline{N}$, can be calculated for both of the monomers from the expressions:

$$\overline{N}_M = \frac{F_M}{F_{MG}} = \frac{F_M}{F_{GM}} \qquad \overline{N}_G = \frac{F_G}{F_{GM}} = \frac{F_G}{F_{MG}}$$

where F's are the frequencies of occurrence.

Diad frequencies and block lengths can also be calculated from the ^1H spectrum (FIGURE 2.16). The spectrum is complicated by signals from the interfering "fucan" but the major signals in the low field region may be assigned to alginate.

Signals A and B are due to H-1 of guluronate and H-1 of mannuronate residues respectively.[42] Signal C is assigned to H-5 in a guluronate residue with a neighbouring guluronate residue linked through C-1. The signal due to H-5 in a guluronate with a neighbouring mannuronate residue coincides with peak B at neutral pD. The mole fraction of guluronate F_G and diad frequency F_{GG} are related to the peak areas of the respective signals by the relationship:

$$F_G = \frac{A}{B + C} \qquad F_{GG} = \frac{C}{B + C}$$

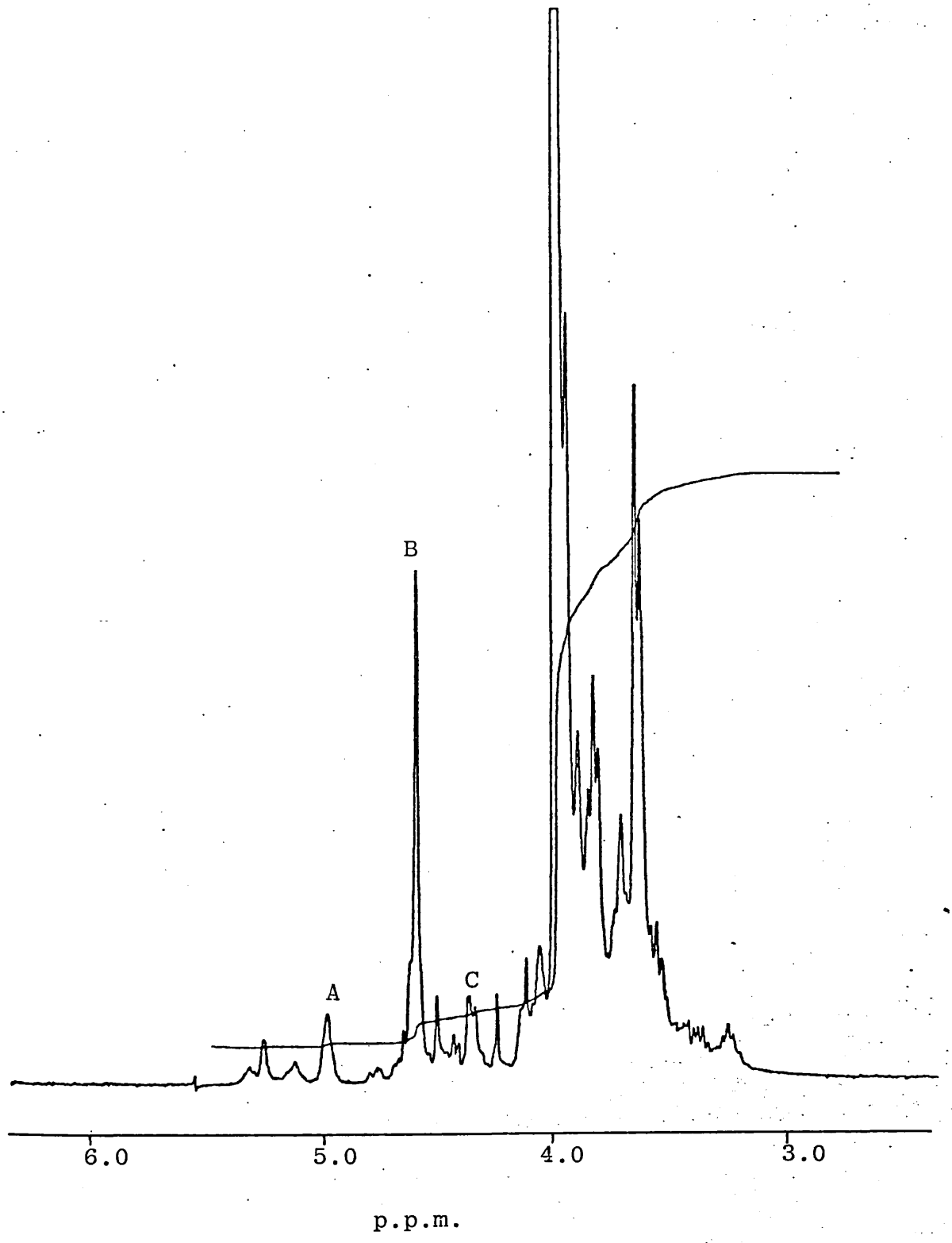
The remaining diad frequencies follow from:

$$F_G + F_M = 1$$

and $F_{GG} + F_{GM} = F_G$, $F_{MM} + F_{MG} = F_M$

For long chains F_{MG} may be taken as equal to F_{GM}.

FIGURE 2.16 ^{1}H N.M.R. SPECTRUM OF ASCOSEIRA FRACTION 3A



Calculation by this method for Fraction 3A gave diad frequencies of:

F_{MM} = 0.76, $F_{MG}(=F_{GM})$= 0.03, F_{GG}= 0.18 and block lengths $\overline{N}_M$ = 26 and $\overline{N}_G$ = 7.

Though lending support to the predominance of polymannuronate sequence these data are clearly significantly different from those in TABLE 2.16. A possible explanation for the lower F_{MG} may be the inherent error in taking the difference between two similar numbers which are themselves subject to error.

The block lengths shown in TABLE 2.16 are averages for the whole molecule; the homopolymeric sequences must be somewhat longer. It may be that the polymannuronate blocks approach the chain length calculated by gel filtration to be 20-25 units and may even occur as a pure homopolymer.

As indicated earlier the bulk of the alginate (Extract 7) would be expected to be extensively degraded during the Acidic Extraction step. The polyguluronate blocks are the most resistant to acid hydrolysis and hence should survive in preference to the other two block types. The ^{13}C spectrum (FIGURE 2.17, TABLE 2.17) and the calculated diad frequencies (TABLE 2.16) confirm that this is the case. The ^{1}H spectrum (FIGURE 2.18) confirms the predominance of polyguluronate sequences, though

FIGURE 2.17 ^{13}C N.M.R. SPECTRUM OF ASCOSEIRA EXTRACT 7

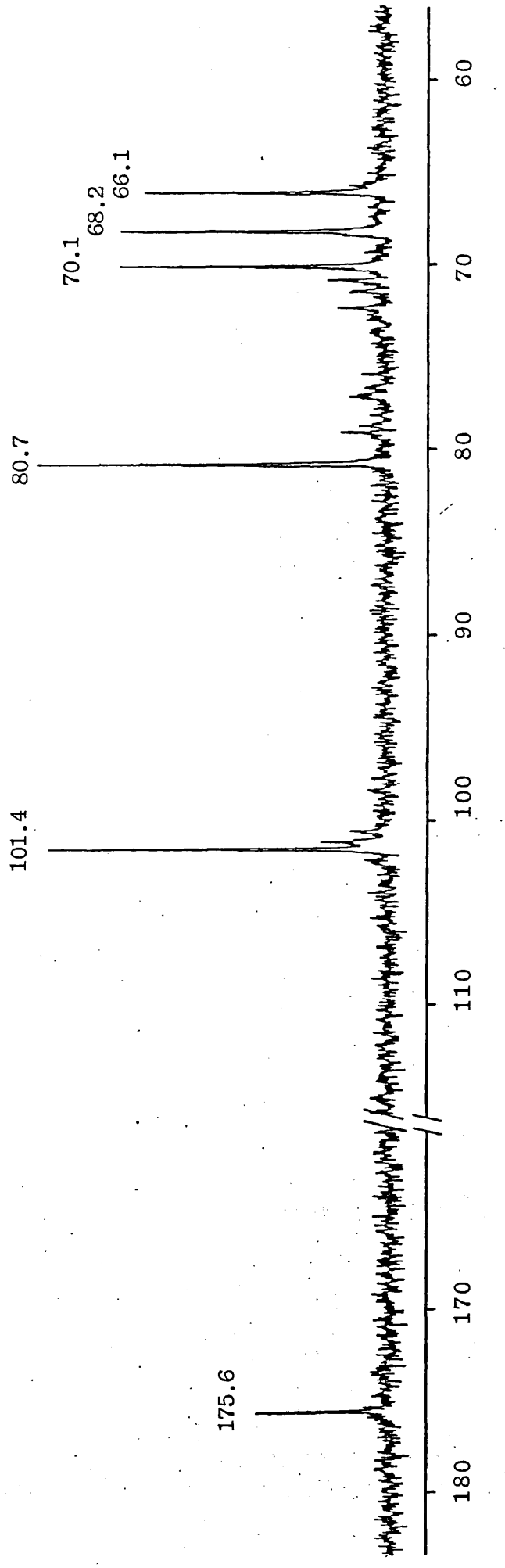
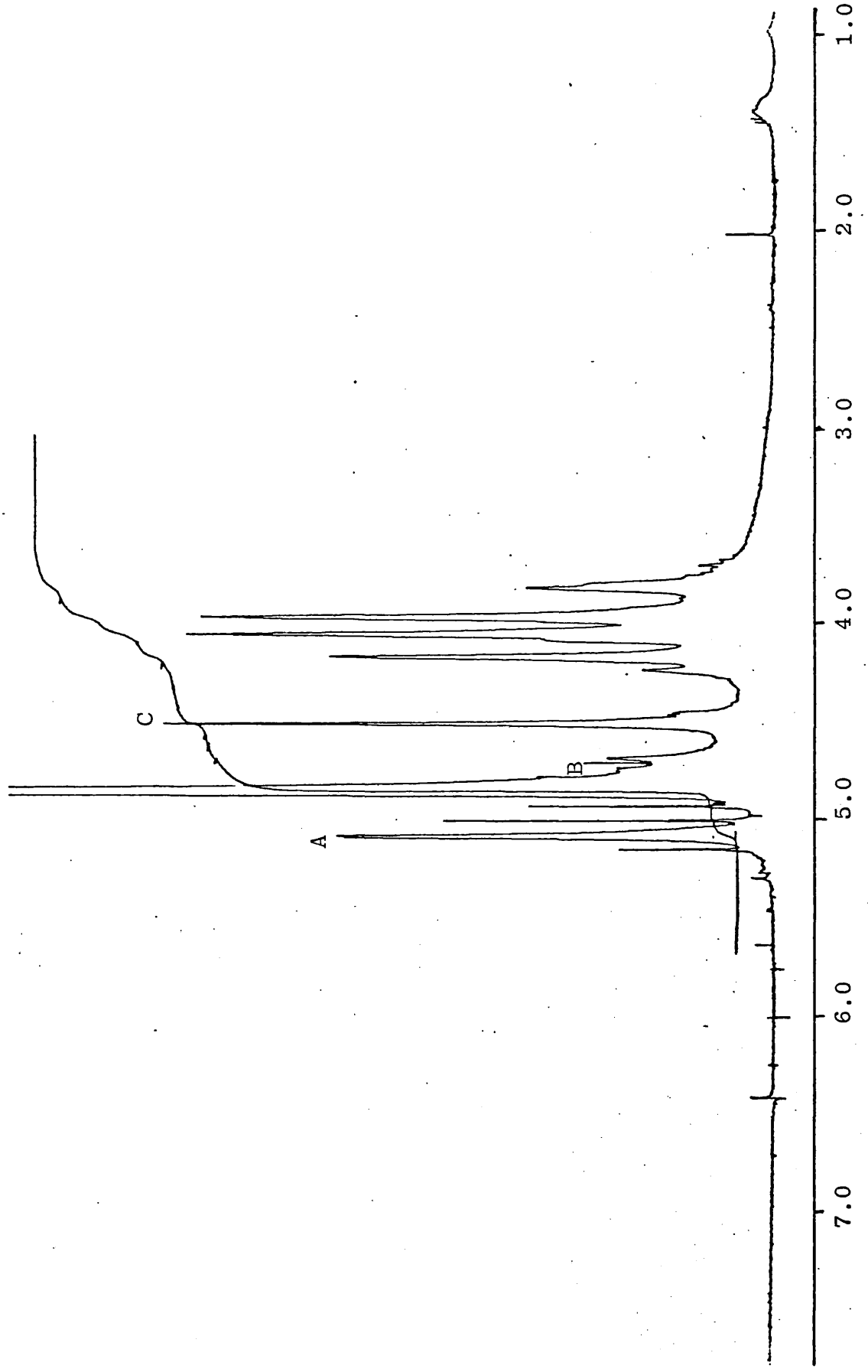


FIGURE 2.18 ^{1}H N.M.R. SPECTRUM OF ASCOSEIRA EXTRACT 7



N.B. This spectrum was recorded at ambient temperature.

again the diad frequencies (F_{MM} = 0.04, F_{MG}(= F_{GM}) = 0.12, F_{GG} = 0.72) differed from those in TABLE 2.16.

TABLE 2.17

ASSIGNMENT OF PEAKS IN ^{13}C N.M.R. SPECTRUM OF ASCOSEIRA EXTRACT 7 (a)

TRIAD SEQUENCE	Carbon atom in central residue of triad					
	C-1	C-2	C-3	C-4	C-5	C-6
MMM	101.0	70.8	72.3	79.0	77.1	175.4
MMG	102.2	70.9	(b)	79.0	(b)	(b)
GMM	101.0	70.8	(b)	(b)	(b)	(b)
GMG	102.2	70.9	(b)	(b)	77.1	(b)
GGG	101.4	66.1	70.1	80.7	68.2	175.6
GGM	100.4	65.7	(b)	80.7	68.2	(b)
MGG	101.4	66.1	(b)	80.7	68.4	(b)
MGM	100.4	65.7	70.3	80.7	68.4	(b)

(a) Shifts in ppm., downfield from external TMS at room temperature.

(b) No assignment made.

The low proportion of alternating sequences in Fractions 3A and 7 cannot be taken as proof that they are in low abundance in the native alginate. The directly extracted Alginate 1 should give a better representation of the composition of the native alginate, with degradation of alternating

sequence blocks being avoided. In fact, apart from a higher proportion of MM blocks, the ^{13}C spectrum (FIGURE 2.19, TABLES 2.18, 2.19) closely resembles that of Extract 7. The resolution was sufficient to allow calculation of diad frequencies from the signals assigned to C-4 in mannuronate residues. The agreement between these and values calculated from the ^{1}H spectrum (FIGURE 2.20) is reasonable.

TABLE 2.18

ASSIGNMENT OF PEAKS IN ^{13}C N.M.R. SPECTRUM OF ASCOSEIRA ALGINATE 1[a]

TRIAD SEQUENCE	Carbon atom in central residue of triad					
	C-1	C-2	C-3	C-4	C-5	C-6
MMM	100.8	70.7	72.1	78.8	76.9	175.3
MMG	101.9	71.2	10	78.8	(76.3)[b]	(c)
GMM	100.8	(c)	(c)	78.4	(77.1)	(c)
GMG	101.9	71.3	72.4	78.4	76.4	175.8
GGG	101.2	65.9	69.9	80.5	68.0	175.5
GGM	100.3	65.6	(c)	80.5	(c)	(c)
MGG	101.2	65.9	(c)	80.5	68.0	(c)
MGM	100.2	65.6	70.0	80.5	(c)	175.8

(a) Shifts in ppm., downfield from external TMS at room temperature.

(b) Values in parentheses denote tentative assignments.

(c) No assignment made.

FIGURE 2.19 ^{13}C N.M.R. SPECTRUM OF ASCOSEIRA ALGINATE 1

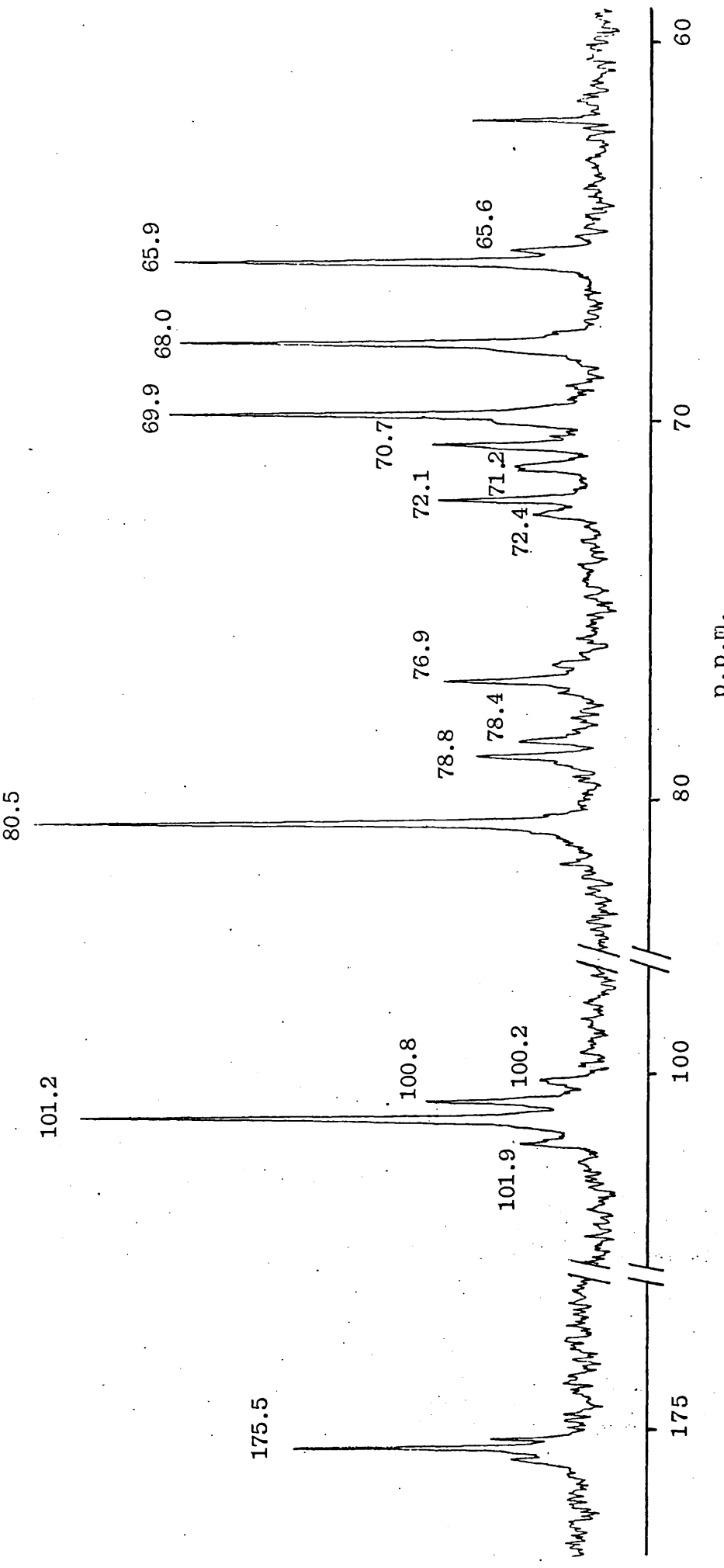


FIGURE 2.20 ^{1}H N.M.R. SPECTRUM OF ASCOSEIRA ALGINATE 1

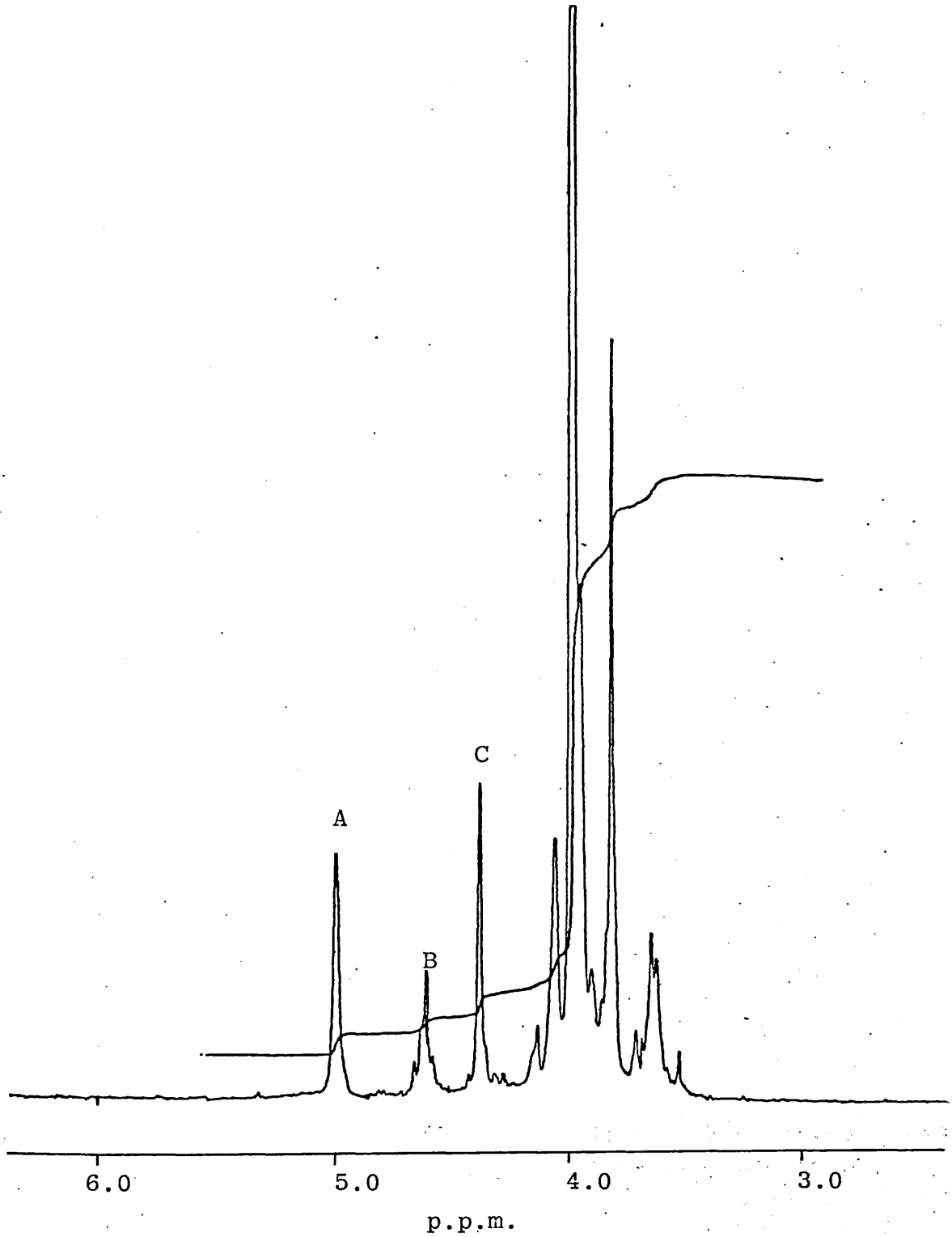


TABLE 2.19

DIAD FREQUENCIES AND BLOCK LENGTHS IN ASCOSEIRA ALGINATE 1

SPECTRUM	Diad						Block Length (a)	
	F_M	F_G	F_{MM}	F_{MG}	F_{GM}	F_{GG}	N_M	N_G
^{13}C (C-1)	0.31	0.69	0.20	0.11	0.11	0.58	1.8	5.3
^{13}C (C-4)	0.30	0.70	0.20	0.10	0.10	0.60	2.0	6.0
^{1}H	0.32	0.68	0.26	0.06	0.06	0.62	4.3	10.3

(a) See text.

To check the veracity of the calculated block distribution a sample of Alginate 1 was subjected to partial acid hydrolysis[41,45] Under the conditions used the M-G and G-M glycosidic linkages are particularly labile and the MG blocks are hydrolysed giving acid-soluble low oligomers and leaving the acid-resistant MM blocks and GG blocks essentially intact and insoluble in acid. Hence the block distribution can be determined after calculation of the relative proportions of MM and GG blocks by fractionation[45] or n.m.r.[41] The breakdown of molecules should eliminate the possibility of aggregation of GG blocks and thus any loss of signal in FIGURES 2.19, 2.20 caused by aggregation in the intact Alginate 1 should be apparent by comparison of the block distribution. A possible source of error in the partial hydrolysis method must be noted. The hydrolysis is carried out under

heterogeneous conditions and a sharp division into two distinct fractions cannot be assumed; there may be incomplete solubilisation of MG blocks or partial solubilisation of MM blocks which are more susceptible to hydrolysis than GG blocks. In past studies both features have been evident; in the latter case the M/G ratio in the soluble fraction is commonly in the range 1.2 - 1.4.[46-48] This seems too high to be due simply to the known divergence of MG blocks from perfect alternation. However it should be possible to check for these errors by observation of the n.m.r. spectra of both fractions.

After the hydrolysis the acid-soluble and acid-insoluble fractions were separated and the carbohydrate contents determined, to correct for non-carbohydrate impurities in the isolated solids. The MM and GG block distribution was determined as previously from the ^{1}H spectrum of the acid-insoluble fraction (FIGURE 2.21, TABLE 2.20), and the absence of MG blocks in this fraction confirmed.

The reducing end-group anomeric signals were assigned according to Grasdalen et al.;[43] the signals were not sufficiently well resolved to allow calculation of the relative proportions of M and G residues but nevertheless an approximate average block length of 16 was calculated. It is probable that the GG blocks are somewhat longer than the average. On gel filtration (FIGURE 2.22) there was no apparent separation and the

FIGURE 2.21 ^{1}H N.M.R. SPECTRUM OF ACID-INSOLUBLE FRACTION OF ALGINATE 1

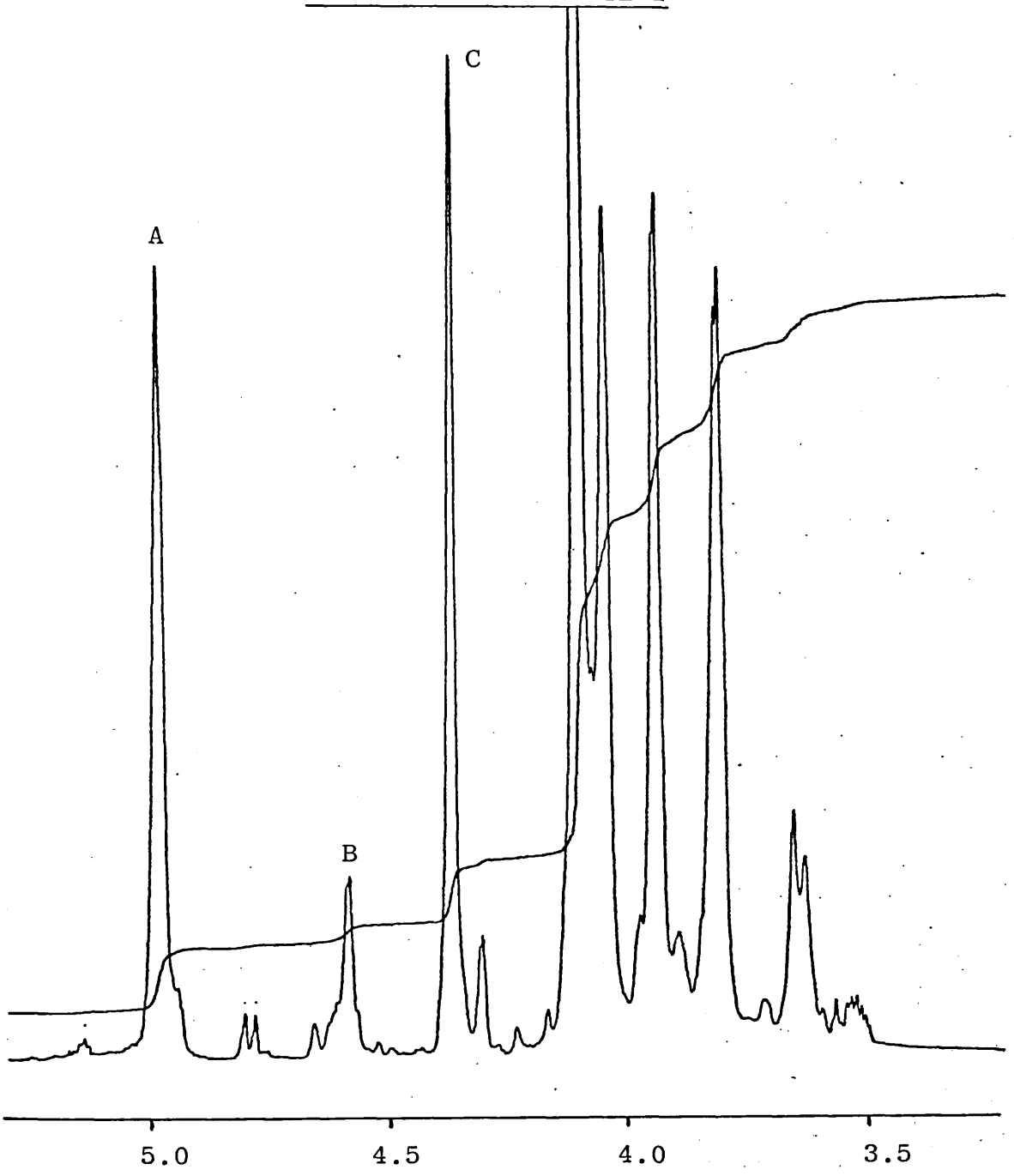
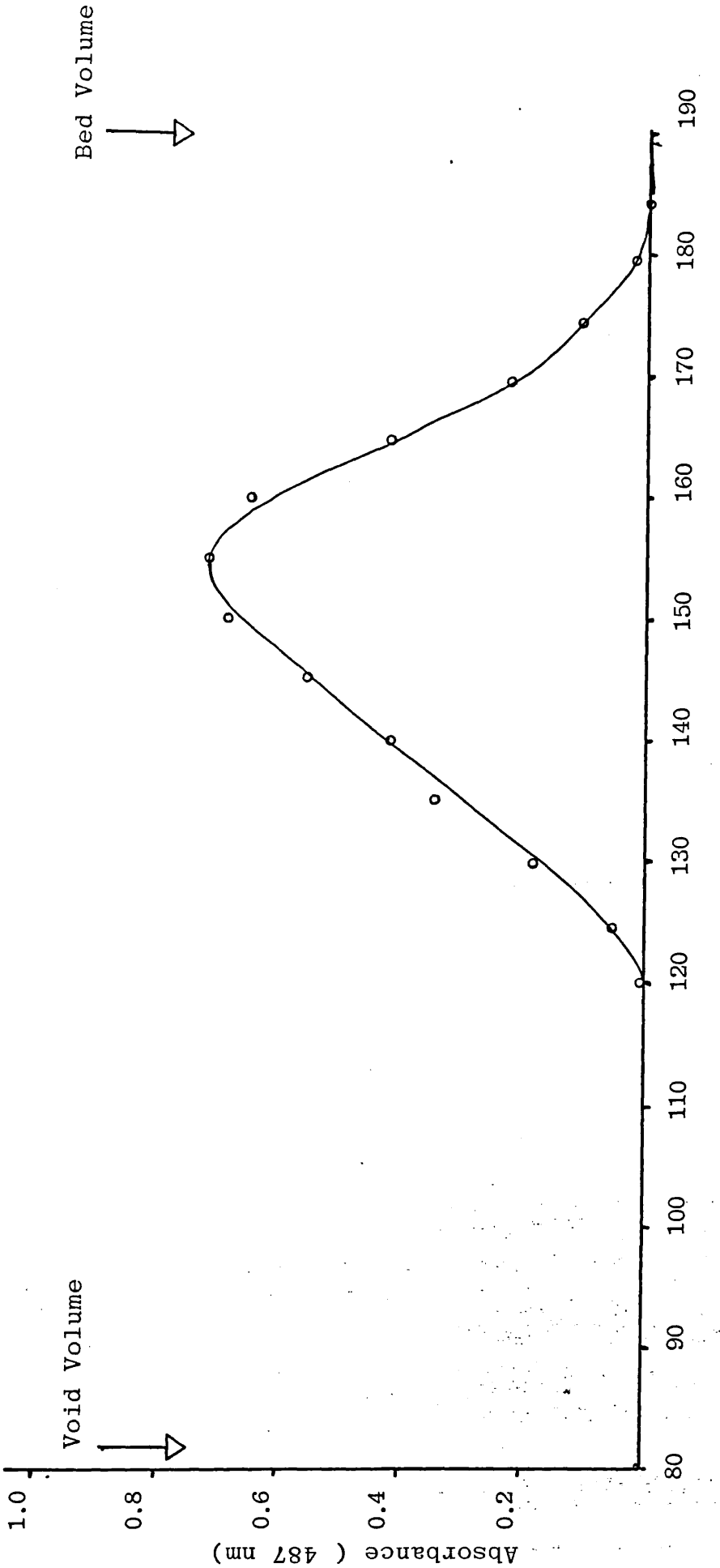


FIGURE 2.22 ELUTION PROFILE OF ACID-INSOLUBLE FRACTION OF ALGINATE 1 ON SEPHADEX G100



average chain length calculated from the number average molecular weight was about 40. It is not possible to ascertain whether this represents the length of a single block; GG and MM blocks may be mutually linked or the gel filtration pattern may represent some molecular association. However it is noteworthy that the calculated chain length is greater than that of the water-soluble polymannuronate-rich material in Fraction 3A (ca. 20 units, Section 2.2.4), but similar to that of the guluronate-rich Extract 7 (Section 2.2.8).

TABLE 2.20

BLOCK DISTRIBUTION IN ASCOSEIRA ALGINATE 1

METHOD	Block Distribution					Block Length	
	F_M	F_G	F_{MM}	F_{GG}	$F_{MG}+F_{GM}$	N_{MM}	N_{GG}
Partial Hydrolysis	0.32	0.68	0.16	0.62	0.22	16	
^{13}C n.m.r.	0.30	0.70	0.20	0.60	0.20	2	6
^{1}H n.m.r.	0.32	0.68	0.26	0.62	0.12	4.3	10.3

Most previous estimates of block lengths have been based on partial hydrolysis[46-49] and hence doubt must remain as to the homogeneity of the blocks on which the calculations have been made. A more reliable method,[50] based on complete enzymic degradation

of MG and GG blocks, gave MM block lengths of about 24 for several samples. A sample from growing tips of Ascosphyllum nodosum was rather polydisperse (d.p. 21-30); with a mannuronate content of 75% it is very similar to the material in Fraction 3A and it is reasonable to conclude that Fraction 3A includes newly synthesised alginate.

The soluble fraction from the partial hydrolysis was extensively degraded; the average degree of polymerisation was about 3, it eluted in the bed volume on Sephadex G100 and the ^{1}H n.m.r. spectrum (FIGURE 2.23) had a high proportion of reducing end-group signals. Overlapping peaks make calculation of the M/G ratio difficult and assignments uncertain (TABLE 2.21)[42] even with resolution enhancement. It is not possible to ascertain whether any of the MM blocks have been solubilised; M-M diuronides may be derived from MM blocks or may simply be irregularities in the MG blocks. The relatively low proportion (0.16) of MM blocks calculated from the insoluble fraction suggests that some stabilisation of MM blocks has occurred and that the partial hydrolysis method gives proportions of MM blocks and MG blocks that are respectively too low and too high. Nevertheless the block distribution is in rough agreement with those determined by n.m.r.

FIGURE 2.23 ^{1}H N.M.R. SPECTRUM OF ACID-SOLUBLE FRACTION OF ALGINATE 1

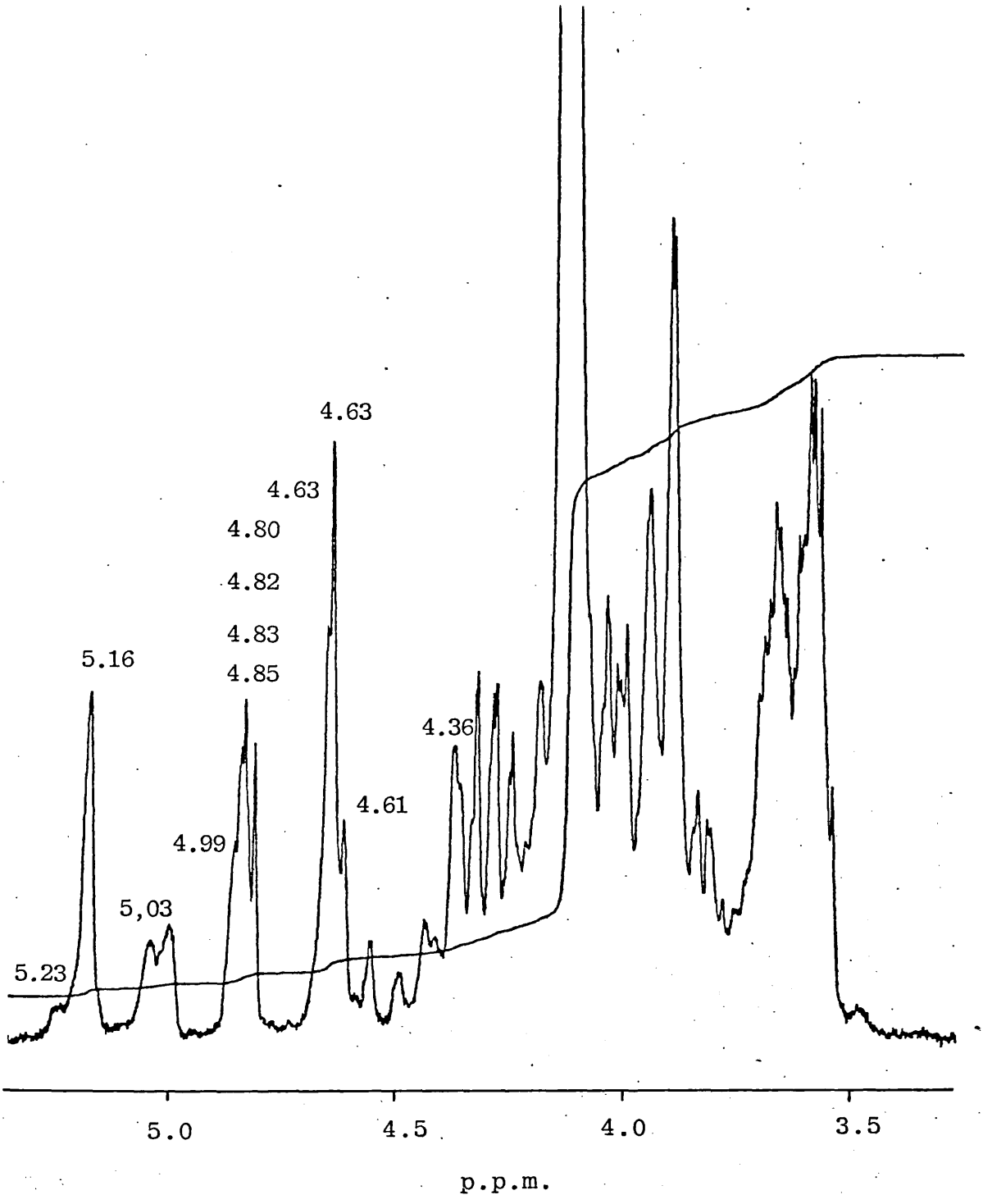


TABLE 2.21.

ASSIGNMENT OF ^{1}H SPECTRUM OF ASCOSEIRA ALGINATE ACID-SOLUBLE FRACTION

Chemical Shift δ	Tentative Assignment
4.36	H-5 of G in -G-G- (*)
4.61, 4.63, 4.65	H-5 of G in -M-G- (*); H-1 of M in -M-M- or -G-M-
4.80, 4.82, 4.83, 4.85	H-1 of M and G reducing end groups (β)
4.99, 5.03	H-1 of G in -G-G- or -M-G- (*)
5.16, 5.23	H-1 of M and G reducing end groups (α)

It may be concluded that (a) there has been negligible suppression of signals in the spectra of the intact alginate and (b) the proportion of MG blocks in the intact alginate is low. In the absence of evidence to the contrary it is safe to assume that the alginate does not differ fundamentally from other alginates and

hence the three types of block are mutually linked. The GG block length in the insoluble fraction is much shorter than the average chain length of alginate determined by gel filtration (d.p. 100). The earlier assertion that the water-soluble alginate may be comprised of separate blocks does not conflict with the concept of mutual linkage since the amount of MM blocks in the water-soluble alginate is insufficent to account for all the MM blocks in the total alginate; some of the MM blocks must be linked to the other block types (this assumes that the composition of alginate 2 is essentially the same as alginate 1).

A low proportion of MG blocks is not uncommon in alginates.[41,45] As described earlier (Section 1.2.3.1) alginate is considered to be synthesised as polymannuronate, individual residues then being converted by C-5 epimerase to guluronates generating either MG or GG blocks. Recent studies[51] suggest that the two block types are generated by different epimerases, the "MG epimerase" being rather more sensitive to calcium ion concentration. When this concentration is high MG blocks are generated preferentially, whereas at low concentration GG blocks are favoured. Thus a form of homeostasis may be operating through the alginate structure; when the calcium ion concentration in the ambient water is too low for adequate gel rigidity an alginate is

synthesised with GG blocks that bind calcium strongly, and when it is high enough to render the gel brittle non-binding MG blocks are generated.

Thus the deficiency of MG blocks in Ascoseira alginate may be an indication of a low concentration of calcium ions in its environment. An alternative explanation may be deduced by the relatively short chain lengths. With extended GG blocks (at least 20 residues[52]) required for gelation the chains may not be able to accommodate a large proportion of MG blocks.

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CHAPTER 3

THE MUCILAGINOUS EXUDATE OF MACROCYSTIS PYRIFERA FROM THE ANTARCTIC AND CALIFORNIA

3.1. Introduction

Many species of brown algae exude a sticky slimy liquid from their surface, a fact that imperils the unwary stroller on a weed-covered rocky shore. It has been suggested that this liquid prevents desiccation, ameliorating the effects of excessive salinity during the long periods when the weed is exposed.[1] For Macrocystis pyrifera and other species of the Laminariacae there would appear to be other functions since these species are rarely exposed. The main polysaccharide constituent of the slime is "fucoidan", a "fucan" of high fucose and sulphate content. As outlined in the Introduction (1.2.2.2.), the "fucan" in brown algae is thought to serve partly as a gelling agent to lend support to alginic acid in protecting the structural integrity of the alga, and partly as a selective ion-exchanger controlling intake and loss of cations from the seawater.[2] It is not clear whether any distinction can be made between "fucan" within the alga and "fucan" on the surface, but it seems reasonable to assume that no sharp change in structure occurs as the slime is exuded from the surface. Thus any division between the two 'forms' of "fucan" must be rather arbitrary.

In the context of adaptation to the Antarctic the significant features, the composition and quantity of the slime, and how they change with lowering temperature, are obviously influenced by more than just the polysaccharide content. The total solids, salt composition and the presence of small organic molecules may all be relevant. To this end the detailed composition of the slime of the Antarctic sample of Macrocystis pyrifera has been determined and compared with that of the same alga growing in the temperate waters of Southern California.
The scope of this sort of study is severely constrained by several factors. All the parameters to be measured are likely to vary both seasonally and with plants in differing stages of development. A comprehensive study would involve monitoring the composition of the slime throughout the year, an impractical proposition. To minimise differences studies were made on batches of algae harvested in the same season.

The Antarctic sample of exudate was obtained from a batch of whole plants collected from kelp beds on South Georgia during late October 1978 (i.e. early Spring). The weed was immediately frozen and kept at -20^{o}C during transportation and storage. After about 12 months the weed was allowed to thaw and the liquid draining off was collected. (Expt. 5.37).

The Southern California exudate was taken from

a batch of weeds harvested for commercial purposes by Kelco of San Diego during March 1980 (Spring). The weed is harvested by a reaping machine which cuts the stems about 1m below the surface, leaving the lower part of the stems and the holdfast behind. Since these contribute little to the total surface area of the weed they probably accommodate little exudate and therefore any variation in exudate composition from that of the fronds is unlikely to be reflected in differing composition of the exudates from the two geographical sources. The exudate was collected from drainage tanks for the harvested weed, weighed and freeze dried for transportation. This difference in treatment of the two exudates was unavoidable and must be borne in mind when comparing properties.

The only previous work on exudate components other than fucoidan in Macrocystis pyrifera was reported by Schweiger, who found mannitol, glycerol, taurine, laminitol, succinic acid and several amino acids.[3]

3.2 Solids Contents (Expts.5.38, 5.43).

Accurate determination of the solids content of the exudates is complicated by the difficulty in distinguishing between exudate and adhering seawater. In the sea it is unlikely that there is a sharp dividing line between the exudate and the seawater, hence although most of the water will drain off immediately after removal of the weed from the sea,

the possible different dilutions of the exudates must be borne in mind when considering the compositions.

After freeze-drying, the Antarctic exudate (AE) contained about 5% w/v of solid, the exact amount varying slightly between different samples. For the California exudate (CE) the concentration was 6.5% w/v from the weight and volume quoted by the supplier. Seawater contains about 3.4% of solids. 71% (AE) and 66% (CE) of the solid remained after ashing; since at least 10% of the freeze-dried material may be taken to be water it is apparent that the bulk of the material is inorganic. Titration showed chloride to be the dominant anionic species in both, accounting for just over half the inorganic material; free sulphate was the other significant anion, accounting for 8% of the inorganic in both.

The four principal cations found in seawater were quantified by atomic absorption spectrophotometry, after "ashing" of the solids by two methods, acid digestion and muffle furnace ignition. For sodium, potassium and magnesium both methods gave similar, reproducible results, whereas for calcium the results were variable with the dry ash method giving high results. The results are summarised in TABLE 3.1 both as concentrations and as relative proportions, with figures for seawater listed for comparison.

TABLE 3.1 METAL CATION COMPOSITION OF ANTARCTIC AND CALIFORNIAN MACROCYSTIS MUCILAGES

Metal	Abundance[(a)]			Relative Proportion		
	AE	CE	Seawater	AE	CE	Seawater
Na	5.95	3.60	10.54	11	12	26
K	26.40	15.60	0.38	51	52	1
Mg	0.96	0.56	1.27	2	2	3
Ca	0.28-0.76	0.25-0.35	0.40	1	1	1

(a) mg/ml of mucilage.

The reason for the variable results for calcium is not clear. The tendency of alkali earth metals to form salts which are stable in the air/acetylene flame, thereby suppressing the yield of free atoms, is well known.[4] The addition of La^{3+} ions in sufficient excess should release mostof the calcium by forming compounds of similar stability. Another depressing effect, complexation with protein, should have been eliminated by the ashing. The lower values for wet ashing suggest incomplete digestion, though the method differs only slightly from a recommended method for plant materials, and the consistency of the other results tends to rule out this possibility. Since interference effects in atomic absorption spectrophotometry are generally depressive rather than enhancive it seems likely that the actual calcium

concentrations are probably best represented by the highest observed values.

Regardless of the above, the profound difference in cation concentrations between the exudates and the seawater, and the close similarities of CE and AE reflected in the relative proportions, are quite obvious. The operation of ion "pumps" in algae has been described before[5] and it would appear that Macrocystis pyrifera actively accumulates potassium and excludes sodium, the relative concentrations being unaffected by temperature.

The exudate is a viscous, sticky fluid when it is stripped from the weed. However, reconstitution of the freeze-dried solids to their original concentrations gave solutions of drastically reduced viscosity, which fell even further when the solutions were left standing.

3.3 Carbohydrate Contents (Expts. 5.39-5.42, 5.44-5.47)

The carbohydrate contents (fucose graph) of the two dried solids were 3.5% w/w (AE) and 5.0% w/w (CE), respectively 0.12% and 0.32% solutions of the exudates.

The salts and low molecular weight compounds were separated from the polymer materials by dialysis. 450ml of AE (ca. 0.7g carbohydrate) and 25g of CE reconstituted in 300ml of water (ca. 1.25g of carbohydrate) were separately dialysed until free of

chloride. The material passing through the dialysis membranes was recovered, deionised, the mannitol separated and the reducing sugars examined (TABLE 3.2).

TABLE 3.2. LOW MOLECULAR WEIGHT SUGARS IN MACROCYSTIS MUCILAGES.

Sugar	AE	CE
Fucose	xxxx	xxxx
Xylose	x	x
Mannose	x	x
Glucose	xx	xx
Galactose	x	x
Oligosaccharides	trace	trace

It would appear that in both cases most of the reducing sugar is derived from degradation of "fucan". During the course of dialysis the pH inside the membrane fell in both cases to about 4.5. This increase in acidity is probably caused by the slight acidity of the water used for dialysis, and of the exudates themselves (pH~6.5). Exchange of protons with metallic cations, both free and associated with the "fucan",is initiated and then sustained by each change of dialysis water. The "fucan" is then degraded over several days both by free acid and through autohydrolysis, acid-labile

fucosyl linkages are cleaved releasing the fucose which accounts for most of the free reducing sugar. Later results will support the view that the bulk of the free sugars does not occur as such in the living alga. The similar proportions of the other carbohydrate constituents suggest that none of the small carbohydrates has any significant role in adaptation to the low temperature; in fact a higher proportion of the polyalcohols might have been expected at the lower temperature.

The two dialysed solutions (now apparently devoid of any viscous character) were adjusted to pH 7.0 and separately freeze-dried. The yields and compositions of the two solids, hereinafter referred to as "fucan" AE and "fucan" CE, were determined (TABLES 3.3, 3.4).

TABLE 3.3. RECOVERY AND COMPOSITION OF "FUCAN" FROM MACROCYSTIS MUCILAGES.

	% by weight of the recovered "fucan"	
	AE	CE
Recovery(a)	0.5	0.65
Carbohydrate(b)	35	38
Uronic acid(c)	5.6	4.9
Sulphate(d)	25(71)	27(71)
Protein	12	15
Ash	25	20
Na	0.51	0.67
K	2.01	2.15
Mg	2.15	1.50
Ca	0.82-2.05	1.00-1.47

(a) Percentage w/v of the mucilage

(b) Fucose graph

(c) Glucuronic acid graph

(d) Figures in parenthesis are percentages of the carbohydrate

TABLE 3.4. RELATIVE PROPORTIONS OF SUGARS IN MACROCYSTIS "FUCANS"(a)

Fucan	Fuc	Xyl	Man	Glu	Gal	GluA
AE	75	5	4	2	8	6
CE	74	3	6	4	8	5

(a) Neutral sugars determined by g.l.c., uronic acid determined colorimetrically; total set to 100%

The similarity between the two fucans is evident. Of particular significance is the distribution of the cations, where the predominance of potassium in the mucilages, (which would be expected after oxygen deprivation)[5] has clearly not been sustained; the divalent calcium and magnesium ions are preferentially bound during dialysis. This marked affinity (for divalent ions) has been observed before by Lestang and Quillet[6] in the "fucan" from Pelvetia canaliculata. They noted that the "fucan" fixed magnesium preferentially, so that when the fronds of the alga are in contact with seawater the half-ester sulphate groups are largely associated with magnesium ions. They suggested that the magnesium ions are highly hydrated, thereby retaining water in the fronds.

This preference for magnesium and, to a lesser extent, calcium, appears to be repeated in Macrocystis pyrifera, though more detailed experiments would be necessary to confirm this. The higher concentration of magnesium in "Fucan" A is probably not significant.

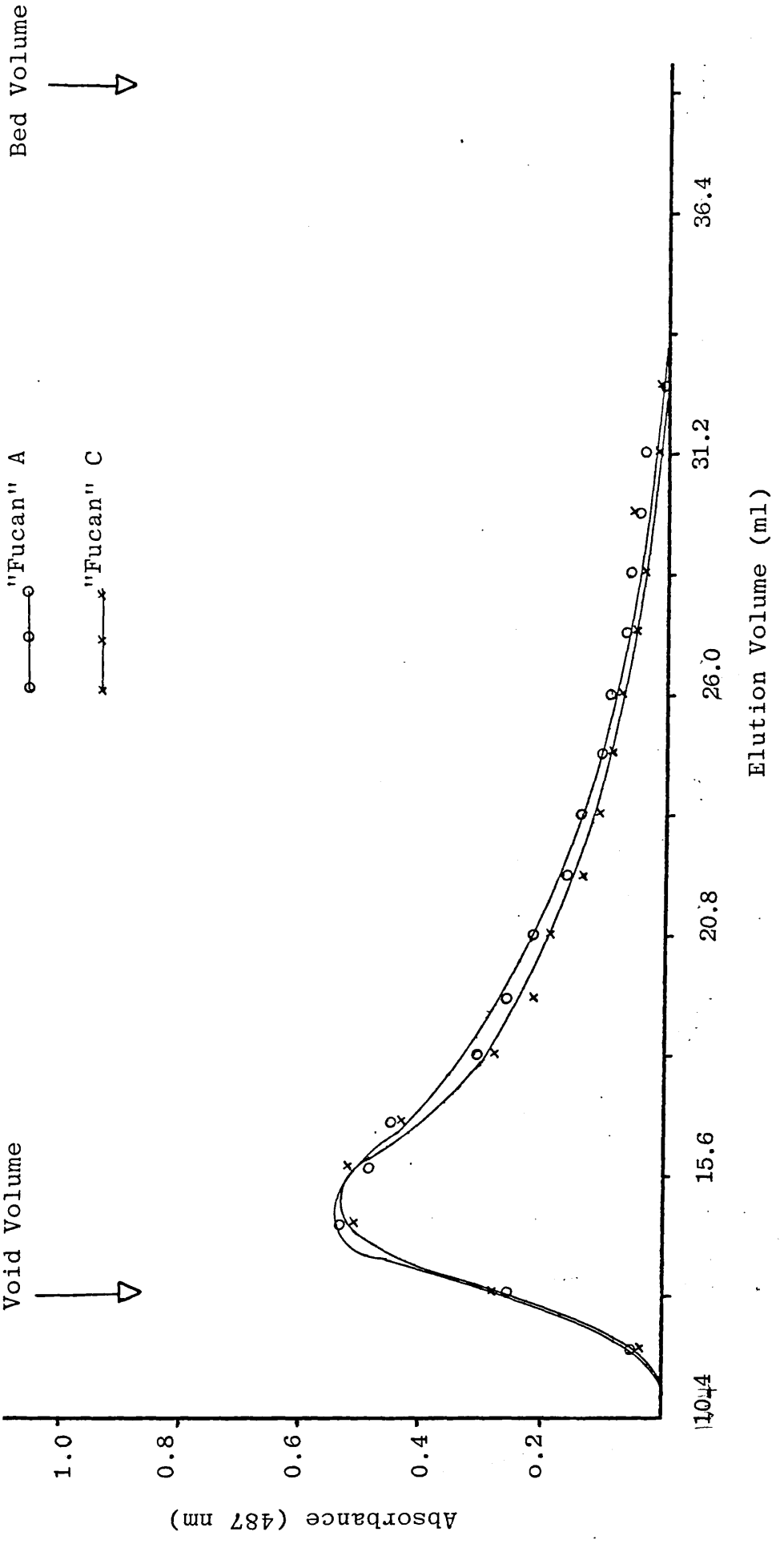
Attempts were made to differentiate the two M.pyrifera "fucans" at the macromolecular level on the basis of heterogeneity, size and charge. Electrophoresis on both cellulose acetate at pH 5.1 and 8.0, and on polyacrylamide gels at pH 8.5 gave

single bands of identical mobility for the two "fucans"; in both a coincident band which stained for protein was observed. In view of the compositional similarity of the two "fucans", particularly the sulphate: fucose ratio, this was to be expected. A more likely area for divergence is in molecular size; however gel filtration on Sepharose 4B (FIGURE 3.1) indicated no apparent difference, both being highly polydisperse with average molecular weight probably in excess of 10^6.

It might be expected that the viscosity of the mucilage will depend on the molecular size of the constituent "fucan" as well as its concentration and the prevailing temperature. The Antarctic sample at lower temperature and similar concentration would require the "fucan" to impart less viscous character to the mucilage, assuming an inverse dependence of viscosity and temperature. The similarity in apparent size of the two "fucans" implies that the viscosity is not controlled by the size; the differing concentrations of "fucan" (20% lower in the Antarctic mucilage) may be more significant.

Experiments to measure the temperature and concentration dependence of the viscosity of "fucan" solutions were inconclusive; as has been previously observed[7], the viscosities of "fucan" solutions

FIGURE 3.1 ELUTION PROFILES OF MACROCYSTIS PYRIFERA "FUCANS" A AND C ON SEPHAROSE 4B



were considerably lower than those of the native mucilage and also fell substantially on standing. Attempts to reconstitute the mucilages by addition of the appropriate salts served only to reduce viscosities even further, presumably through electroviscous effects.

The polysaccharide conformation may have some bearing on the viscosity. Lestang and Quillet[6] assumed that the "fucan" was essentially composed of α-(1→2)-L-fucose residues carrying sulphate at C-4. They proposed a helical conformation with the half-ester sulphate groups on the outer surface of the helix, carrying partially hydrated magnesium ions. This rather simple model fails to account for a degree of sulphation in excess of 1.0 and precludes linkage through other ring carbon atoms. Until detailed structural information on the "fucan" is available it is unwise to speculate on likely molecular conformations.

It must be concluded that the composition of the mucilage is not profoundly affected by climate, at least not in terms of the carbohydrate components.

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CHAPTER 4

CARBOHYDRATES OF MACROCYSTIS PYRIFERA FROM THE ANTARCTIC

4.1 Sequential Extraction of Macrocystis pyrifera

The exudate has yielded a substantial amount of "fucan" and some low molecular weight carbohydrate. To recover the remaining carbohydrate a sequential extraction was carried out. The intact alga (about 0.5Kg after removal of exudate) was ground, reground and dried to a powder (60 g. dry weight). Since some low molecular weight material had already been removed an ethanol extraction was omitted and the low molecular weight carbohydrate recovered from the supernatant of the Cold Water Extract. The extraction procedure is outlined in FLOW CHART 4.1 (Expts.5.48 - 5.52).

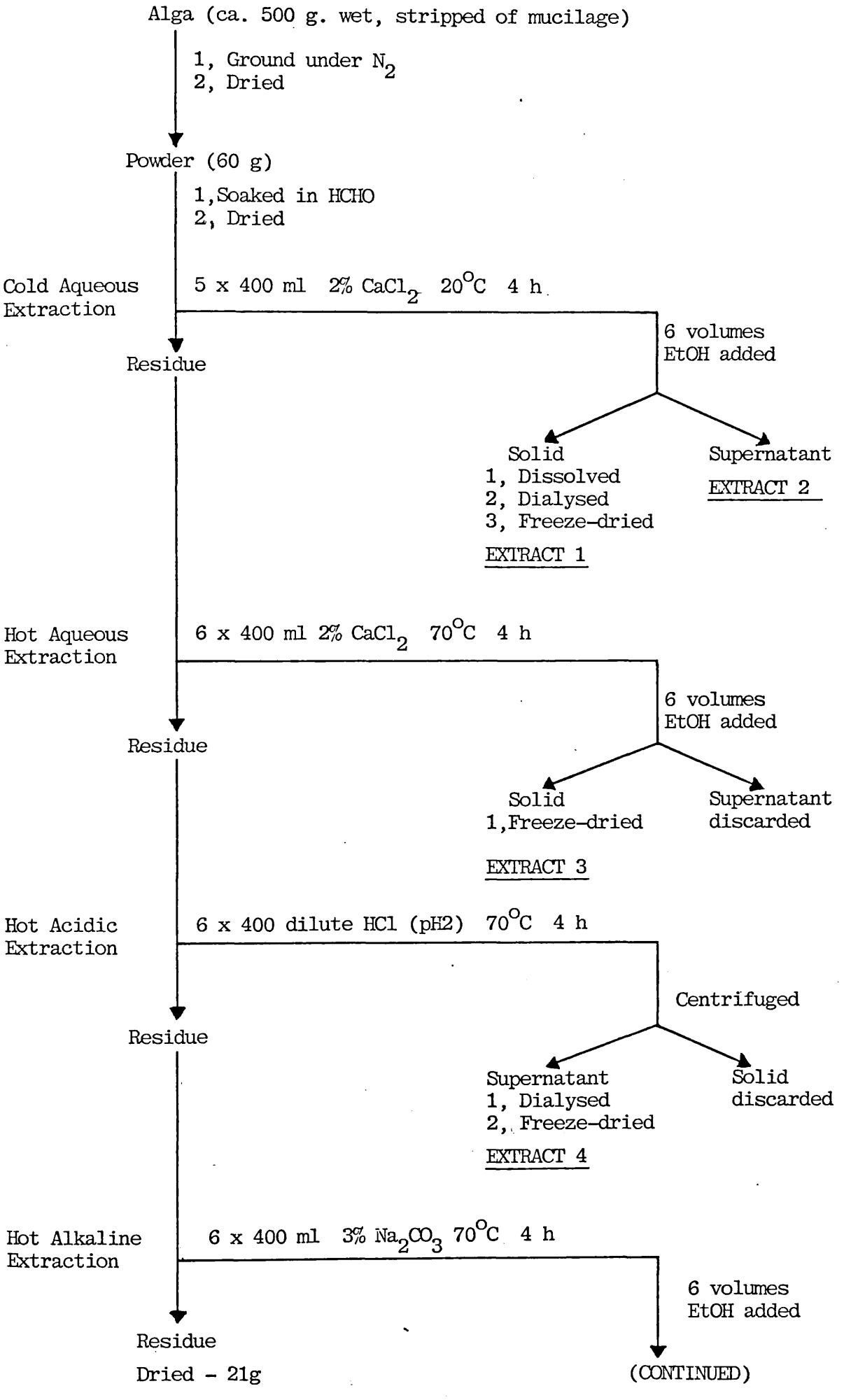
4.1.1. Low Molecular Weight Carbohydrates (Expt.5.52)

The supernatant from the Cold Water Extract was concentrated and the salts removed. About 200 mg of mannitol was isolated and characterised. This amounts to only 0.3% of the dry weight; in combination with the small amount found in the exudate it is obvious that the alga contained little mannitol when harvested. This is consistent with the general observation for Laminariales of a low mannitol content in early spring, prior to the rapid accumulation by photosynthesis.[1]

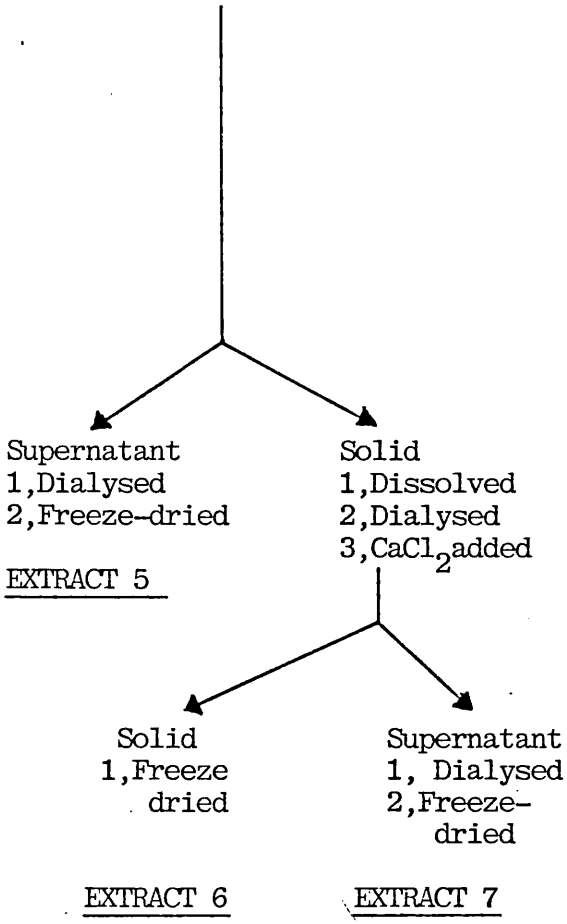
The residual syrup yielded about 25 mg of carbohydrate (glucose graph) comprising fucose, glucose

FLOW CHART 4.1

SEQUENTIAL EXTRACTION OF MACROCYSTIS PYRIFERA



FLOW CHART 4.1 (continued)



and lesser quantities of xylose, mannose and galactose, identified by paper chromatography and g.l.c. These are probably derived from degradation of polysaccharides, during either storage or the grinding and drying.

4.1.2. Examination of the Polymeric Extracts

(Expts. 5.54 - 5.59)

All the polymeric extracts were buff coloured, indicating that the formaldehyde treatment had not been fully successful in preventing extraction of pigments (TABLE 4.1)

TABLE 4.1

RECOVERY OF POLYMERIC EXTRACTS FROM MACROCYSTIS PYRIFERA

EXTRACTANT	EXTRACT	WEIGHT	% OF DRY WEIGHT
Cold Water	1	1.82	3.0
Hot Water	3	0.23	0.4
Hot Acid	4	2.52	4.2
Hot Alkali	5	0.03	-
Hot Alkali	6	3.70	6.2
Hot Alkali	7	0.66	1.1
	TOTAL	8.96	14.9

Extract 6, the calcium alginate, was converted to its sodium salt; no material was solubilised during the conversion to the free acid. After hydrolysis no neutral sugars appeared on paper chromatography, confirming that no "fucan" had been precipitated by the calcium ions. In view of the likely degradation during extraction the alginate was not studied further.

The four remaining major polymeric extracts (the "fucans") were examined for carbohydrate, uronic acid, protein and sulphate. Portions of Extracts 1, 4 and 7 were hydrolysed with 2M trifluoroacetic acid and 90% formic acid and the monosaccharides in each hydrolysate identified by paper chromatography and quantified by g.l.c. Only one hydrolysis was carried out on Extract 3 in view of the small amount.

The results are summarised in TABLES 4.2 and 4.3. The carbohydrate contents were calculated from the standard graphs quoted in the footnotes. The proportions in the graphs do not correspond exactly with those determined by g.l.c. but given the differences between the two hydrolysis methods, the ambiguity in the proportions of hexose and hexuronic acid and the fact that hydrolysis was probably incomplete in all cases, any putative better "fit" of standard graph is of doubtful value. The incomplete hydrolysis was apparent from paper chromatograms where small amounts of oligomeric material, probably aldobiuronic acids,

were detected for all the hydrolysates except for those of Extract 1. In addition, the extent of any destruction of sugars is uncertain. In a repeat of the hydrolysis of Extract 4 about 20% and 33% of the carbohydrate was destroyed during hydrolysis with trifluoroacetic acid and formic acid respectively. Addition of three volumes of ethanol to each hydrolysate precipitated respectively 29% and 43% by weight of the original material. Both precipitates contained about 5% of the original carbohydrate. Evidently there has been both destruction of carbohydrate and incomplete hydrolysis and thus the results in TABLE 4.3 must be taken as only indicating the approximate proportions of each monosaccharide present.

TABLE 4.2 COMPOSITIONS OF POLYMERIC EXTRACTS

EXTRACT	CARBOHYDRATE % (a)	URONIC ACID % (b)	SULPHATE % (c)	PROTEIN % (d)
1	48	8	64	10
3	60	24	(e)	0
4	50	12	26	5
7	48	31	27	7

(a) Fucose standard graph for 1, Fucose:Mannose:Glucuronic Acid (1:1:1) for 3,4 and 7.

(b) Glucuronic acid standard graph and expressed as percentage of the carbohydrate.

(c) Expressed as a percentage of the carbohydrate.

(d) Expressed as a percentage of the extract.

(e) Not determined.

TABLE 4.3

RELATIVE MOLAR PROPORTIONS OF SUGARS IN MACROCYSTIS EXTRACTS

EXTRACT	ACID[a]	SUGAR DISTRIBUTION[b]							
		FUC	XYL	MAN	GLU	GAL	GLUA	MANA	GULA
1	1	75	3	6	2	5	8	-	-
"	2	82	2	3	1	3			
3	1	20	5	5	5	8	8	35	5
4	1	27	9	5	15	14	4	20	4
"	2	42	9	5	6	9			
7	1	13	2	5	8	14	8	40	4
"	2	21	5	3	4	13			

(a) 1: 2M trifluoroacetic acid (GM 5.3.3).

2: 90% formic acid (GM 5.3.1).

(b) Neutral sugars determined from g.l.c., mannose estimated visually from paper chromatograms. Uronic acid estimated by carbazole method. Total set to 100%.

The above caution must be extended to consideration of the quoted sulphate contents; however they are consistent with the view that only fucose units carry sulphate in significant quantity.

The uronic acid contents suggest that about 30% of the carbohydrate of Extract 4 and 50% of Extract 7 is alginic acid, solubilised either by acid degradation during the extraction or by virtue of a high mannuronic acid content. After correction to a weight basis the

alginic acid represents about 15% and 25% respectively. Adding the appropriate quantities to the calcium alginate (Extract 6) gives a total alginate content of 7.1% of the dry weight of the alga.

The "fucan" content must be corrected accordingly, giving a total of 7.8% of the dry weight. Two assumptions are made here, firstly that the content of laminaran is negligibly small, and secondly that the protein in each extract is considered as part of the "fucan". The laminaran content in each extract is probably negligible since the glucose content was small in each case. The validity of the second assumption is considered in the next section.

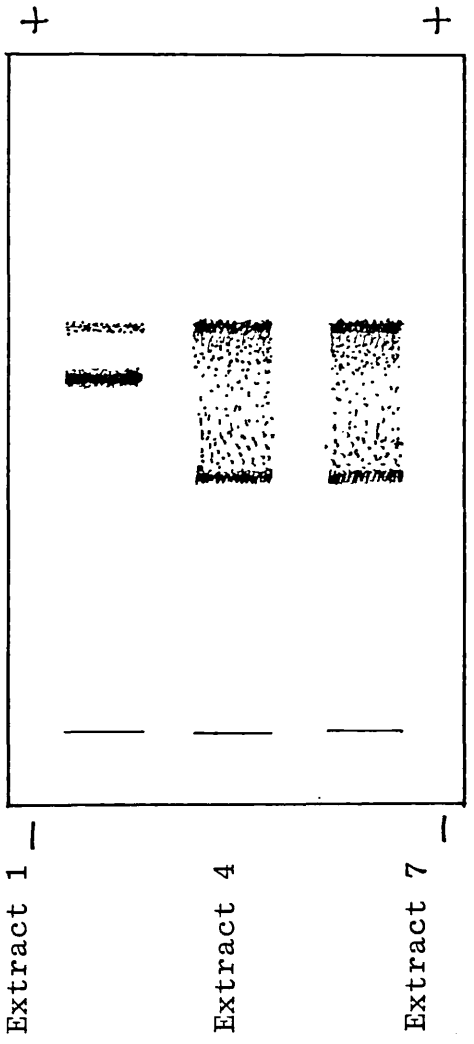
The recovery of alginic acid is exceptionally low, the more so because Macrocystis pyrifera is known to have a high content of this material.[2]

4.1.3. Analytical Fractionation of the Extracts (Expts. 5.54 - 5.59).

Having established the composition of each extract, the size, heterogeneity and nature of any linkage of the various components was investigated.

The behaviour of Extracts 1,4 and 7 on cellulose acetate electrophoresis at pH 5.1 is shown in FIGURE 4.1. The slower bands in the latter two extracts are coincident with that from a genuine sample of alginic acid, indicative of non-linkage of "fucan" and alginate. Evidently Extract 1 migrates as a single polysaccharide.

FIGURE 4.1 ELECTROPHORETIC PATTERNS OF MACROCYSTIS PYRIFERA EXTRACTS ON CELLULOSE ACETATE AT PH 5.1



Protein bands could not be visualised, presumably because of the low percentage of protein in the extracts.

On gel electrophoresis, where greater loadings are possible, no protein band could be visualised for Extract 4 but Extracts 1 and 7 showed single coincident protein and polysaccharide bands at pH 8.5. The electrophoretic patterns at pH 6.5 are shown in FIGURE 4.2. The behaviour of the extracts is similar to that found earlier for the various extracts of Ascoseira. Extract 1 barely penetrates the gel, suggesting a high molecular weight. For Extracts 4 and 7 the patterns are consistent with:

(a) non-linkage of alginate and "fucan" by virtue of the occurrence of bands coincident with those for genuine alginic acid.

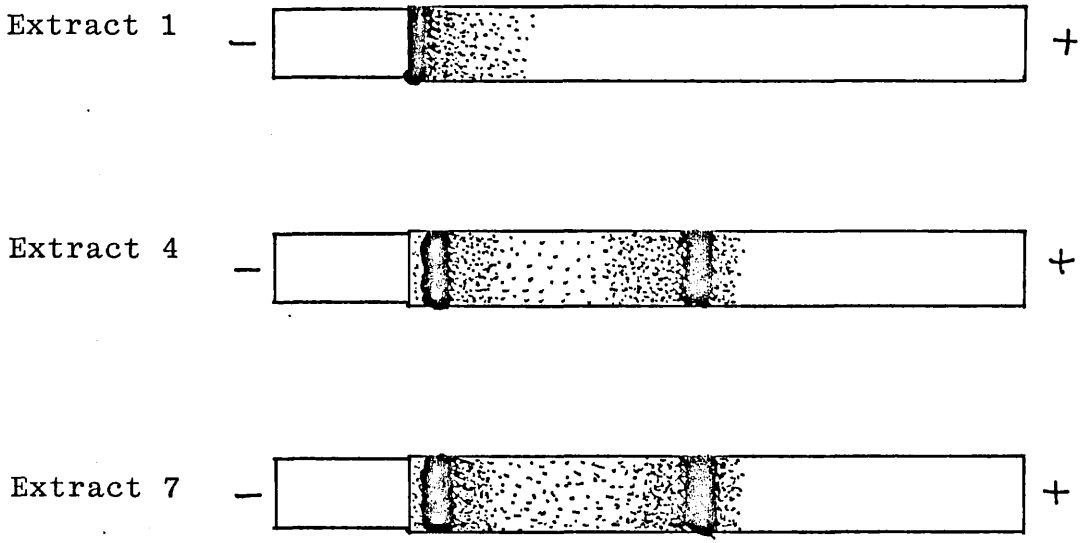
(b) the occurrence of "fucan" of lower molecular weight than in Extract 1 as shown by its greater penetration.

No protein bands were visible, indicating that they may have become diffuse along with the "fucan" band.

The failure to generate gel systems of higher porosity or of lower buffer pH was described earlier.

These electrophoretic results point to, but do not confirm, the mutual linkage of protein and "fucan". Supporting evidence comes from gel filtration studies. The elution profiles of Extracts

FIGURE 4.2 POLYACRYLAMIDE GEL ELECTROPHORESIS OF MACROCYSTIS PYRIFERA EXTRACTS AT PH 6.5



1,4 and 7 on Sepharose 4B and Sephadex G100 are shown in FIGURE 4.3 and 4.4. The former indicates that Extract 1 is a "fucan" of considerable polydispersity with the bulk of the material having a molecular weight greater than 10^6, though whether this is a genuine result or a reflection of molecular aggregation is not clear. Any aggregation caused by divalent ions should be suppressed by the presence of the complexing agent, EDTA, in the eluant.

Isolation of the material from Extract 1 eluting in the void volume on both columns and subsequent analysis showed that most of the protein remained associated with the "fucan" during elution.

The elution profiles of Extracts 4 and 7 are similar to those obtained for the corresponding extracts from Ascoseira, with a polydisperse "fucan" eluting prior to a low molecular weight alginate fraction. To support this assertion the run of Extract 4 on Sephadex G100 was repeated and the material eluting under the two separate peaks (shown in FIGURE 4.4) isolated by dialysis and freeze-drying. The recoveries and the approximate composition of each fraction are shown in TABLE 4.4.

FIGURE 4.3 ELUTION PROFILES OF MACROCYSTIS PYRIFERA EXTRACTS ON SEPHAROSE 4B

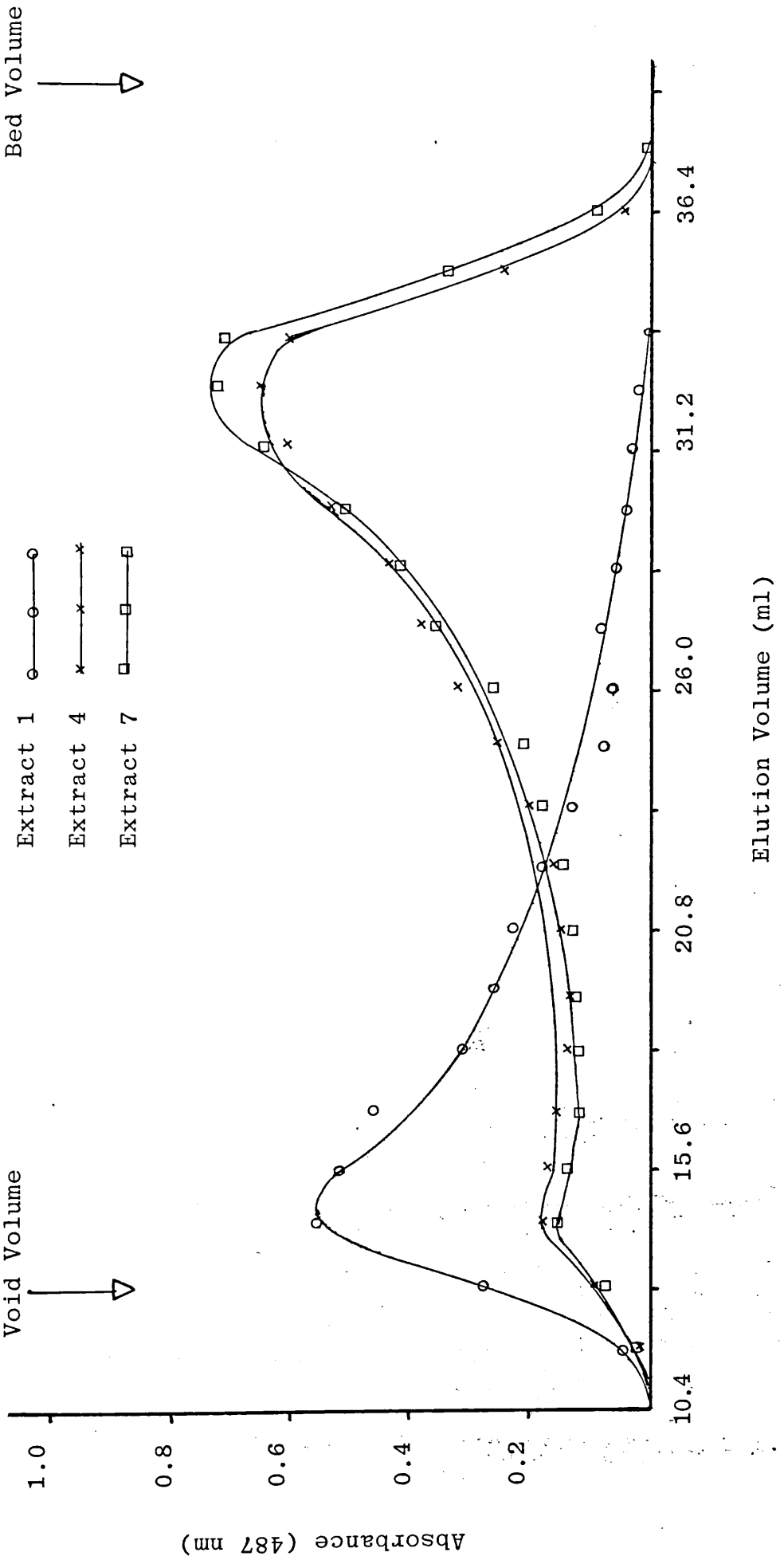


FIGURE 4.4 ELUTION PROFILES OF MACROCYSTIS PYRIFERA EXTRACTS ON SEPHADEX G100

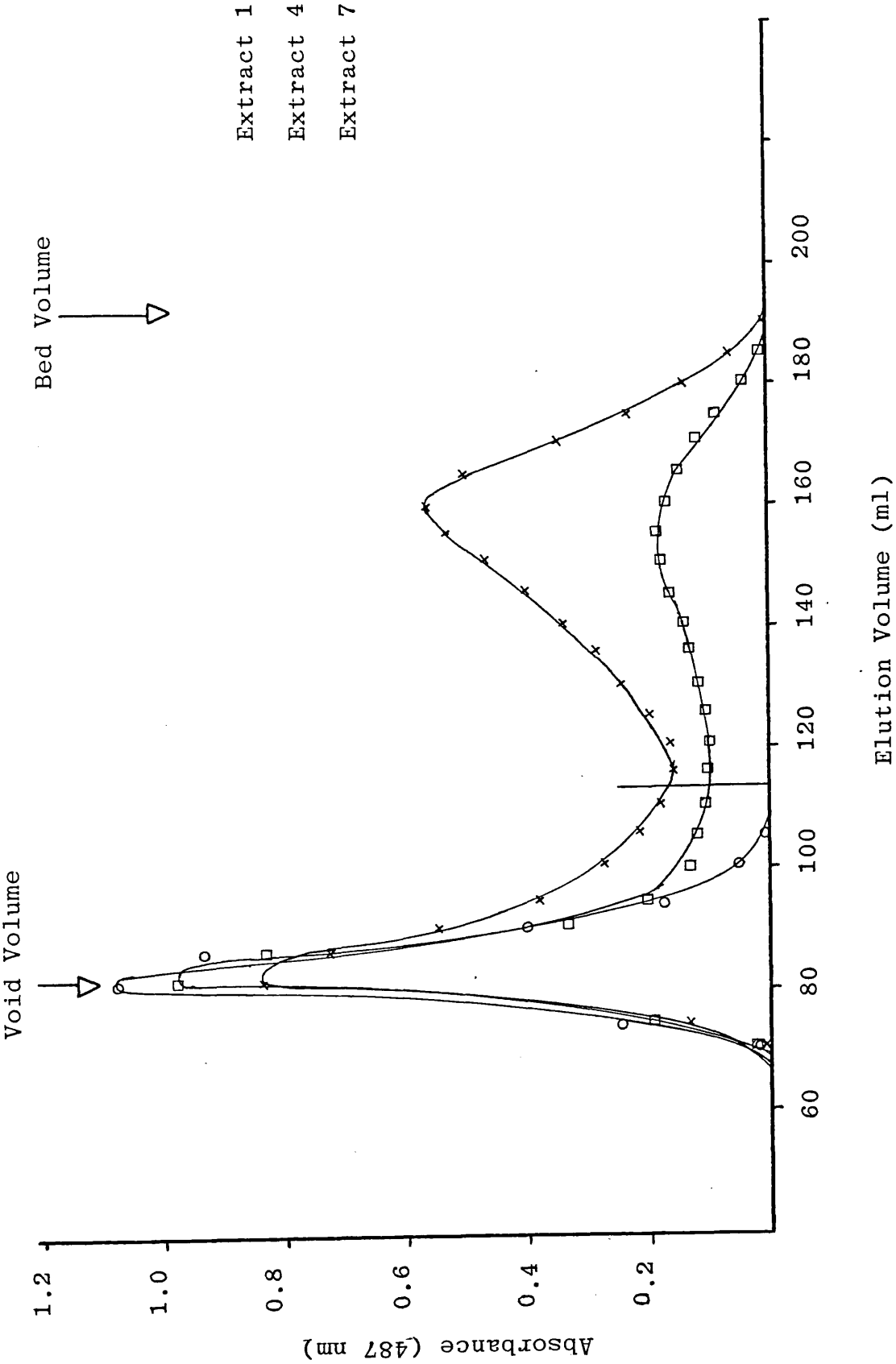


TABLE 4.4.

SUGAR COMPOSITION OF G100 COLUMN FRACTIONS OF MACROCYSTIS EXTRACT 4

FRACTION	SUGAR DISTRIBUTION[a]							
	FUC	XYL	MAN	GLU	GAL	GLUA	MANA	GULA
High Molecular Weight	30	16	3	23	19	5	2	2
Low Molecular Weight	20	5	8	10	9	3	40	5

(a) See footnote (b) to TABLE 4.3

These data are consistent with the electrophoresis results, i.e. the occurrence of a broad range of molecular weight for the "fucan", with probably most of the protein covalently linked. It therefore seems reasonable to consider the protein as part of the "fucan" as suggested in the previous section.

It is possible that the low fucose content of the "fucans" from Extract 4 and 7 is due to extensive cleavage of acid-labile fucosyl links during the acid extraction. However examination of the dialysis water during isolation of Extract 4 revealed only small amounts of carbohydrate, insufficient to alter the monosaccharide composition of the "fucans" significantly. Negligible carbohydrate was detectable in the dialysis water from Extract 7. It therefore seems reasonable to conclude that the monosaccharide compositions of the Extracts are similar to those of the native polymers.

4.1.4. Mild Acid Treatment of the "Fucan" (Expt.5.60).

In studies on "fucan" extracts from Ascophyllum nodosum and Fucus vesiculosus, Medcalf and Larsen found that some apparently electrophoretically pure fractions could be broken into two discrete components by mild acid hydrolysis (0.02M hydrochloric acid, 1 h, 80°C). The two components were respectively rich in uronic acid and protein and rich in fucose.[3]

In an attempt to achieve a similar effect 750 mg of Extract 1 was treated in the same manner. 50 mg of material was rendered insoluble and this was removed by centrifugation (Fraction C). The remainder was passed down a Sephadex G100 column, giving two bands (FIGURE 4.5) which were isolated separately. The higher molecular weight band (Fraction A) was then passed down a Sepharose 4B column, giving three overlapping bands (FIGURE 4.6) which were isolated separately. The compositions of the five fractions (A1, A2, A3, B and C) are summarised in TABLES 4.5 and 4.6.

FIGURE 4.5 ELUTION PROFILE OF SUB-FRACTIONS OF EXTRACT 1 ON SEPHADEX G100

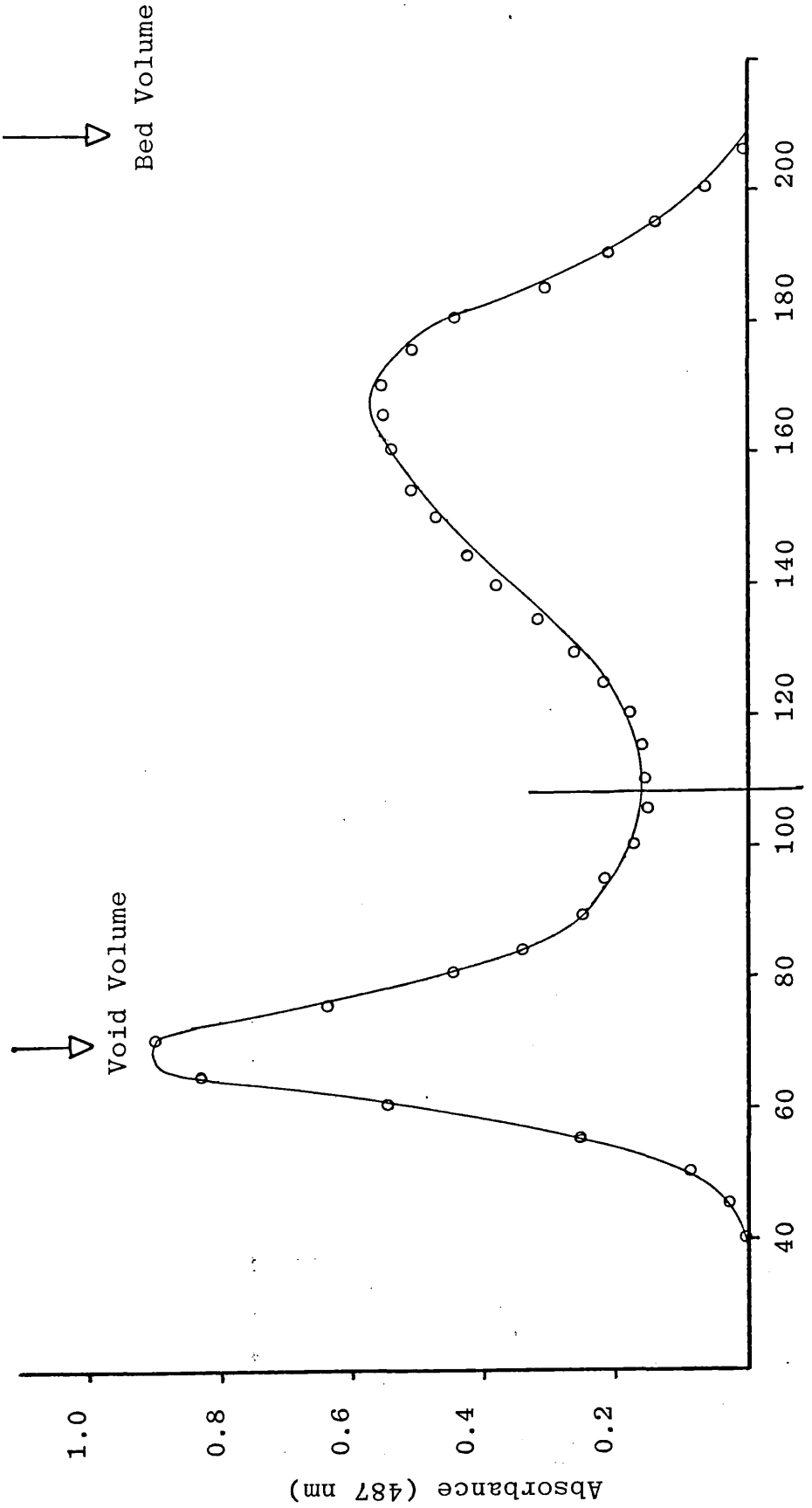


FIGURE 4.6 ELUTION PROFILE OF HIGH MOLECULAR WEIGHT SUB-FRACTION OF EXTRACT 1 ON SEPHAROSE 4B

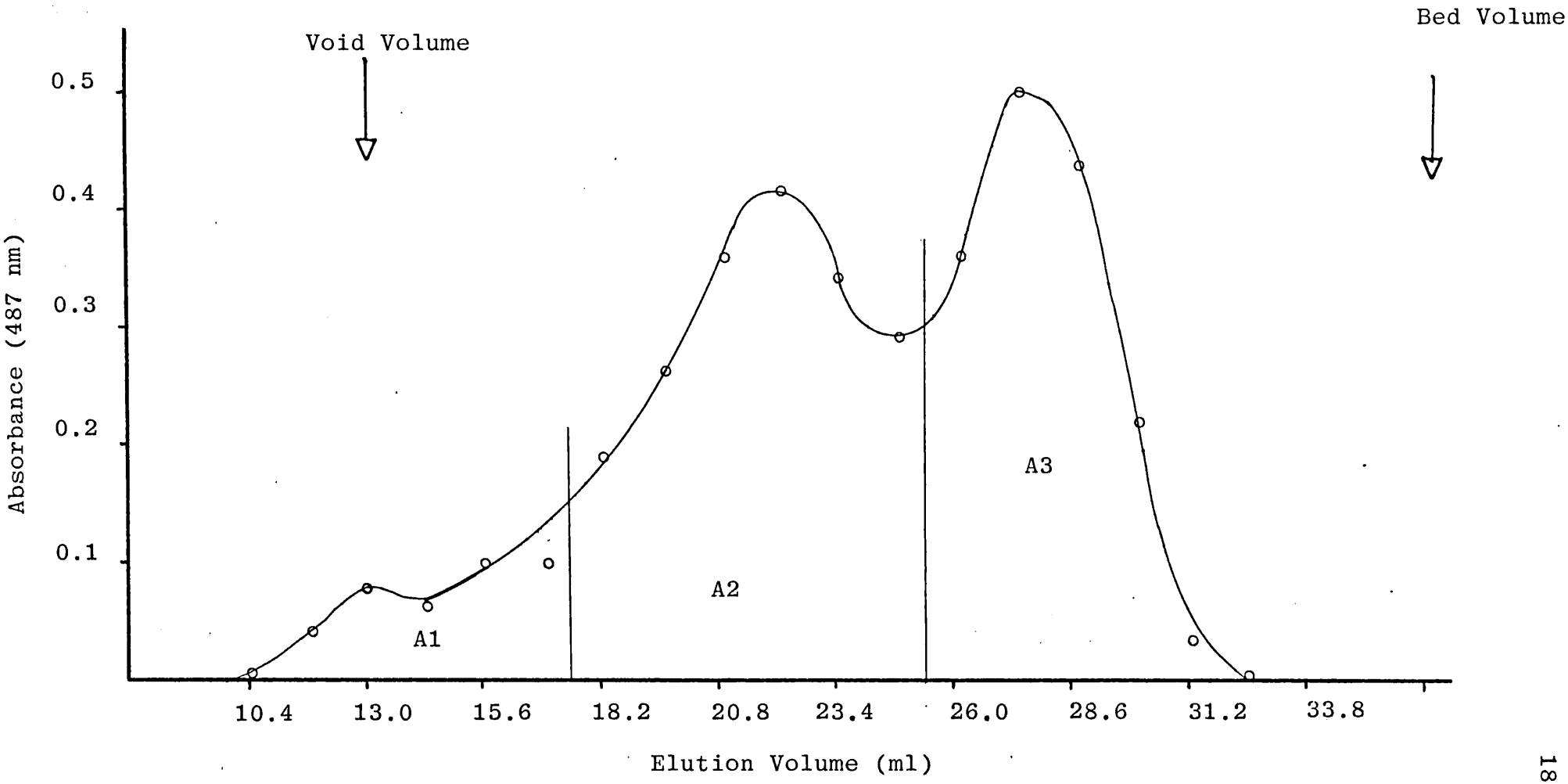


TABLE 4.5

FRACTIONS FROM MILD ACID TREATMENT OF "FUCAN"

FRACTION	YIELD	CARBOHYDRATE	URONIC ACID	PROTEIN	MOLECULAR WEIGHT (d)
	%(a)	%(b)	%(b)	%(b)(c)	
A1	6	8	10	6(6)	$>10^6$
A2	24	30	36	22(5)	5×10^5
A3	30	35	30	28(7)	8×10^4
B	30	20	16	26(8)	8×10^3
C	7	5	9	18(27)	(e)

(a) Weight as a percentage of the original.

(b) As a percentage of the amount in the "fucan".

(c) Figures in parenthesis are percentages of the weight of the fraction.

(d) Calculated from dextran standards on Sepharose 4B (A1,A2,A3) and Sephadex G100(B).

(e) Not determined.

TABLE 4.6 RELATIVE SUGAR PROPORTIONS IN "FUCAN" FRACTIONS.

FRACTION	SUGAR DISTRIBUTION(a)					
	FUC	XYL	MAN	GLU	GAL	GLUA
A1	77	3	3	3.5	4.5	6
A2	73	3	3	3	8	6
A3	74	3	3	3	7	5
B	75	3	10	3.5	2	4
C	43	7	22	6	13	9

(a) Neutral sugars determined by g.l.c., uronic acid by m-hydroxydiphenyl method; total set to 100%.

The electrophoretic behaviour on cellulose acetate of Fractions A1, A2, A3 and B (FIGURE 4.7) is not surprising in view of the similar proportions of fucose and suggests a similar degree of sulphation in all of them.

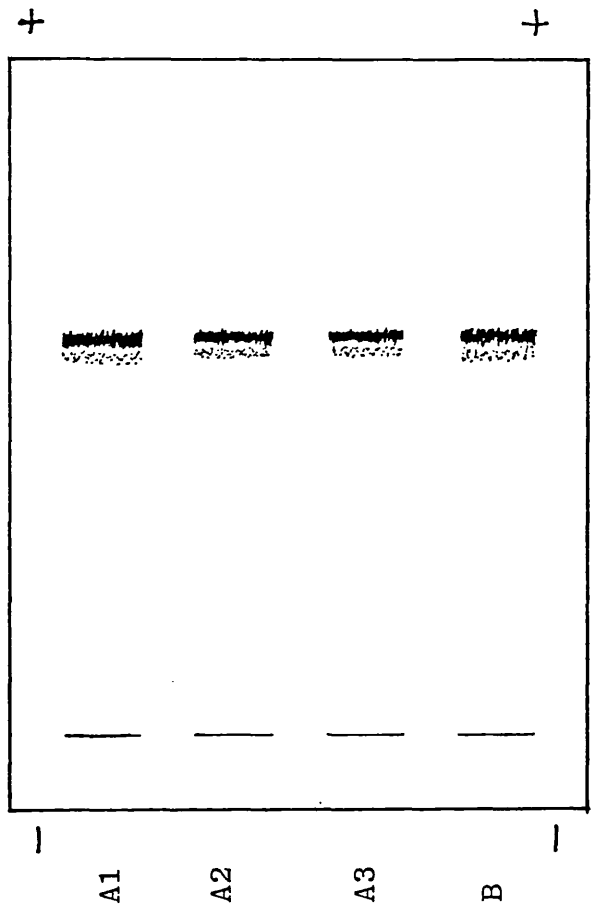
These results point to a "fucan" macromolecular structure built up of several subunits of varying size and of slightly differing compositions; those of higher molecular weight apparently rich in galactose and deficient in mannose.

The low fucose content of the protein-enriched fraction C suggests that the protein is not linked directly to the fucose-containing chains.

With a molecular weight of about 8000, Fraction B has an average degree of polymerisation of rather less than 50, which tends to preclude the possibility of the sugars other than fucose occurring in homopolymeric sequences. For the more massive fractions such a conclusion cannot be drawn. The absence of any fractionation on the electrophoretograms into fucose- and uronate-rich fractions appears to rule out the occurrence of a pure fucan sulphate in any of the fractions.

The exact nature of the linkages between the various sub-units in the "fucan" macromolecule is not clear. The acid-lability argues against a glycosidic linkage, particularly since acid hydrolysis

FIGURE 4.7 ELECTROPHORETIC PATTERNS OF SUB-FRACTIONS OF EXTRACT 1 ON CELLULOSE ACETATE AT PH 5.1



would be expected to release some free sugar from the ends of chains; no free sugar was detectable in the dialysis water during recovery of the acid treated "fucan". The possibility of protein bridges between components was considered by Medcalf and Larsen[3] but ruled out after alkaline degradation removed some protein from one fraction but failed to break it into two components, leading them to conclude that the protein is located at the periphery of the "fucan" fraction.

The overall similarities of the various fractions suggest that attempts to study the small amounts of each fraction separately would have no advantage over experiments on a larger amount of the intact "fucan".

4.1.5 Alkaline Degradation of the "Fucan" (Expt.5.61)

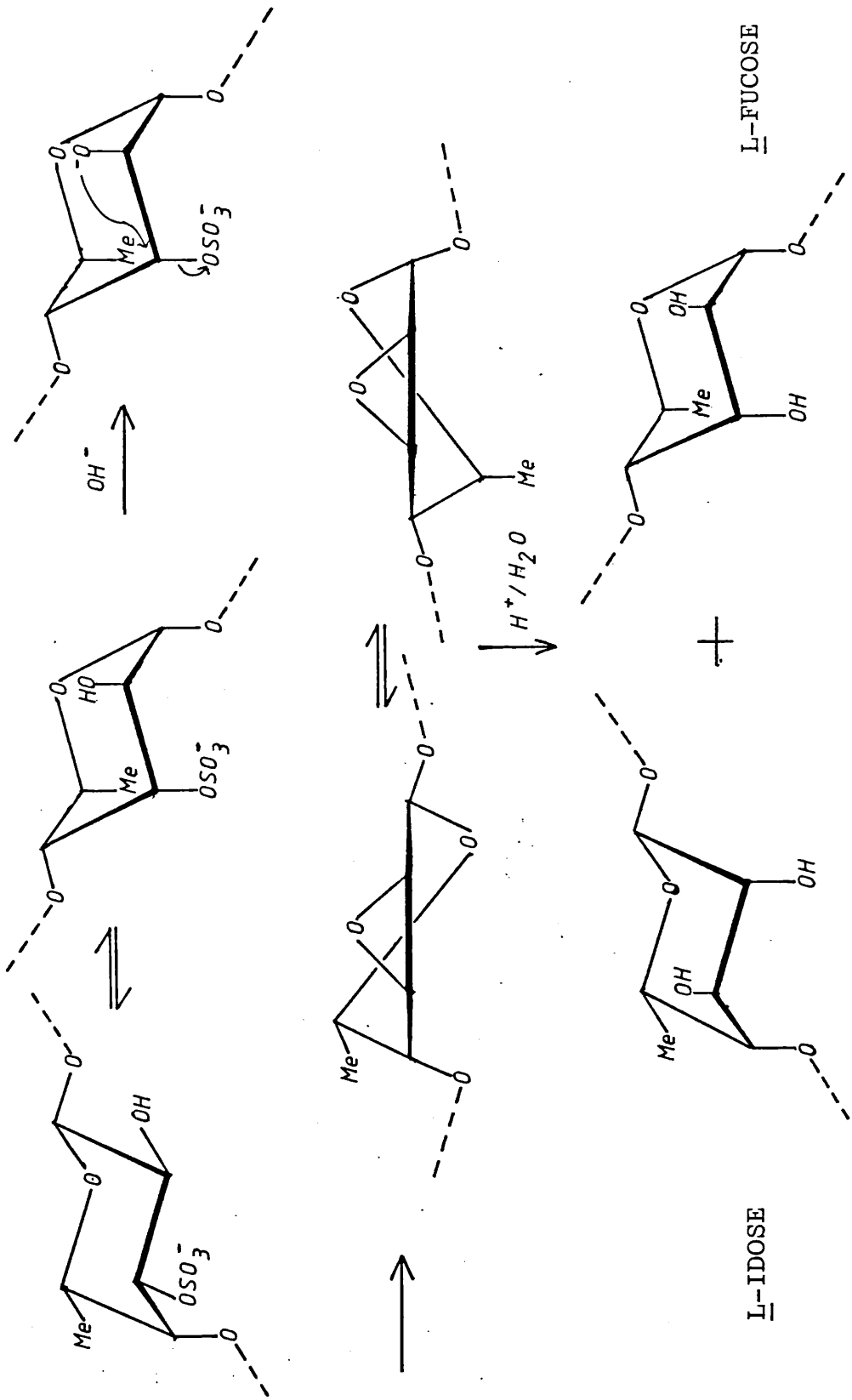
Treatment of polysaccharides with alkali is a common method of structural analysis. Two effects may be identified for "fucans".

(a) Desulphation of sulphated residues.

(b) β-elimination of uronic acids.

(a) Half-ester sulphate groups may be displaced by intramolecular nucleophilic substitution. This occurs only when the carbon adjacent to the ester-substituted carbon carries a hydroxyl group in trans-disposition to the sulphate ester (FIGURE 4.8).[4] The trans- hydroxyl group may be unsubstituted, sulphated,

FIGURE 4.8 ALKALINE DESULPHATION OF FUCOPYRANOSIDE-3-SULPHATE

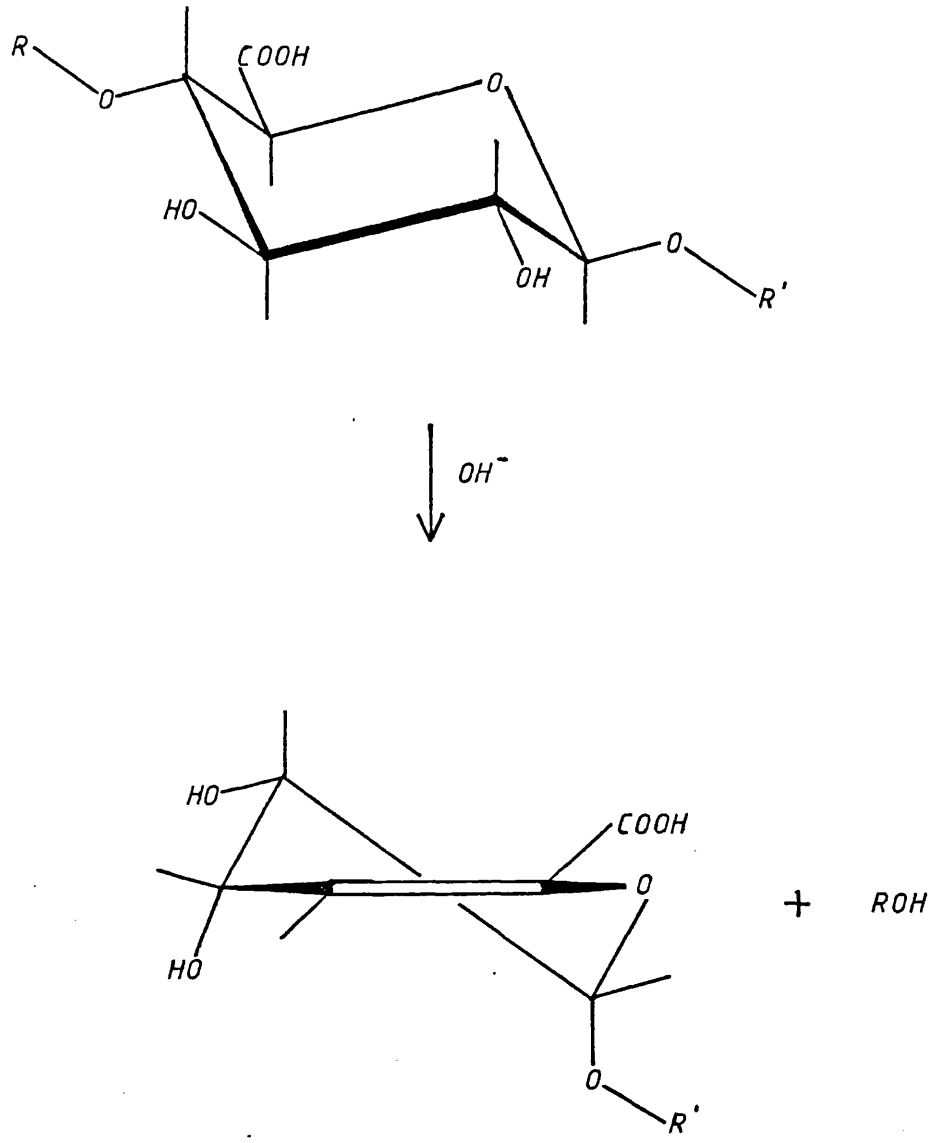


acetylated or benzoylated. After displacement an epoxide ring is formed, with inversion of configuration at the formerly substituted carbon; subsequent hydrolysis of the epoxide ring may therefore lead to formation of a new monosaccharide residue - as illustrated in FIGURE 4.8 for a typical "fucan" residue, fucose-3-sulphate. Hence the distribution and position of sulphate groups can be determined from the loss of sulphate and the possible formation of new sugars.

(b) Uronate residues substituted at C-4 commonly undergo β-elimination under alkaline conditions, cleaving the polymer chain and generating 4,5-unsaturated end-group residues (FIGURE 4.9).[5] The size and composition of the consequent polymer fragments gives information on the position of uronic acid residues.

After addition of $NaBH_4$ to render reducing-ends immune to alkaline "peeling",[6] Extract 1 (170 mg) was treated with alkali and the polymeric and oligomeric products were separated by dialysis. 4,5-unsaturated acid was confirmed in both fractions, indicating elimination of (1→ 4)-linked glucuronic acid. About 12% of the carbohydrate passed through the dialysis membrane and of this 14% was undegraded glucuronic acid, the remainder being roughly similar quantities of fucose, xylose, mannose, glucose and galactose.

FIGURE 4.9 β-ELIMINATION OF (1 → 4)-LINKED URONIC ACID



The recovered polymer (70% yield) had a higher fucose proportion (84%) than the original "fucan" (75%) and a lower proportion of sulphate (41% compared to 64% relative to carbohydrate). No new sugar was observed on paper chromatograms or g.l.c., though one may be masked by the fucose. Gel filtration (FIGURE 4.10) suggested a molecular weight of about 10^6.

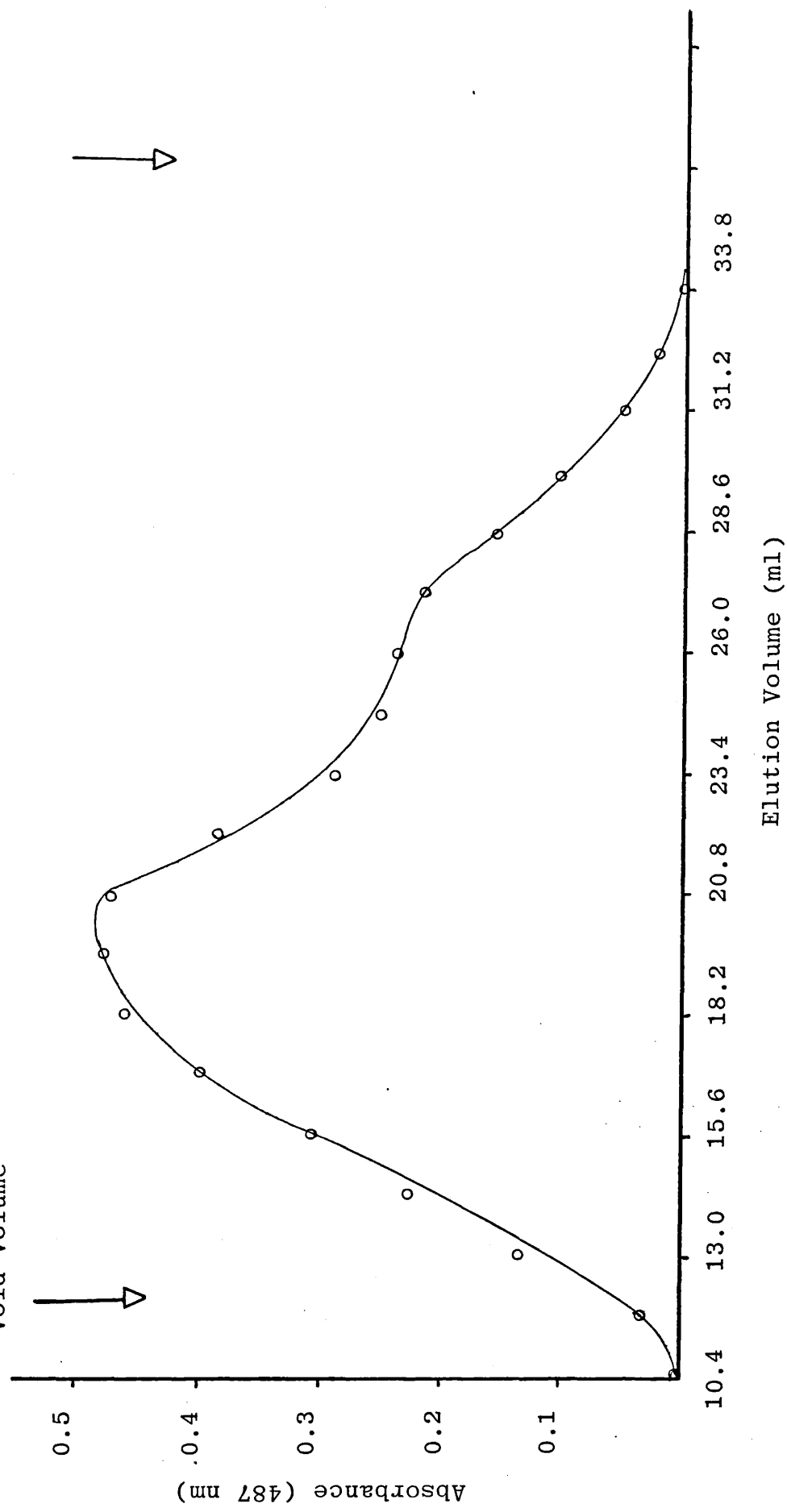
These results point to the fucose and uronic acid being located in different parts of the macromolecule, with the other monosaccharides being mostly closely associated with the latter. About one third of the sulphate appears to be linked to either C-2 or C-3 of fucose.

4.2. Direct Alkaline Extraction of Macrocystis pyrifera (Expts. 5.62, 5.63).

As with Ascoseira, a sample of the dried, ground alga (30 g) was extracted under mild alkaline conditions (FLOW CHART 4.2). The resultant extracts were extremely viscous, immediately indicating an alginate of high molecular weight.

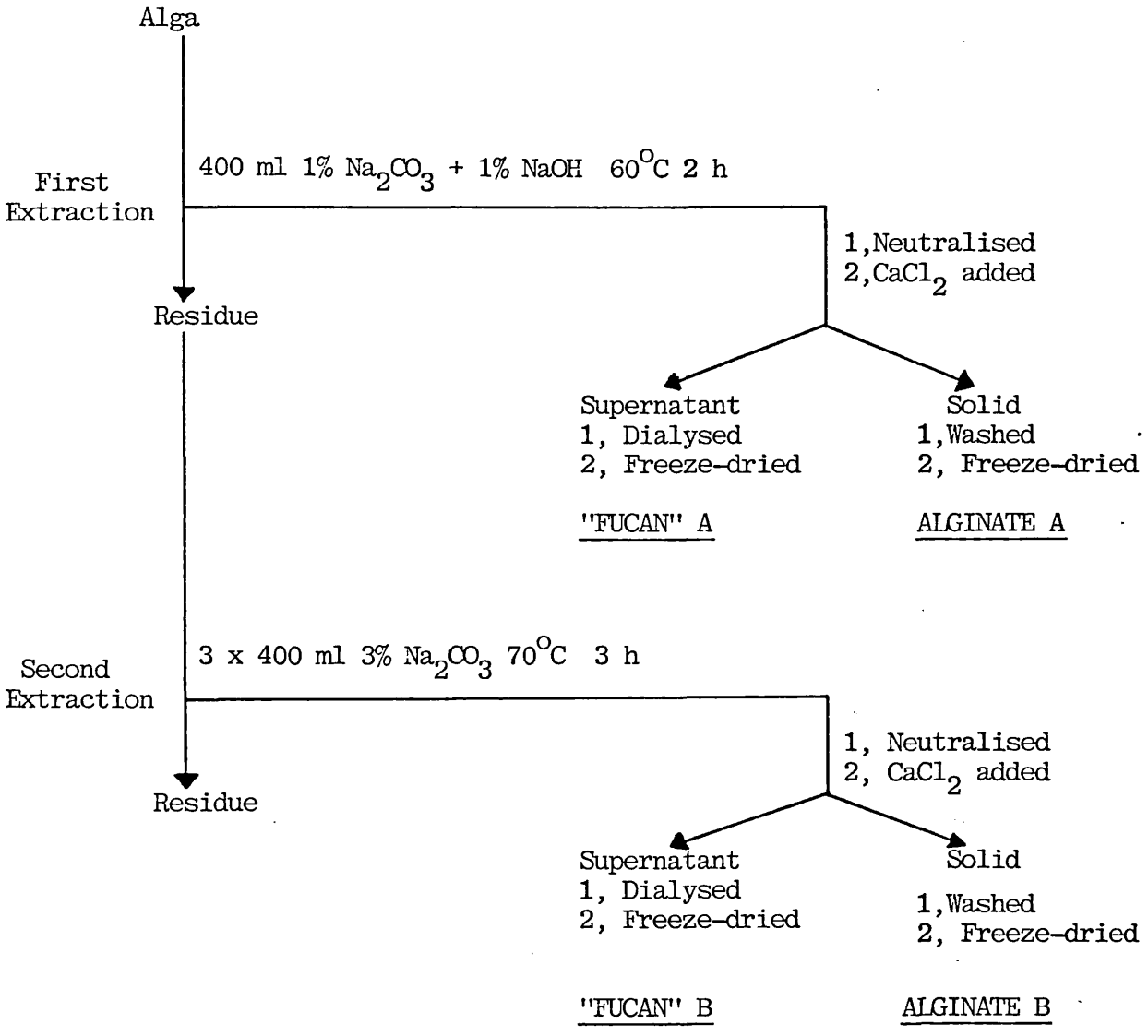
The recoveries of both "fucan" and alginic acid were both higher than for the sequential extraction. However the carbohydrate contents of the "fucans" were lower than previously, suggesting rather impure extracts. Similarly, on conversion

FIGURE 4.10 ELUTION PROFILE OF ALKALI-DEGRADED "FUCAN" ON SEPHAROSE 4B



FLOW CHART 4.2

DIRECT ALKALINE EXTRACTION OF MACROCYSTIS PYRIFERA



of Alginate A to its sodium salt. some colouring matter was solubilised during acidification (TABLE 4.7).

TABLE 4.7

ALKALINE EXTRACTS FROM MACROCYSTIS PYRIFERA

EXTRACT	CALCIUM FORM WEIGHT g.	%(a)	SODIUM FORM WEIGHT g.	%(a)
Alginate A	6.80	22.7	5.90	19.7
Alginate B	6.32	21.0	5.42	18.1
TOTAL	13.12	43.7	11.32	37.8
"Fucan" A	1.24	4.1	-	-
"Fucan" B	2.40	8.0	-	-
TOTAL	3.64	12.1		

(a) Percentage of the dry weight.

The viscosity of the resultant sodium alginate was measured as 177 relative to water for a 1% solution at 25°C. The high viscosity is not unexpected in view of the fact that it is typical for alginate from Macrocystis pyrifera harvested commercially. Since the alga was harvested, stored and subsequently extracted under the same conditions as the Ascoseira, the occurrence of an essentially undegraded alginate lends support to the argument in Section 2.3 that the alginate in Ascoseira was of naturally low molecular weight and had not been degraded prior to extraction.

4.2.1. N.m.r. of Sodium Alginate (Expts.5.63, 5.64)

At the concentration required for n.m.r. spectroscopy (ca. 50mg/ml) the sodium alginate solution was too viscous to give well-resolved spectra. The alginate was therefore partially depolymerised under the conditions used by Grasdalen et al. (dilute hydrochloric acid, pH 3.0, 100°C for 30 min).[7] They found by reducing power that the resultant alginate had a D.P. of 20-30, but here gel filtration on Sephadex G100 (FIGURE 4.11) suggested an average molecular weight of about 5×10^5, though this may be an overestimate for the reasons suggested earlier (Section 2.3). Nevertheless acceptable spectra, with partial resolution of diads in the ^{13}C spectrum, were obtained at 400 MHz (^1H) and 100 MHz (^{13}C) (FIGURES 4.12, 4.13, TABLE 4.8.) The spectrum closely resembles that of an alginate recorded by Grasdalen et al. and was assigned by comparison. The diad frequencies and number-average block lengths were calculated, in the same manner as for the Ascoseira alginate, from the resonance integrals of the signals from the anomeric carbons in the ^{13}C spectrum and the signals from the anomeric and C-5 protons in the ^1H spectrum (TABLE 4.9). The agreement is not too close, probably reflecting the incomplete resolution due to signal broadening in the ^{13}C spectrum. The high proportion of alternating sequences accounts for the low number-average block lengths.

FIGURE 4.11 ELUTION PROFILE PARTIALLY-DEGRADED MACROCYSTIS PYRIFERA ALGINATE A ON SEPHAROSE 4B

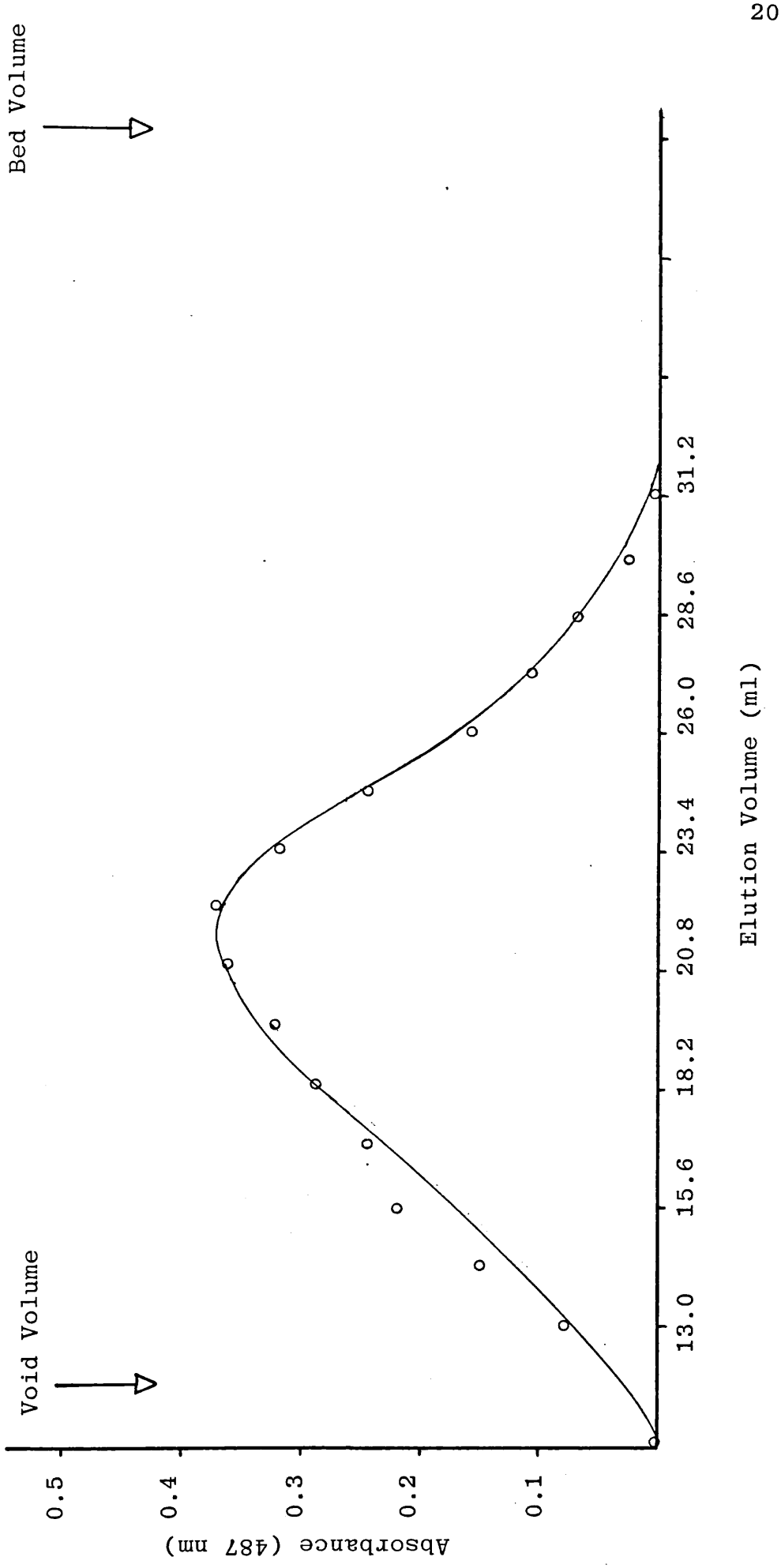


FIGURE 4.12 ^{13}C N.M.R. SPECTRUM OF MACROCYSTIS PYRIFERA ALGINATE A

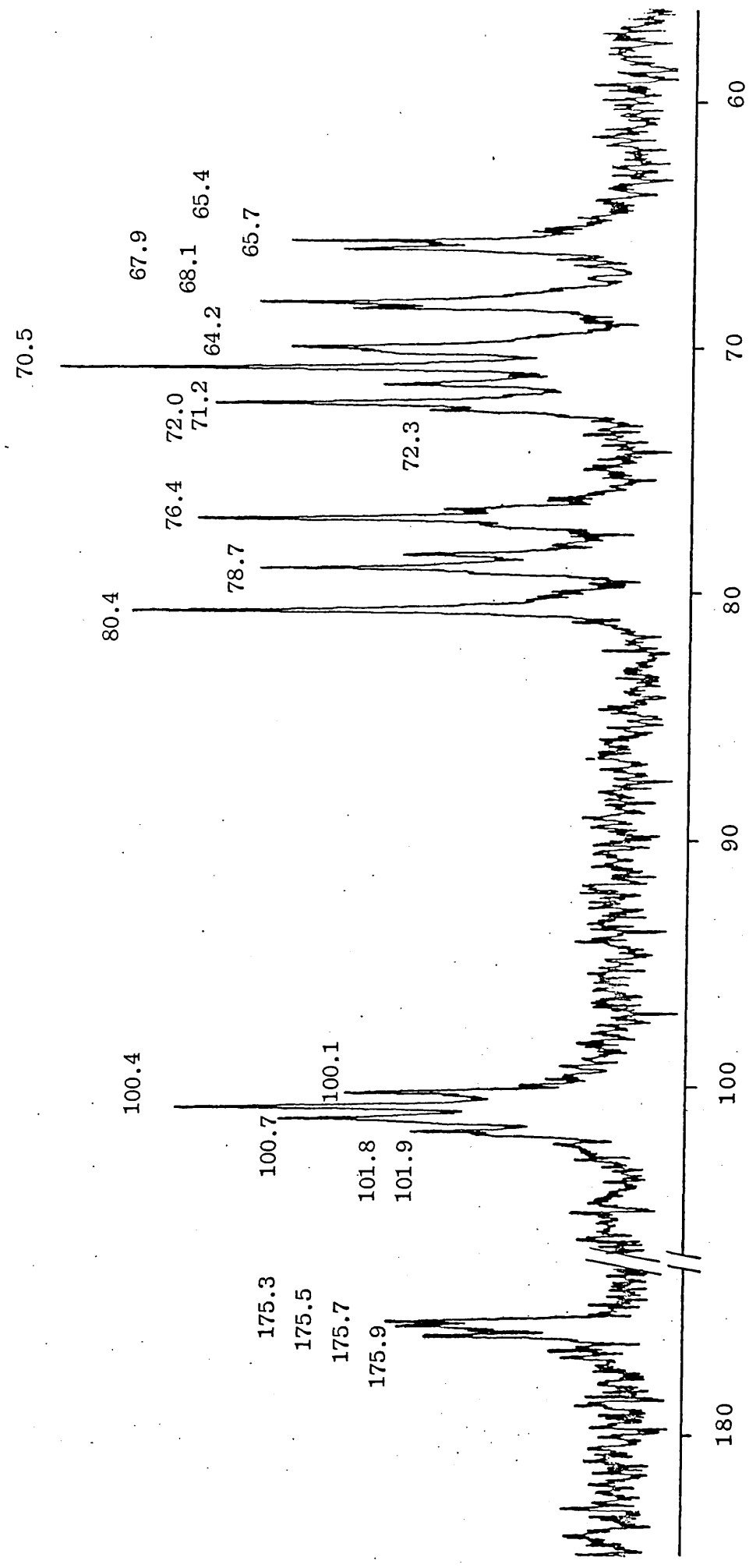


FIGURE 4.13 ^{1}H N.M.R. SPECTRUM OF MACROCYSTIS PYRIFERA ALGINATE A

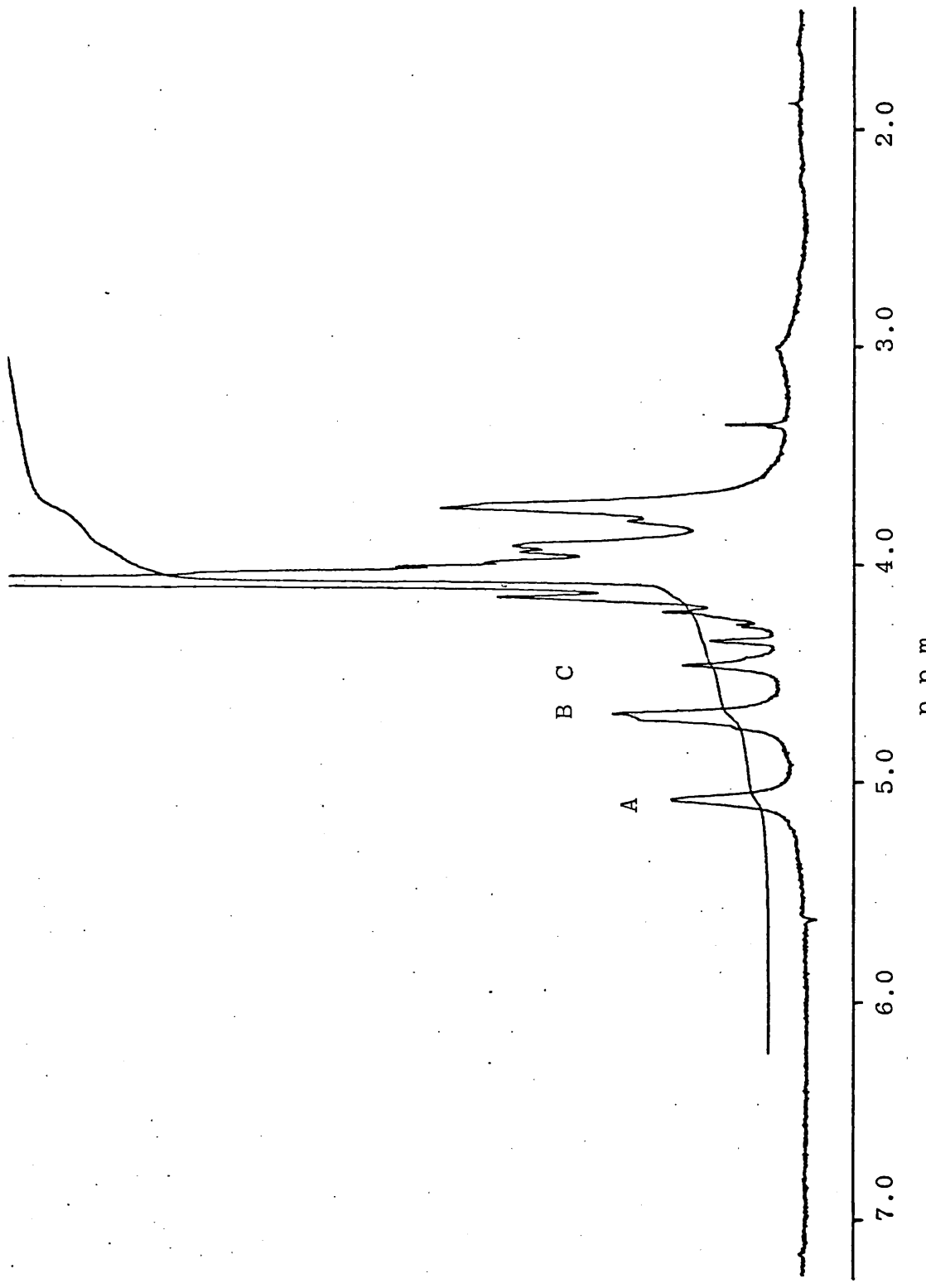


TABLE 4.8

ASSIGNMENT OF PEAKS IN ^{13}C SPECTRUM OF MACROCYSTIS ALGINATE A[a]

Triad Sequence	Carbon atom in central residue of triad					
	C-1	C-2	C-3	C-4	C-5	C-6
MMM	100.7	70.5	72.0	79.9	76.7	175.3
MMG	101.8	(71.2)[b]	(c)	79.9	(76.3)	(c)
GMM	100.7	(70.9)	(c)	78.7	(76.9)	(c)
GMG	101.7	71.2	72.3	78.7	76.4	175.9
GGG	101.9	65.7	69.2	80.4	67.9	175.5
GGM	100.4	65.4	(c)	80.4	68.1	(c)
MGG	101.9	65.7	(c)	80.4	67.9	(c)
MGM	100.1	65.4	69.8	80.4	68.1	175.7

(a) Shifts in ppm., downfield from external TMS at room temperature.

(b) Values in parenthesis denote tentative assignments.

(c) No assignment made.

TABLE 4.9

BLOCK DISTRIBUTION AND LENGTHS IN MACROCYSTIS ALGINATE A

Method			Block distribution			Block Length	
	F_M	F_G	F_{MM}	F_{GG}	$F_{GM}+F_{MG}$	N_M	N_G
Partial Hydrolysis	0.50	0.50	0.23	0.30	0.47	15	
^{13}C n.m.r.	0.49	0.51	0.31	0.34	0.34	2.9	3.0
^{1}H n.m.r.	0.49	0.51	0.25	0.27	0.48	2.1	2.0
(Australian Alginate[8])	0.58	0.42	0.41	0.17	0.42	(a)	(a)

(a) Not determined.

The partial hydrolysis and fractionation into acid-soluble and acid-insoluble fractions gave a block distribution in good agreement with that determined from the ^{1}H spectrum. (TABLE 4.9). The ^{1}H spectrum of the acid-soluble fraction (FIGURE 4.14) is closely similar to that of the corresponding fraction in the Ascoseira alginate (FIGURE 2.23, TABLE 2.21) and hence it is reasonable to conclude that this fraction includes fragments from MM blocks. However since the spectrum indicates a substantial proportion of monomeric and dimeric species it is impossible to quantify the proportion derived from MM blocks and hence no allowance has been made in the calculation of block distribution. For the acid-insoluble fraction (FIGURE 4.15) calculation indicated that about 20% of the material was MG blocks which had not been solubilised, a reflection perhaps of the initial high molecular weight of the intact alginate. The presence of MG blocks interferes with the calculation of the average homopolymeric block length (i.e. the average for MM and GG blocks) from the ratio of the major anomeric signals to the reducing end anomeric signals, but sections of over 15 units were suggested. Gel filtration (FIGURE 4.16) gave no apparent separation of MM and GG blocks and the number average chain length was about 45 units.

FIGURE 4.14 ^{1}H N.M.R. SPECTRUM OF ACID-SOLUBLE FRACTION OF MACROCYSTIS PYRIFERA ALGINATE A

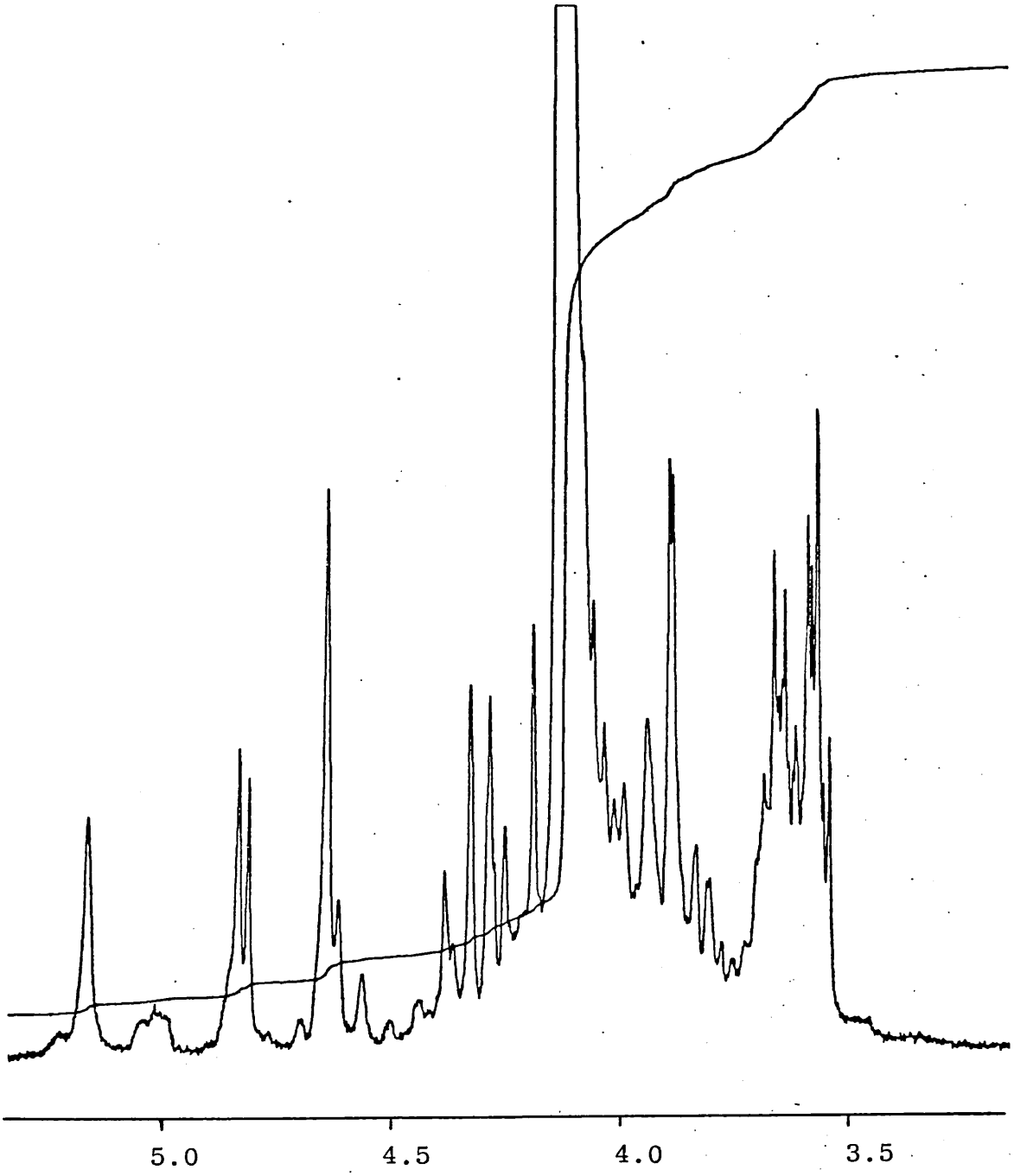


FIGURE 4.15 ^{1}H N.M.R. SPECTRUM OF ACID-INSOLUBLE FRACTION OF MACROCYSTIS PYRIFERA ALGINATE A

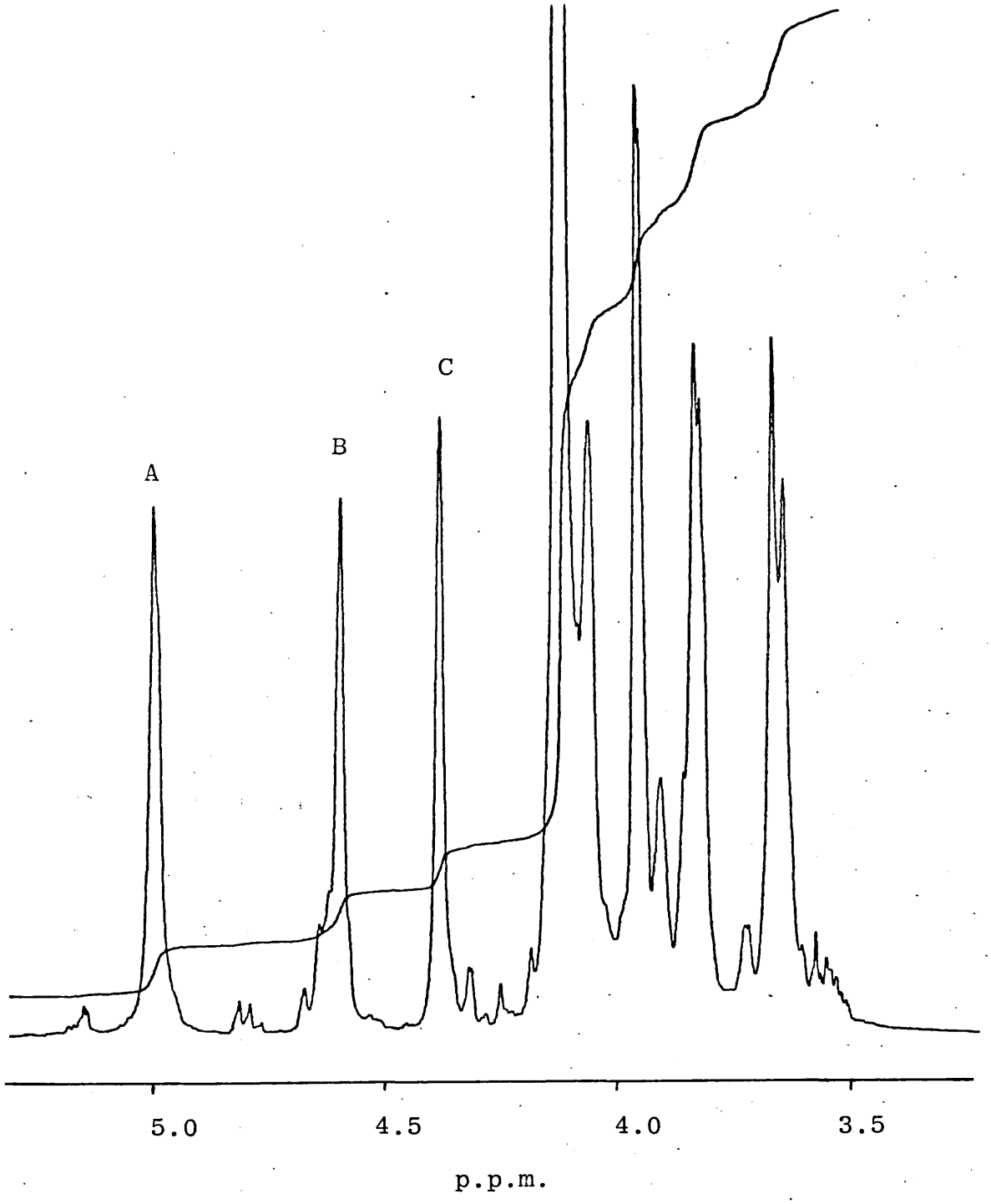
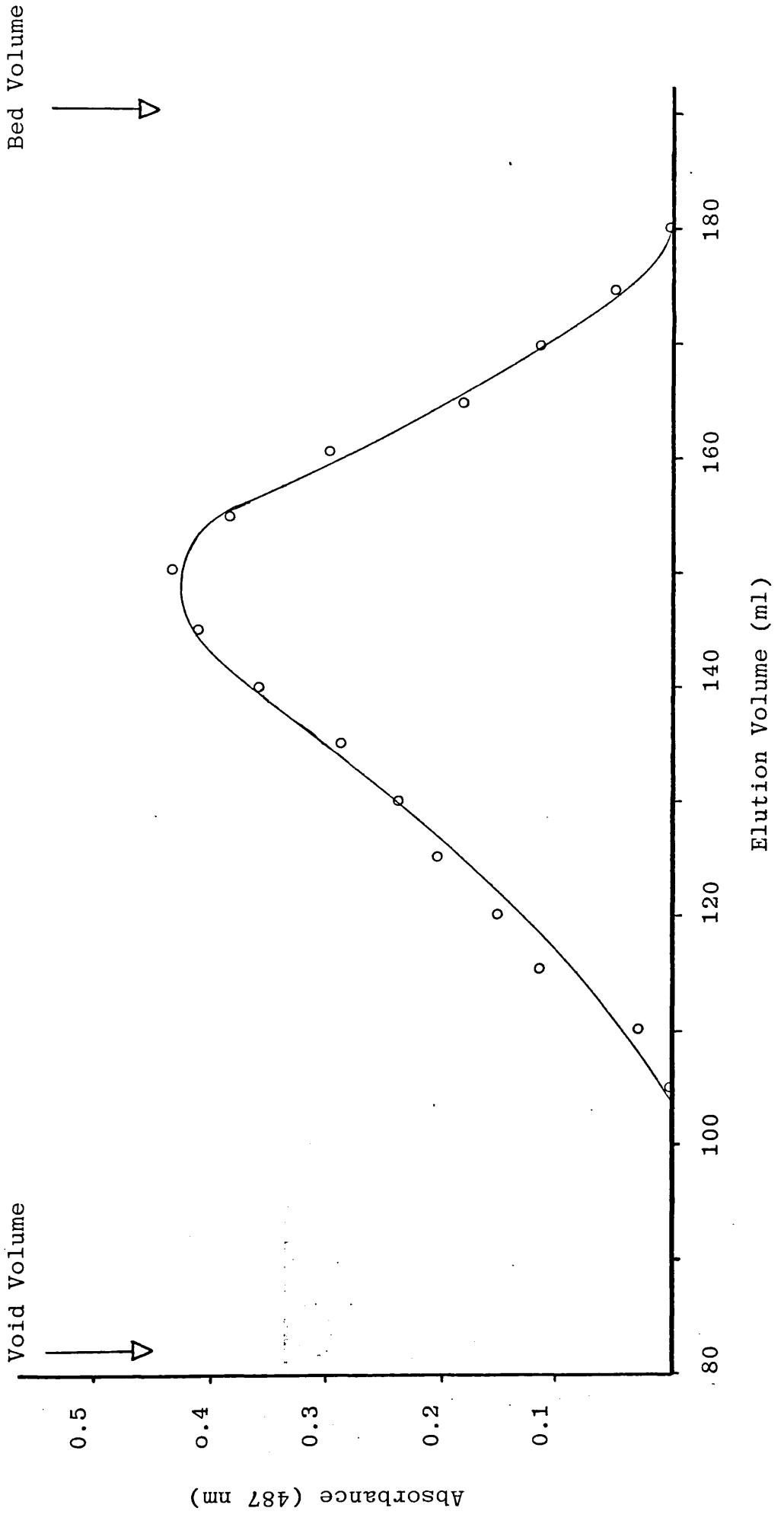


FIGURE 4.16 ELUTION PROFILE OF ACID-INSOLUBLE FRACTION OF MACROCYSTIS ALGINATE A ON SEPHADEX G100



Penman and Sanderson[8] calculated the block distribution for alginate from M.pyrifera from a temperate zone (Australia). In comparison (TABLE 4.9) the major difference is a lower proportion of GG blocks in the Australian sample. The latter was a commercial sample and presumably derived from commercially harvested weed, which would be devoid of the lower stems and the holdfast. These stiffer sections of the weed are rich in GG blocks and their absence might account for the lower proportion of GG blocks in the harvested bulk of the weed. This inference can only be tentative since other factors, particularly the age of the plants, may be significant. Any correlation of block distribution with the prevailing water temperature must be similarly tentative.

4.3. Large Scale Extraction of Macrocystis pyrifera (Expt.5.65)

The bulk of the "fucan" in the alga is easily extracted in a virtually undegraded form, is free of alginate and is contaminated with only a small amount of laminaran. These facts make it particularly suited for detailed structural studies.

A large scale extraction was carried out on 4 Kg of the alga (from which the exudate had been stripped). Grinding under liquid nitrogen on a large scale, particularly of the stems, proved difficult and time-consuming, so the alga was

macerated in a blender before soaking overnight in 40% formaldehyde. After removal of the formaldehyde in a stream of dry air the alga was extracted eight times with 4 l portions of 2% $CaCl_2$ solution and the extracts combined and concentrated under reduced pressure. The resultant viscous liquor (10 l) was dialysed for 5 days with frequent changes of water and finally isolated by freeze-drying, yielding 28 g of buff-coloured material (Extract 5).

Analysis by the techniques used previously gave the composition shown in TABLE 4.10; the data for the previous Cold Water Extract (Extract 1) are shown for comparison.

TABLE 4.10

COMPOSITION OF LARGE SCALE EXTRACT FROM M.PYRIFERA

EXTRACT	CARBOHYDRATE	SULPHATE	PROTEIN	SUGAR DISTRIBUTION					
	%(a)	%(b)	%(c)	FUC	XYL	MAN	GLU	GAL	GLUA
Extract 5	28	17(63)	7	72	2	6	5	7	8
Extract 1	48	31(64)	10	75	3	6	2	5	8
Pure Fucan (Extract 5A)	56	34(60)	15	73	2	6	3	8	8

(a) Fucose standard graph.

(b) Percentage of total; figures in parentheses are percentage of carbohydrate.

(c) Percentage of total.

(d) Neutral sugars by g.l.c., uronic acid by m-hydroxydiphenyl method; total set to 100%

The low figures for carbohydrate, sulphate and protein indicate an impure extract; the material was darker than previously, suggesting that the formaldehyde treatment has been less effective in preventing extraction of pigment.

Electrophoresis experiments gave the same results as for Extract 1. Gel filtration on Sephadex G100 effected a separation of polysaccaride and colouring matter but was not practicable as a purification technique for the whole extract.

4.3.1. DE52 Cellulose Column Fractionation (Expts. 5.65-5.67).

1 g of the Extract was fractionated on a DEAE-cellulose column. Elution with water recovered 15 mg of neutral polymer along with a trace of free mannitol (10 mg) which had obviously not been completely removed by dialysis. The neutral polymer (5N) gave only glucose on hydrolysis, but was insufficient for structural study. On the assumption that this is laminaran it represents, by comparison with the previous extraction, only 0.05% of the dry weight of the alga. Even assuming that all the glucose detected in the Acid and Alkali Extracts derives from laminaran, the total laminaran content is still less than 0.3% of the dry weight. This low quantity is in agreement with the common observation that laminaran is at a low level in early spring,

the alga having been harvested in late October.[1]

The electrophoretic behaviour suggested that there would be no fractionation of the acidic material with a salt gradient so the column was eluted with M KCl; 400 mg of polysaccharide were recovered (Extract 5A). This was only 40% of the original extract but analysis showed it to contain respectively 80% and 84% of the original carbohydrate and protein. A substantial amount of colouring remained adsorbed to the column. The column obviously provides a good method of purifying the extract but it is not practicable for use with over 20 g of extract. The composition of the purified fucan is shown in TABLE 4.10 .

4.3.2. Periodate Oxidation of the Purified "Fucan" (Expt.5.68).

The "fucan" recovered from the DEAE-cellulose column (Fraction 5A) was subjected to periodate oxidation. After 80 h consumption of periodate had ceased at 0.24 moles per anhydro unit. 84% of the carbohydrate remained uncleaved and of this one fifth passed through the dialysis membrane during isolation of the polyalcohol; these fragments were predominantly free fucose, with traces of galactose, glucose, mannose and glucuronic acid. The polyalcohol was recovered in 71% yield. Analysis of the composition allowed calculation of the approximate

proportion of each monosaccharide which remained uncleaved. Given the inherent difficulty in accurate determination of the monosaccharide composition, particularly where one sugar dominates, these results (TABLE 4.11) can only be taken as approximate. However it is apparent that virtually all the fucose residues are uncleaved as would be expected since each residue probably carries at least one sulphate ester group. The remaining sugars would all appear to have a variety of linkages.

TABLE 4.11

COMPOSITION OF PERIODATE OXIDISED POLYALCOHOL AND FRAGMENTS FROM THE PURIFIED "FUCAN"

FRACTION	CARBOHYDRATE[a]	SUGAR DISTRIBUTION[a][b]					
		FUC	XYL	MAN	GLU	GAL	GLUA
Fucan	159	116	3	9.5	4.8	12.7	12.7
Polyalcohol	100(63)	84(72)	1(33)	3(32)	2(42)	4(31)	6(47)
Fragments	30(19)	23(20)	1(33)	2(21)	1(21)	2(16)	1(8)
(Total uncleaved)	(82)	(92)	(66)	(53)	(63)	(47)	(55)

(a) Weight in mg; figures in parentheses denote percentage of original "fucan".

(b) See footnote (d) to Table 4.10.

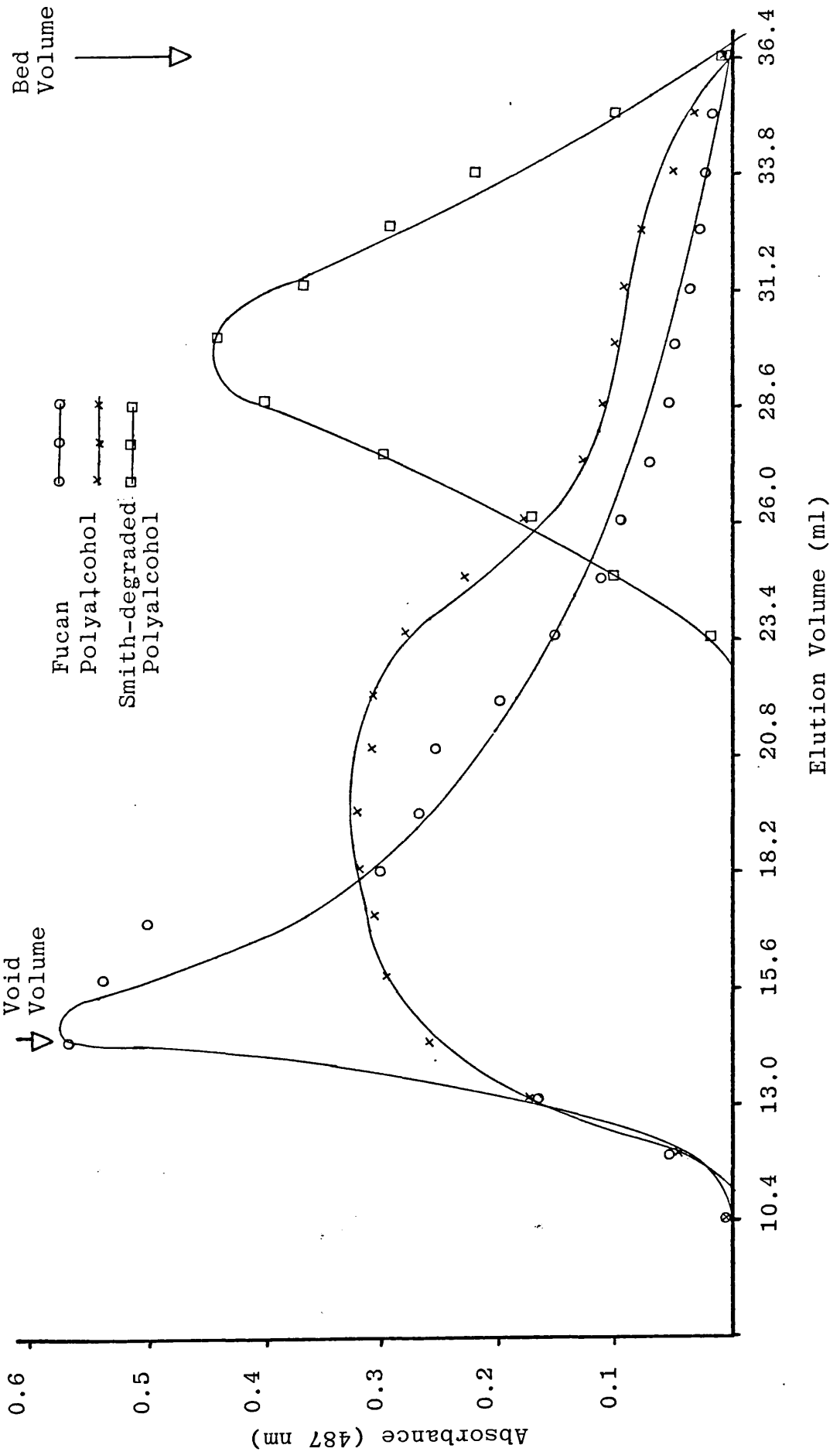
Unequivocal identification of small fragments derived from sugar cleavage was difficult; the acid hydrolysis itself produced small fragments by

degradation of sugars. However glycerol and erythritol were identified, the latter being indicative of (1→ 4)-linked mannose or glucose.

Smith degradation of the polyalcohol should give information on the position of the cleaved units within the macromolecule by selective hydrolysis of the derived acetal linkages. Treatment of the polyalcohol with 0.5M trifluoroacetic acid at 20°C produced appreciable degradation; after precipitation of the polymeric residue with 10 volumes of ethanol, one third of the carbohydrate remained in solution. Paper chromatography showed no reducing sugars (ruling out glycosidic hydrolysis) but did show mixture of oligomers from which no discrete species could be isolated. Hydrolysis of both the mixture of oligomers and the polymeric material revealed monosaccharide distributions similar to that of the parent polyalcohol.

The gel filtration behaviour of the "fucan", the oxidised polyalcohol and the degraded polymer (FIGURE 4.17) showed a substantial reduction in size of the polymer during the Smith degradation suggesting that the cleavable sugars are located in central positions in the macromolecule.

FIGURE 4.17 ELUTION PROFILE OF PERIODATE-OXIDISED "FUCAN" ON SEPHAROSE 4B



4.4. Partial Acid Hydrolysis of the "Fucan" (Expts.5.69-5.75).

To try and obtain a better idea of the fine structural details in the "fucan" a partial acid hydolysis was carried out on 20 g of the unpurified material. Three treatments with 0.04M oxalic acid at 100°C for 20 minutes were made; in each case roughly 20% of the carbohydrate was broken down sufficiently to pass through a dialysis membrane. The dialysis waters were combined and concentrated after neutralisation of the acid (Fraction A). The dialysis sac contained some material precipitated during the hydrolysis; this was recovered by centrifugation and freeze-drying (Fraction B). The supernatant was freeze-dried (Fraction C). The composition and the monosaccharide distribution of each was determined (TABLES 4.12, 4.13). In contrast to the results for extracts from Ascoseira, the choice of acid hydrolysis conditions did not significantly alter the observed monosaccharide distribution.

TABLE 4.12 COMPOSITION OF PARTIAL HYDROLYSIS FRACTIONS

FRACTION	RECOVERY		CARBOHYDRATE		SULPHATE		PROTEIN	
	g.	%	%(a)	%(b)	%(c)	%(b)	%(d)	%(b)
A	(e)	(e)	(e)	61	(e)	80	(e)	10
B	5.20	26	6	6	10	1	18	67
C	1.66	8	60	18	15	5	9	11

(a) Fucose: Mannose: Galactose: Glucuronic Acid (1:1:1:1) standard graph.

(b) Percentage of amount in original "fucan".

(c) Percentage of the carbohydrate content.

(d) Percentage of the weight.

(e) Not determined; fraction in solution.

TABLE 4.13 RELATIVE PROPORTIONS OF SUGARS IN PARTIAL HYDROLYSIS FRACTIONS

FRACTION	RELATIVE PROPORTIONS(a)					
	FUC	XYL	MAN	GLU	GAL	GLUA
A	93(78)	(32)	(12)	(22)	(16)	(7)
B	22(2)	3(9)	22(21)	10(11)	22(18)	19(13)
C	24(6)	4(36)	14(41)	8(29)	24(61)	26(58)
(TOTAL)	(86)	(77)	(74)	(61)	(95)	(77)

(a) Calculated by g.l.c. and m-hydroxydiphenyl method after hydrolysis with 2M trifluoroacetic acid (GM 5.33). Figures are percentage of carbohydrate and, in parentheses, percentage of original sugar in "fucan".

4.4.1. Fraction A. (Expt.5.70-5.73).

Paper chromatography showed a dominant fragment ($R_{GLUCOSE}$~ 0.8 in solvent 18:3:1:4), with small quantities of fucose, xylose, galactose and some oligomeric material. The high mobility of the major fragment in a mobile phase containing the detergent cetylpyridinium chloride,[10] suggested that it was a mixture of sulphated derivatives.

The fragments were fractionated on an anion-exchange column. Elution with water removed 16% of the carbohydrate which was further separated into monomeric and oligomeric material by paper chromatography. The monosaccharides (85% of the total) comprised fucose, xylose and galactose in the ratio 10:1:2. The oligomeric material could not be separated into discrete species but appeared to be a mixture of di- and tri-saccharides of, in order of decreasing amounts present, glucose, galactose, mannose, fucose and xylose. As these fragments constituted only a tiny proportion of the whole "fucan" no further study was made.

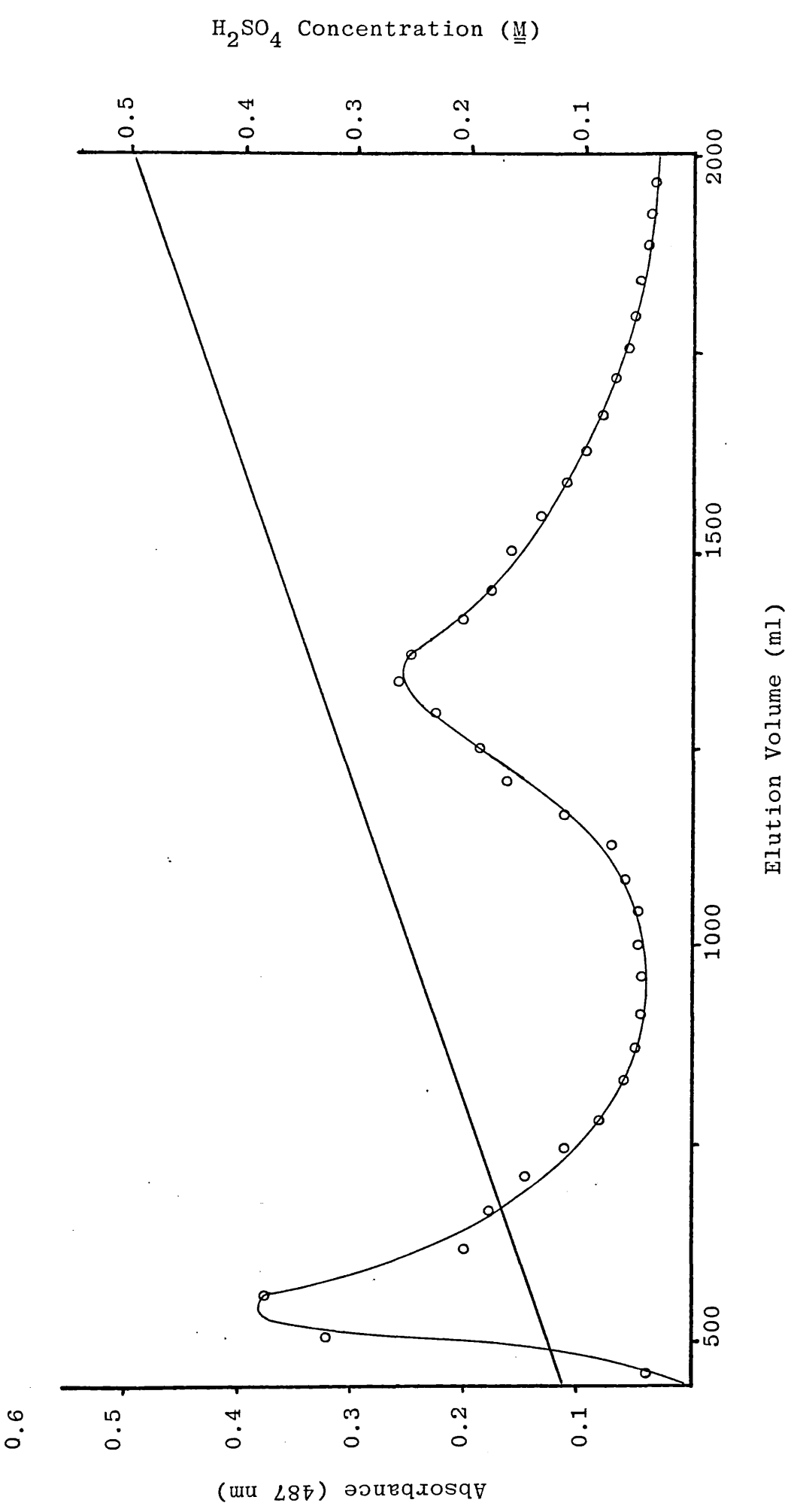
Uronic acid-containing fragments were eluted with 2M formic acid. 75 mg of carbohydrate (glucuronic acid graph) was removed; fractionation on paper gave 22 mg of monosaccharide comprised of glucuronic acid, its lactone and traces of neutral monosaccharides, and two oligomeric bands ($R_{GLUCOSE}$ ~ 0.30 and ~ 0.15 in 18:3:1:4) of 32 mg and 11 mg respectively. Esterification,

reduction and hydrolysis of both bands suggested aldobi- and aldotriuronic acids, with fucose, mannose, galactose and xylose all possibly linked to uronic acid. Again these fragments cannot be taken as representative of the bulk of the uronic acid-containing material.

Finally a gradient of sulphuric acid running from 0 to 1$\underline{\underline{M}}$ was applied to the column, removing 1.3 g of carbohydrate (fucose graph). The remainder of the carbohydrate (ca. 1.5 g 26% of the original "fucan" carbohydrate) was strongly bound to the column and was only slowly removed by further elution with sulphuric acid. With hindsight this form of ion-exchange column is not a good method of fractionating sulphated sugars. The material which was recovered eluted in two broad, overlapping bands (FIGURE 4.18) and these were isolated separately, after neutralisation of the acid with barium hydroxide, as sodium salts. Some further loss of carbohydrate occurred through precipitation of barium salts but, after desalting by passing through a column of Sephadex G25, 780 mg of A (430 mg of carbohydrate) and 650 mg of B (360 mg of carbohydrate) were obtained. (Theoretical carbohydrate for sodium fucose monosulphate = 61%).

Paper chromatography, electrophoresis, hydrolysis/g.l.c. and degree of polymerisation measurement all indicated that A and B were single species containing monomeric fucose with approximately one sulphate group

FIGURE 4.18 ELUTION PROFILE OF FRACTION A ON DE52 CELLULOSE ELUTED WITH SULPHURIC ACID



per fucose unit; despite the different elution positions on the gradient A and B appeared identical.

The possible difference in position of sulphate was probed by periodate oxidation. Treatment with 0.015M sodium metaperiodate at 20°C for 48 h in the dark led to periodate consumption and release of acetaldehyde and formic acid (TABLE 4.14).

TABLE 4.14

PERIODATE OXIDATION OF SULPHATED SUGARS FROM FRACTION A

SUGAR	PERIODATE CONSUMPTION[a]	FORMIC ACID RELEASED[a]	ACETALDEHYDE RELEASED[a]
A1	2.46	1.30	0.29
A2	2.50	1.25	0.28

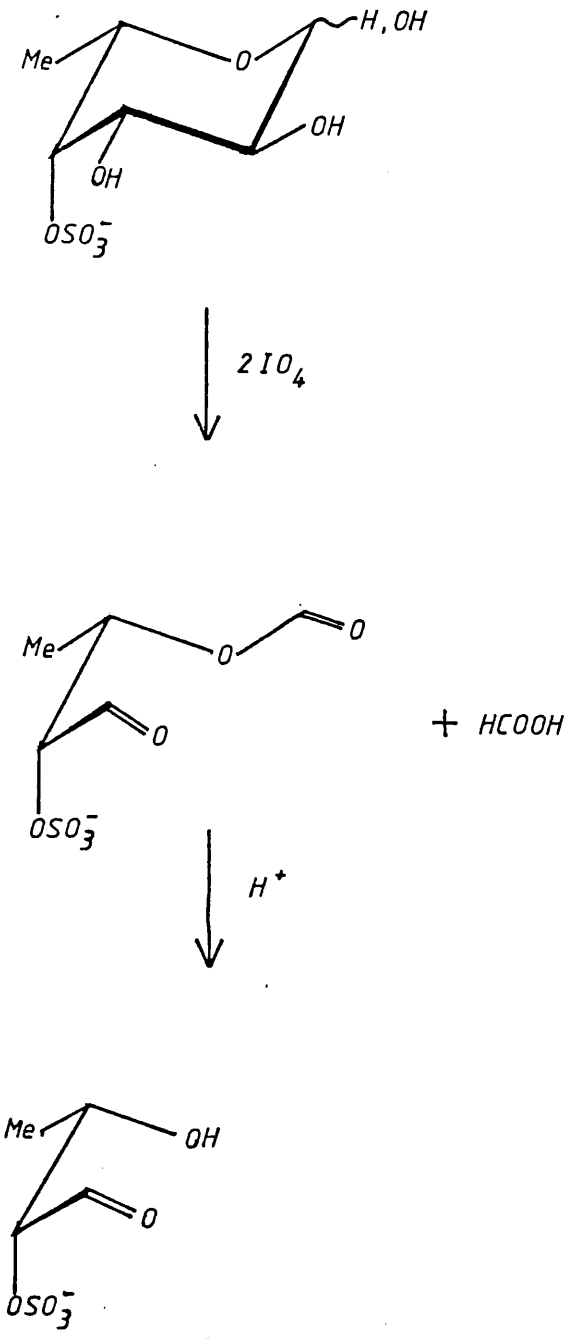
(a) Moles per sugar residue.

The low yield of acetaldehyde is consistent with sulphate at position 4, as commonly found in "fucans"; sulphate at 2 or 3, or overoxidation may account for the acetaldehyde generated. Two moles of formic acid would be expected from fucose-4-sulphate, though the values may have been depressed here by incomplete hydrolysis of the intermediate formyl ester (FIGURE 4.19). In unbuffered periodate solution hydrolysis is known to be slow.[11]

These results suggest that both A and B are mixtures of the various possible monosulphated forms of fucose.

The conclusion must be drawn that the two peaks on the elution gradient are due to an artefact.

FIGURE 4.19 PERIODATE OXIDATION OF FUCOPYRANOSIDE-4-SULPHATE



4.4.2. Fraction B. (Expt. 5.74)

From the low carbohydrate and protein contents and the dark colour the insoluble residue appears to be predominantly phenolic material rendered insoluble during hydrolysis. The co-occurrence of protein and polysaccharide reinforces the view that they are covalently linked, cleavage of most of the polysaccharide from the protein rendering the latter insoluble.

An attempt to isolate aldobiuronic acids from a hydrolysate of the fraction gave a complex mixture of oligomers irresolvable on paper chromatograms. Esterification, reduction and hydrolysis revealed all five neutral sugars, with mannose and glucose (from glucuronic acid) predominant.

4.4.3. Fraction C. (Expt. 5.75)

The monosaccharide distribution is similar to that of Fraction B, the solubility presumably being dependent on the low protein content. The gel filtration pattern (FIGURE 4.20) indicates considerable heterogeneity of structure. This was confirmed by cellulose acetate electrophoresis (FIGURE 4.21) where it ran as a broad band of mobility between those of a uronate-rich polymer (alginic acid) and the "fucan" fractions derived from mild acid treatment (Section 4.1.4). Analysis of separate fractions along the elution profile (FIGURE 4.20) did not suggest any

FIGURE 4.20 ELUTION PROFILES OF FRACTIONS C AND F ON SEPHADEX G100

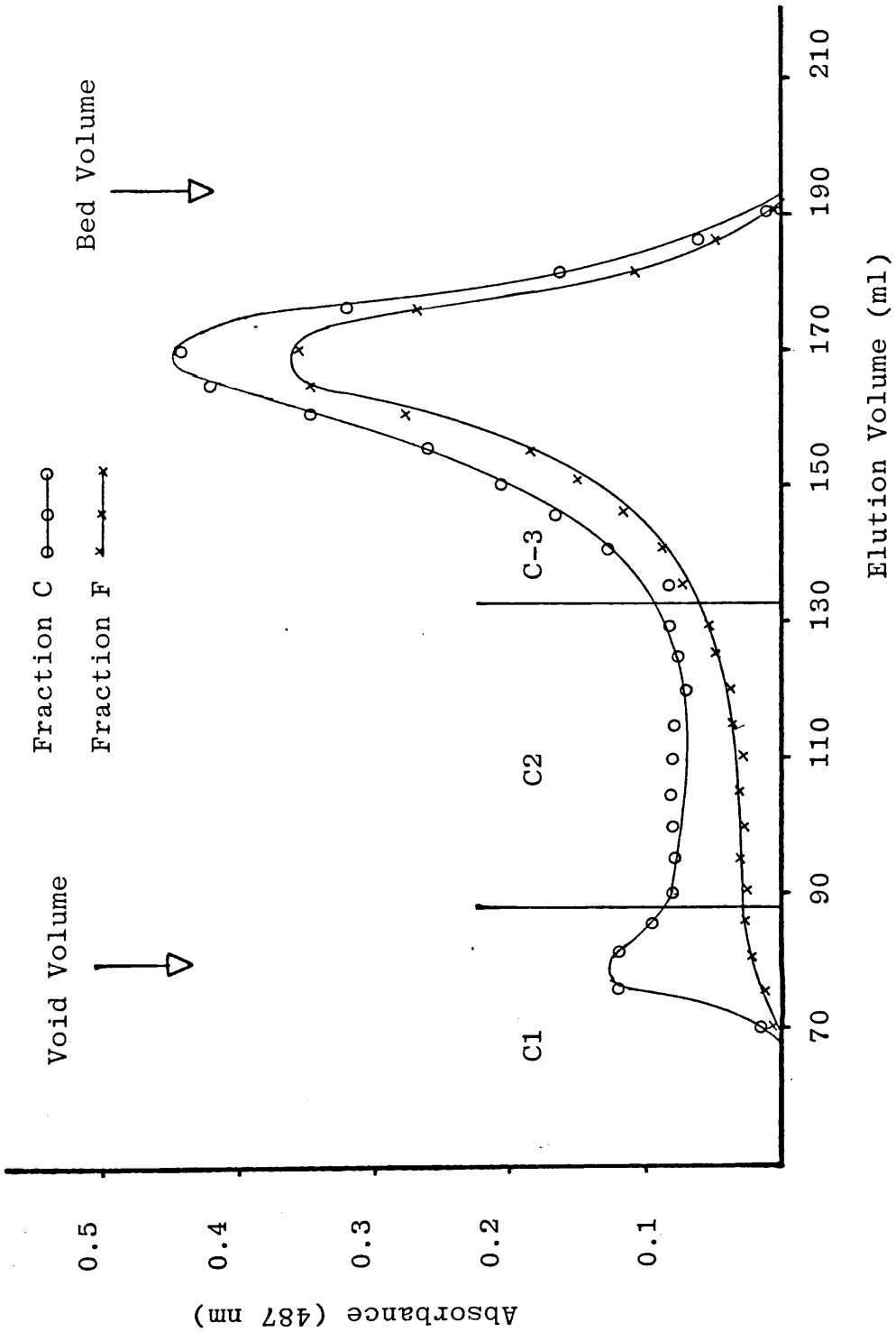
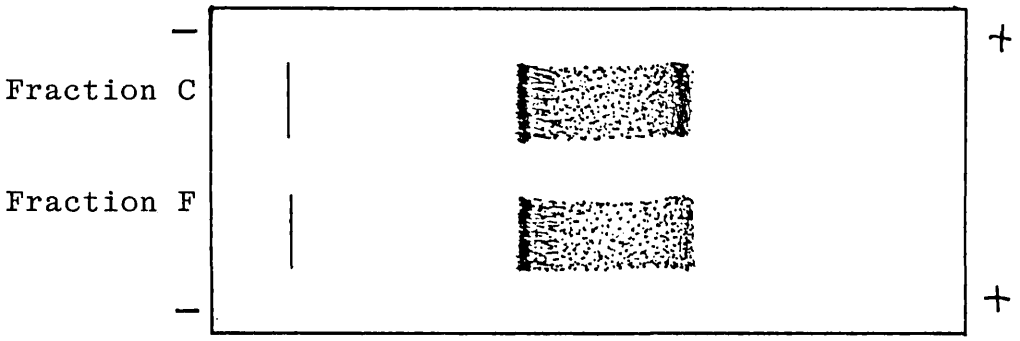


FIGURE 4.21 ELECTROPHORETIC PATTERNS OF FRACTIONS C AND F ON CELLULOSE ACETATE ACETATE AT PH 5.1



separation into discrete fractions by gel filtration (TABLE 4.15).

TABLE 4.15

RELATIVE PROPORTIONS OF SUGARS IN SUB-FRACTIONS OF FRACTION C

Subfraction	Monosaccharide Distribution[a]					
	FUC	XYL	MAN	GLU	GAL	GLUA
C-1	28	3	10	5	34	22
C-2	22	5	20	8	19	26
C-3	24	4	21	10	9	32

(a) See footnote to TABLE 4.14

This broad dispersion of structure is consistent with the greater degree of cleavage obtained here, whereby fucose is extensively removed from the "fucan" giving a range of uronate-rich fragments with varying amounts of fucose still attached. It seems clear that the fucose residues are virtually all in a separate part of the macromolecule from the other sugars.

The breakdown of the "fucan" may still be interpreted in terms of either a fucose-containing backbone with uronate-rich sidechains or a uronate-rich backbone with sidechains of fucose residues.

4.5. Partial Acid Hydrolysis of Fraction C (Expts.5.76-5.82).

Fraction C was complex in form and so a further partial hydrolysis was carried out on 1.5 g to try and obtain simpler fragments. After treatment with 0.1M oxalic acid for 1 h at 100°C the small

fragments, insoluble and soluble polymers were recovered in the same manner as above (Section 4.4).

The composition and monosaccharide distribution of the three fractions (D,E,F) were determined (TABLE 4.16).

TABLE 4.16

COMPOSITION OF SECOND PARTIAL ACID HYDROLYSIS FRACTIONS

FRACTION	RECOVERY %(a)	CARBOHYDRATE %(b)	SULPHATE %(c)	PROTEIN %(d)	SUGAR DISTRIBUTION(e)					
					FUC	XYL	MAN	GLU	GAL	GLUA
D	(f)	(f)(50)	5	0	13	3	13	13	35	23
E	15	27(6)	20	45	19	5	22	15	24	16
F	18	68(19)	13	9	7	7	18	18	29	20

(a) Percentage of the original weight of Fraction C.

(b) Mannose: Glucose: Galactose: Glucuronic Acid (1:1:1:1) standard graph.

(c) Percentage of the carbohydrate.

(d) Percentage by weight of the fraction.

(e) See footnote (a) to TABLE 4.13.

(f) Not determined; fraction in solution.

4.5.1. Fraction D (Expts. 5.77-5.80)

On paper chromatograms the fragments ran as a long streak ranging from monosaccharides to virtually immobile oligosaccharides, with no distinct spots visible. With a low fucose content a fractionation on an anion-exchange column was considered justified so the remainder (450 mg of carbohydrate) was separated on such a column.

The major part (360 mg of carbohydrate) eluted

with water and had a similar complex chromatographic pattern to the total fragments. Monosaccharides (280 mg) and oligosaccharides (80 mg) were separated on paper, the latter being mostly di- and tri-saccharides though no discrete species could be separated. After hydrolysis of the oligosaccharide fraction the monosaccharide distribution was similar to that of the free monosaccharides; glucose, galactose and mannose dominated with traces of fucose and xylose. No firm conclusions can be drawn from these results on whether the hexoses occur in homo- or hetero-polymeric chains and how long the chains might be.

Elution of the column with 2M formic acid removed material containing about 30 mg of carbohydrate. Esterification, reduction and hydrolysis showed this material to be a mixture of aldobiuronic acids of similar composition to those found for the earlier partial hydrolysis.

Finally elution with 0.5M sulphuric acid removed sulphated species (30 mg of carbohydrate as fucose). Fucose was the only sugar present. No further study was made on this fraction.

4.5.2. Fraction E. (Expt. 5.81)

The monosaccharide distribution and low carbohydrate content imply little difference from Fraction B; again the polysaccharide appears to be carried out of solution by the protein. The molar ratio of sulphate to fucose (ca. 1.6) is higher

than in the original "fucan". This may indicate that other sugars carry sulphate or that the more highly sulphated fucose residues are associated with this material. A more likely explanation is that cleaved sulphate groups are not removed by dialysis, remaining associated with the polymer.

4.5.3. Fraction F. (Expt. 5.82).

This material was virtually white, though it still contained protein. On cellulose acetate electrophoresis its behaviour was similar to that of Fraction C, though with less fast-moving material (FIGURE 4.21). A similar result was obtained by gel filtration, with rather less high-molecular weight material, but no significant change in the average molecular weight of the bulk of the material. (FIGURE 4.20). Both results are consistent with the view that fucose occurs in long chains distinct from the remainder of the sugar units in the macromolecule.

The periodate oxidation of the intact "fucan" reported earlier (Section 4.3.2) suggested a variety of linkages for the sugars other than fucose. A second attempt in the absence of most of the fucose should give a clearer idea of the linkage and position of the other sugars.

A portion was oxidised, consuming 1.1 moles of periodate per anhydro unit in 95 h with cleavage of about 60% of the sugar residues, all the sugars being cleaved to some extent, as indicated by hydrolysis of

the derived polyalcohol (recovered in 72% yield) (TABLE 4.17).

TABLE 4.17. SUGARS UNCLEAVED AFTER PERIODATE OXIDATION OF FRACTION F.

	UNCLEAVED CARBOHYDRATE[a]						
	TOTAL	FUC	XYL	MAN	GLU	GAL	GLUA
Polyalcohol	37	31	5	48	22	47	36

(a) Calculated by comparison of sugar composition of polyalcohol with that of Fraction F; composition determined as in footnote (a) to TABLE 4.14.

Given the high uronic acid content of the initial polymer and the polyalcohol it is inevitable that hydrolysis of glycosidic linkages will not have been complete and some accompanying degradation may have taken place, and hence the data in TABLE 4.17 can only be taken as approximate values. Nevertheless the high survival of carbohydrate can only be correlated with the high consumption of periodate by assuming that the degree of branching is high, giving a high proportion of non-reducing terminal units. The high loss of glucose rules out the possibility that it all derived from laminaran. The earlier observation that xylose occurs predominantly as a non-reducing terminal unit appears to be confirmed.

The loss of fucose rules out lengthy chains of sulphated fucose residues, the partial hydrolysis

having probably cleaved the chains down to single, terminal residues. These remaining units appear to be highly sulphated (TABLE 4.16); the minimal amount of butan-1,2,3-triol (indicative of substitution at C-4) in the polyalcohol hydrolysate suggested that some of the unoxidised residues may be disulphated.

The presence of threitol confirmed (1→4)-linked galactose residues though it was insufficient to account for all the cleaved residues; (1→2)- and (1→6)-linked residues could not be ruled out. The amount of erythritol was more than sufficient to account for all the cleaved glucose and mannose, the excess probably being derived from erythronic acid (from (1→4)-linked glucuronic acid) which had lactonised and been reduced during derivatisation for g.l.c. in analogous manner to the uronic acids. Experiments with periodate oxidised alginic acid showed that this was possible.

The uncleaved residues may be (1→3)-linked or occur at branch points. Whichever is the case it is clear that almost all the linkages in the molecule are through 3- or 4-.

An attempted Smith Degradation proved too drastic, cleaving glycosidic as well as acetal linkages. 12% of the carbohydrate passed through the dialysis membrane during isolation, predominantly as momomeric sugars of which glucuronic acid constituted about 50%,

the remainder being glucose and lesser amounts of fucose, mannose and galactose. The remaining polymer was recovered in 66% yield.

To try and confirm these inferences an attempt was made to methylate Fraction F. A portion (30 mg) was first carboxyl-reduced by the carbodiimide method, bringing the glucuronic acid content down from 20% to about 4%. The reduced polymer was methylated twice, hydrolysed and examined by g.l.c. and g.l.c/m.s.[12,13] The methylated products gave a complex pattern of peaks (FIGURE 4.22) and m.s. showed most of the peaks to be mixtures of products. The components identified and their approximate proportions, as determined by m.s., are listed in TABLE 4.18; in some cases no distinction is possible between identically methylated derivatives of different hexoses. In addition about 8% of sugar remained unmethylated; of this galactose was the major component.

FIGURE 4.22 G.L.C. CHROMATOGRAM OF METHYLATED SUGARS FROM FRACTION F

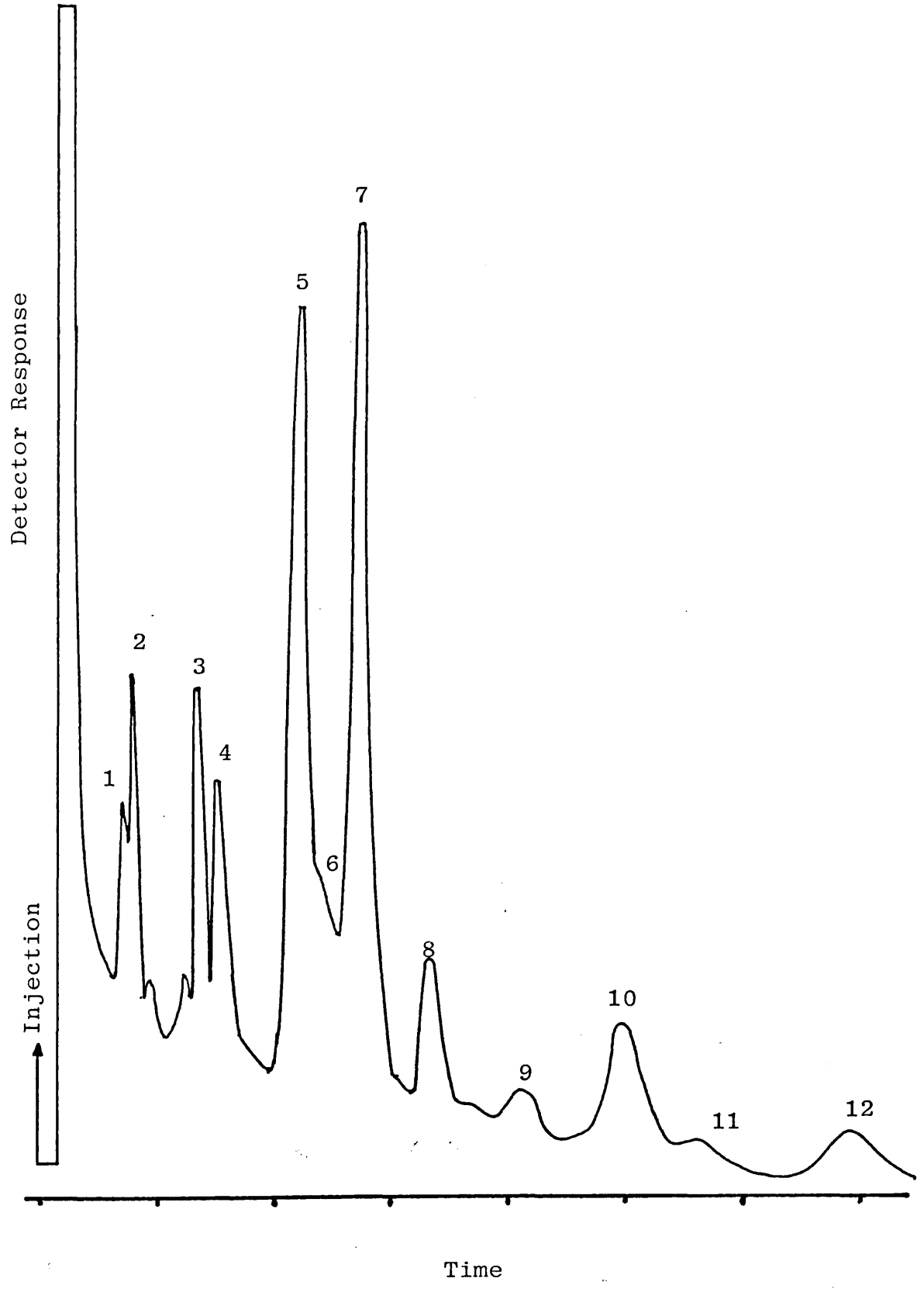


TABLE 4.18

METHYLATED SUGARS FROM FRACTION F

Peak Number	Retention Time(a)	Mole Percentage(b)	Methylated Sugars	
			Major	Minor(c)
1	0.59	2	2,3,4-Xyl	
2	0.64	3	2,3,4-Fuc	
3	1.00	5	2,3,4,6-Glu or Man	2,3-Fuc (20)
4	1.13	4	2,3,4,6-Gal	2,4-Fuc (20)
5	1.73	28	2,4,6-Glu	3,4,6-Glu (20)
				3-Fuc (5)
6	1.86	4	2,4,6-Glu or Man	
7	2.09	24	2,3,6-Man	2,4,6-Gal (20)
8	2.52	5	2,3,6-Glu	2,3,4-Gal (30)
9	3.16	2	2,6-Gal	
10	3.70	8	3,6-Man or Glu or Gal	
11	4.22	2	2,4-Glu	
			3,4-Glu or Man	
12	5.20	5	2,4-Gal	
			3,5-Gal	

(a) Relative to 2,3,4,6-tetramethyl-1,5-diacetylglucitol = 1.00

(b) Approximate; corrected for effective carbon response [14]

(c) Figures in parenthesis denote approximate percentage of the peak.

Discounting the contribution of fucose and xylose intrachain residues it is possible to divide the peaks into non-reducing end units (Peaks 1 - 4), intrachain units (Peaks 5 - 8) and branch point units (Peaks 9 - 12). The large proportion of the latter confirms the earlier conclusion that the molecule is highly branched.

Considering the individual sugars and bearing in mind their relative proportions (TABLE 4.16), fucose and xylose appear to occur predominantly as non reducing terminal units; indeed the substituted residues (in peaks 3,5) may be sulphated terminal units. For mannose, peak 7 ($(1 \rightarrow 4)$-linkage) accounts for most of the sugar in the original polymer, in apparent conflict with the periodate oxidation study which suggested that almost half the mannose residues are resistant to oxidation. However as stated earlier the latter result must be taken as only approximate.

The glucose derivatives identified may be derived from either glucose or glucuronic acid residues (reduction with sodium borodeuteride was not attempted). From the periodate oxidation results it seems reasonable to conclude that both residues contribute to peak 5 ($(1 \rightarrow 3)$-linkage), though the higher proportion of periodate-sensitive glucose

suggests that a substantial amount of this must be accommodated elsewhere, as terminal or (1 → 4)-linked residues. The occurrence of glucuronic acid at branch points is unusual and hence it is probable that the branch point residues are glucose residues.

Despite being the major sugar galactose does not appear in any of the major components. This indicates a multiplicity of linkages, and the proportion of unmethylated material suggests that a lot of the galactose appears at branch points. A significant feature is the apparent occurrence of galactofuranose residues (Peak 12).

4.6. N.m.r. Spectra of "Fucans"

Published work on n.m.r. of algal "fucans" is limited to two papers. Villaroel and Zanlungo[15] recorded a 100 MHz ^{1}H spectrum of a reportedly pure from Lessonia flavicans and assigned three signals: at 5.43 ppm to H-1 of α-L-fucose, at 4.97 ppm to H-4 of α-L-fucose carrying half-ester sulphate at C-4, and at 1.20 ppm to protons on C-6. They did not report any splitting of signals.

Kloareg et al,[16] studying "fucan" from Pelvetia canaliculata and recording spectra at 90MHz, assigned signals at 5.30 ppm and 1.20 ppm to H-1 and H-6 respectively of α-L-fucose. They also noted, but did not assign, a small anomeric signal at 5.13 ppm,

presumably derived from a minor sugar. In a 22.5 MHz ^{13}C spectrum the same authors assigned signals at 16.6 ppm (C-6 of fucose), 99.9 ppm, (C-1 of α-L-fucose), 175.2 and 174.1 ppm (C-6 of glucuronic acid), and a small anomeric carbon signal at 95.5 ppm.

These spectra were evidently poorly resolved and yielded little information. Operation at substantially higher frequency (400 MHz ^{1}H, 100 MHz ^{13}C) might be expected to give better resolution, with splitting of signals in the ^{1}H spectra to assist assignment. However considerable problems were encountered in obtaining good, reproducible spectra, the relative sizes of signals apparently being dependent on concentration and viscosity of the samples. The purified "fucan" (Extract 5A) and the crude "fucan" (Extract 5) gave clearly differing ^{13}C spectra (FIGURES 4.23, 4.24).

The latter is contaminated with mannitol which had not been dialysed away entirely giving three sharp, dominant signals (64.0, 70.6, 72.2 ppm), but even allowing for this the signals from ring carbons (60 - 85 ppm) are substantially different from those of the former. Though in both cases fucose is the dominant residue, the signals which may reasonably be assigned to C-1 of fucose in Extract 5A. (97.0, 96.8 ppm) are absent from the spectrum of Extract 5. This implies that the former spectrum gives a more complete representation

FIGURE 4.23 ^{13}C N.M.R. SPECTRUM OF MACROCYSTIS PYRIFERA EXTRACT 5A

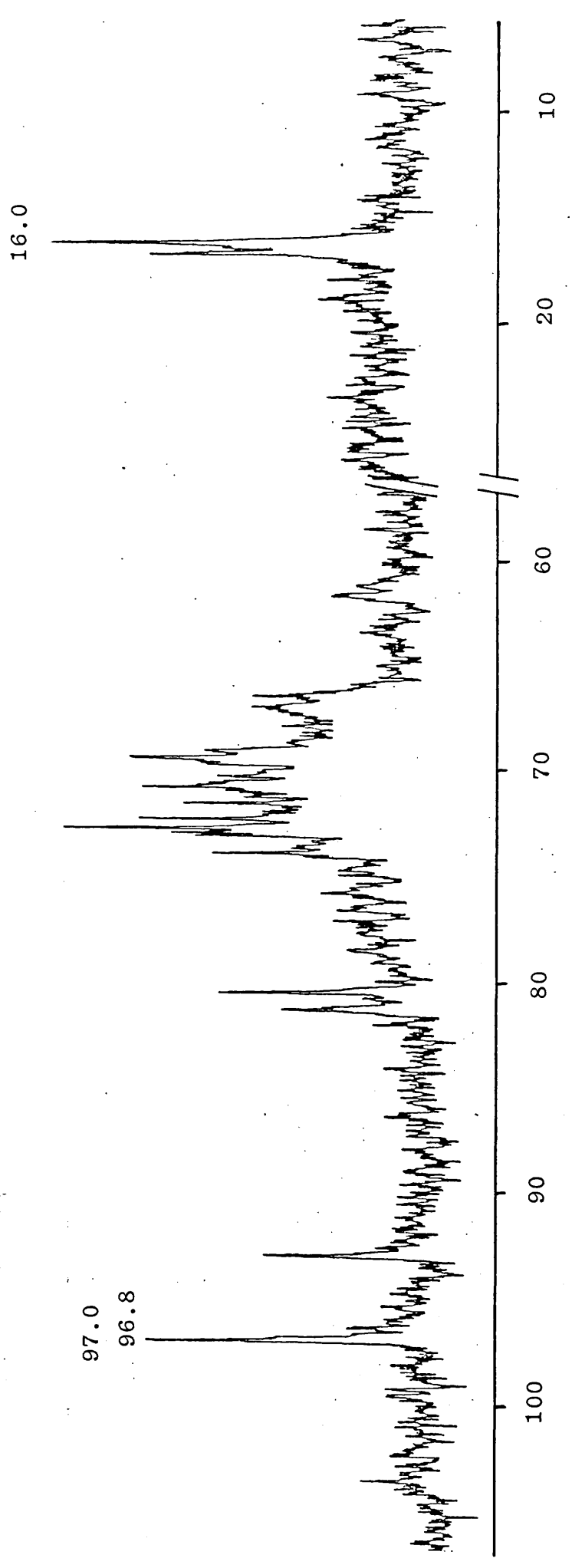
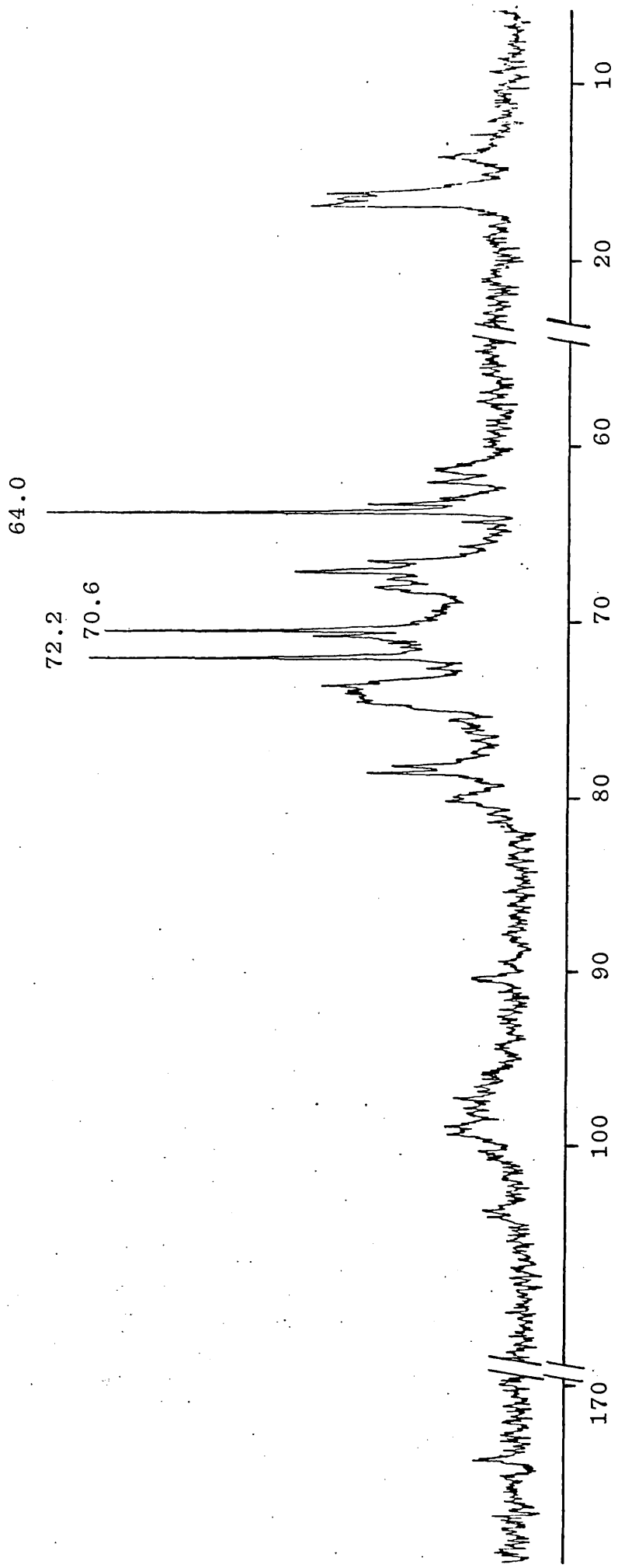


FIGURE 4.24 ^{13}C N.M.R. SPECTRUM OF MACROCYSTIS PYRIFERA EXTRACT 5



of the "fucan", though further assignment brings other anomalous features to light. High field signals in the region 60-63 ppm are generally assigned to unsubstituted C-6 of hexose residues; taking those in FIGURE 4.23 as such, comparison with the signals assigned to fucose C-1 suggests roughly similar proportions whereas chemical analysis has shown fucose to be in several-fold excess of the total hexose residues. This may be a reflection of the effect of different relaxation times on the integrals, though this is not a common observation for these types of carbon atoms.[17] Suppression of signals through selective aggregation of molecules by divalent ions should have been avoided by the prior treatment with chelating resin. Aggregation of the type observed by Rees and coworkers[18] in carrageenans should be ruled out by the elevated temperature, though it does offer a possible explanation, since spectra have been recorded at temperatures in the range 65-80^{o}C, offering some scope for partial aggregation.

Nevertheless some observations may be made from the spectra of Extract 5A (FIGURE 4.23). The resolution of the fucose C-6 signal (~16.0 ppm) into several peaks indicates several types of residue, in line with the chemical results. Similarly the signals in the region 80-85 ppm can, by virtue of their size, only be ascribed to fucose carbon atoms

either substituted by half-ester sulphate groups or glycosidically linked. The ^{1}H spectrum (FIGURE 4.25) supports this, with several doublets ($J_{12} \tilde{=}$ 6Hz) resolved for protons on C-6 of fucose (1.3 - 1.4 ppm) and at least two signals due to H-4 of C-4 substituted fucose (4.9-4.95 ppm).[15]

In addition more than one peak is apparent in the low field signal from H-1 of fucose, though some of these may be due to anomeric protons of other residues. The signal at 1.6 ppm may be due to methyl protons in pyruvate ester groups, which were also identified by g.l.c. of hydrolysates of the "fucan". The signals in the region 2.0 - 2.2 ppm may be due to methyl protons in acetate groups but are too small to be confirmed as being other than impurities.

4.6.1. N.m.r. Spectra of Fraction F.

In view of the above caution must be exercised in interpreting spectra, though most of the fucose and sulphate has been removed, reducing the tendency to aggregation. The ^{13}C spectrum (FIGURE 4.26) shows numerous anomeric carbon signals (95 - 105 ppm) which must derive from both α- and β-linked residues and are in agreement with the complexity established by chemical analysis. The earlier assignments of fucose carbon signals are confirmed by their absence here. Any attempted assignment of signals can only be tentative.

The ^{1}H spectrum (FIGURE 4.27) appears somewhat

FIGURE 4.25 ^{1}H N.M.R. SPECTRUM OF MACROCYSTIS PYRIFERA EXTRACT 5A

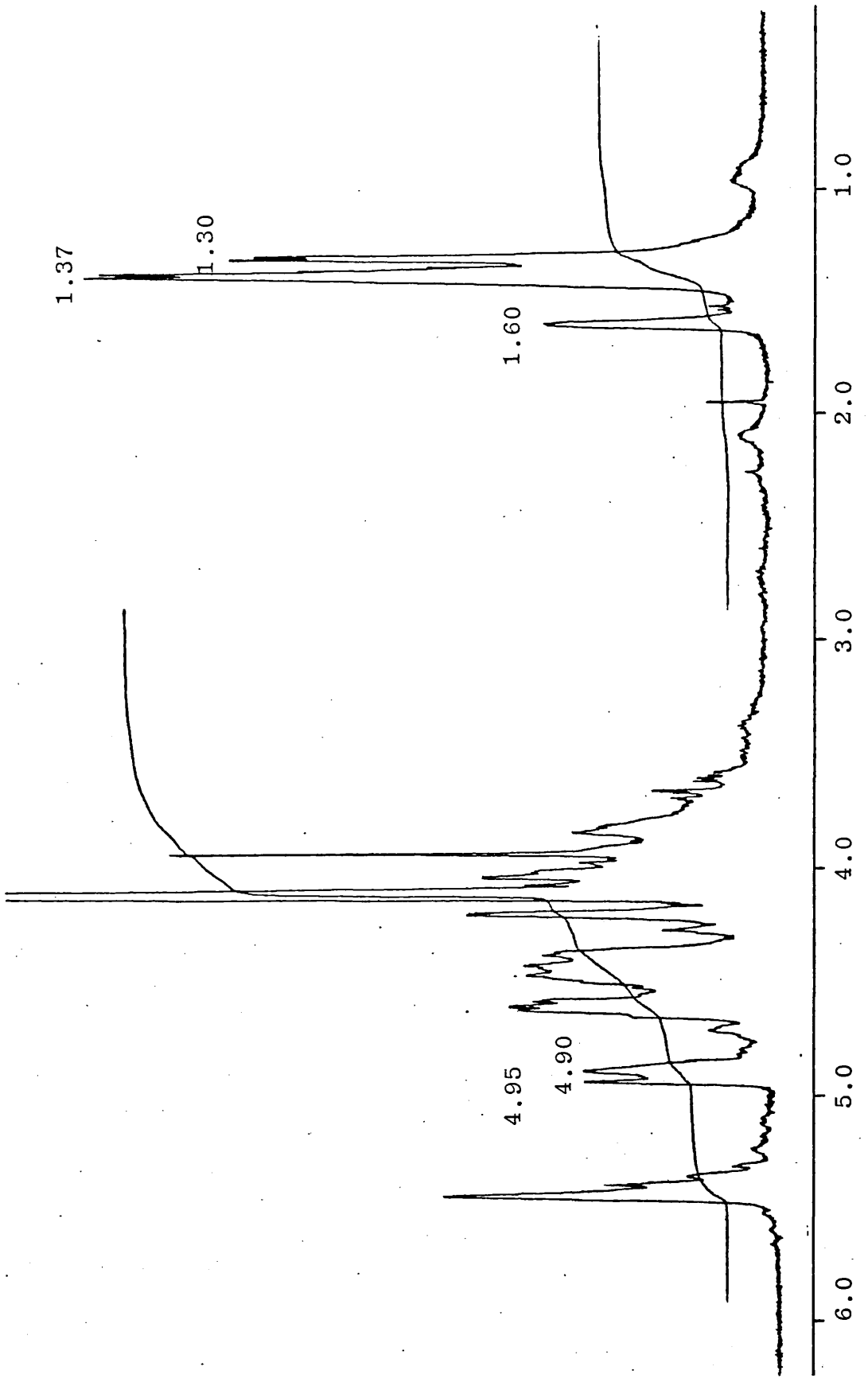


FIGURE 4.26 ^{13}C N.M.R. SPECTRUM OF PARTIAL HYDROLYSIS FRACTION F

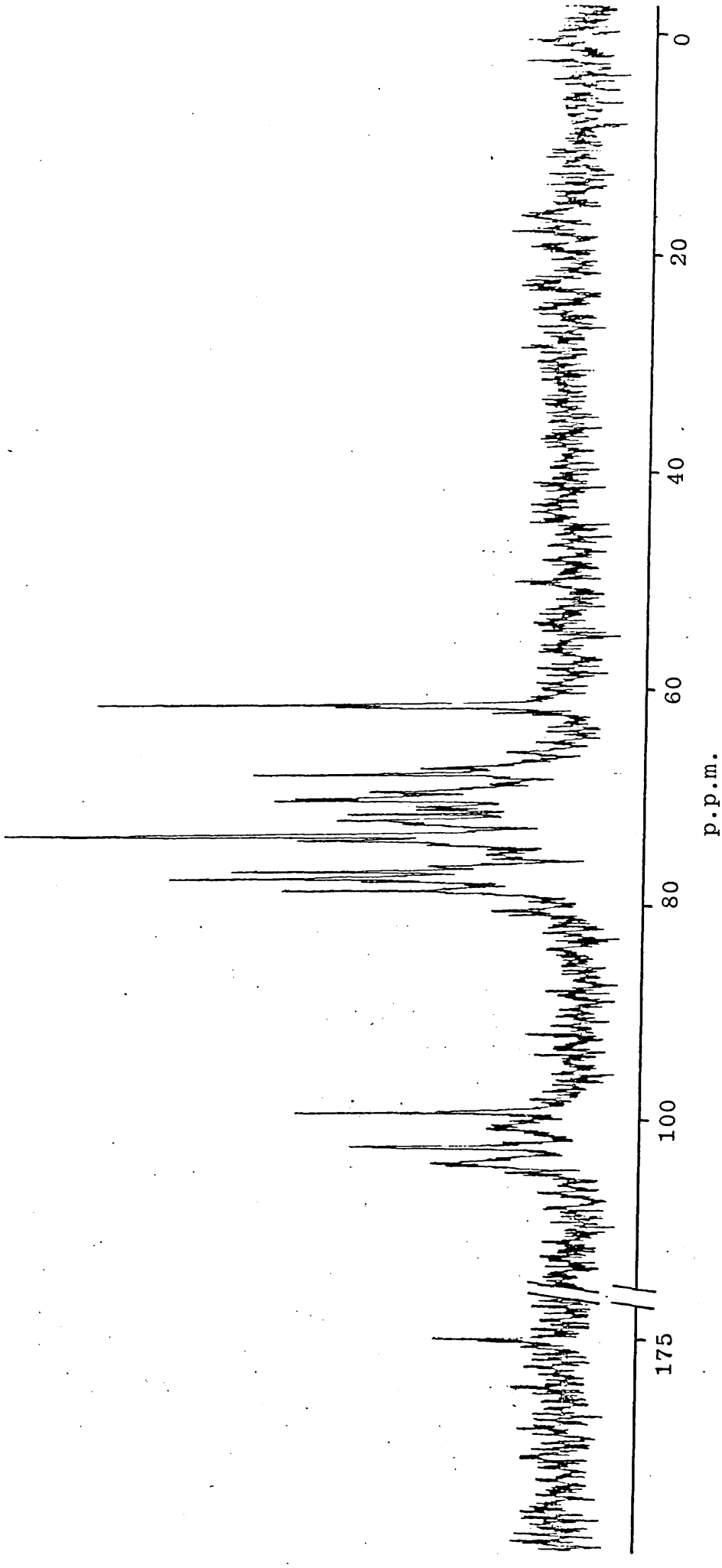
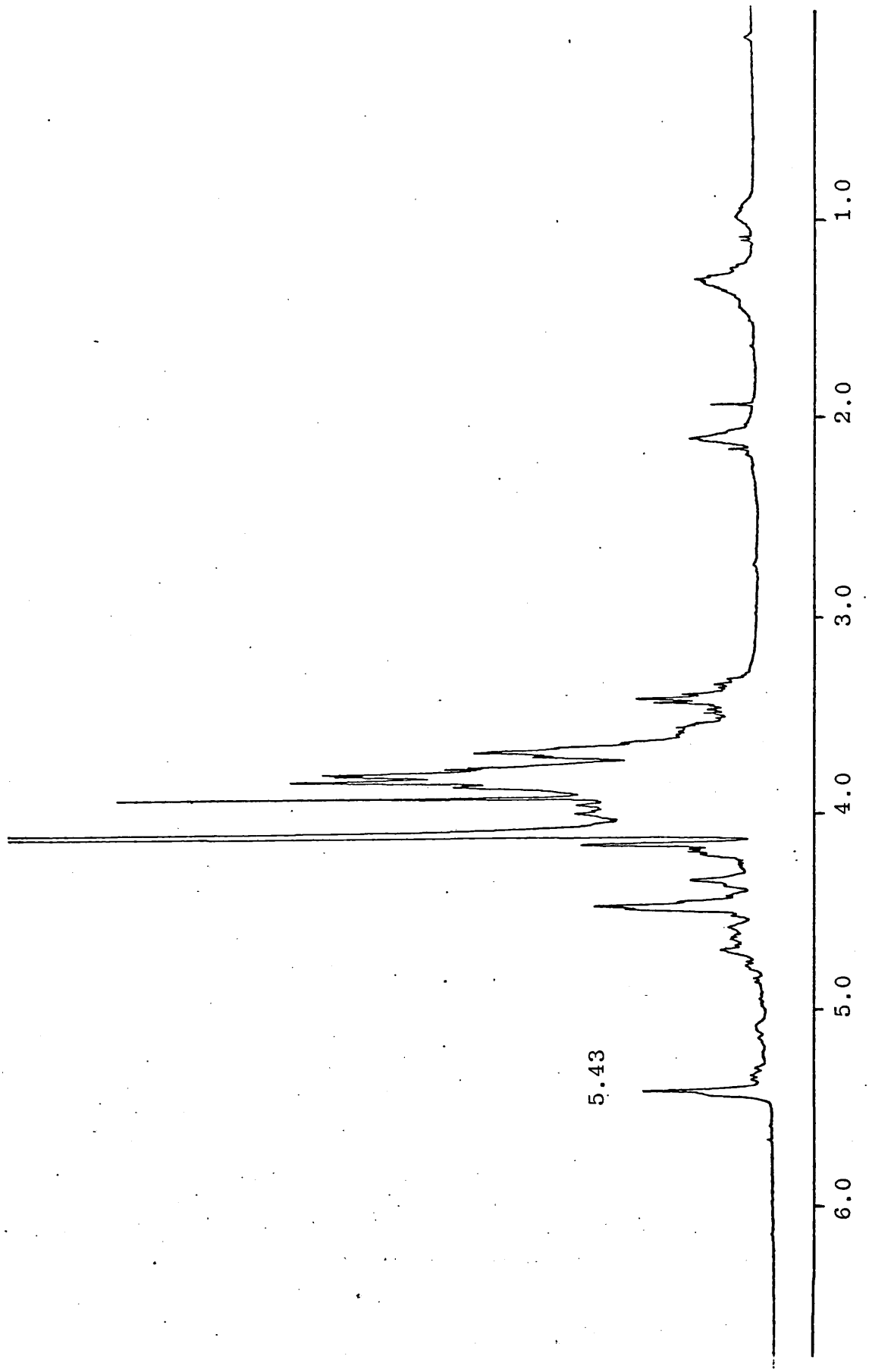


FIGURE 4.27 ^{1}H N.M.R. SPECTRUM OF PARTIAL HYDROLYSIS FRACTION F



simpler; by comparison with the above it is likely that the signal at 5.43 ppm derives from more than one residue. Again the assignment of signals to fucose protons made earlier is confirmed. Unsurprisingly the pyruvate signals have disappeared, these groups being particularly labile to acid hydrolysis . The acetate signals tentatively assigned earlier remain; if they do constitute part of the "fucan" they must be linked preferentially to hexoses other than fucose.

4.7. Summary

The distinctive feature of the "fucan" is the complexity of its structure as defined by the number of different sugar residues and their variety of linkages and neighbours. Given the diverse functions postulated for "fucans" this is not surprising; as described in the Introduction (Section 2.2.2.2) this is commonly the case in brown algae. Though the "fucan" in the Acid and Alkali Extracts has not been examined in detail it seems reasonable to assume that its structural features are of similar complexity.

Where this "fucan" differs from most "fucans" previously studied is in the apparent mutual linkage of six sugars and the absence of any relatively simple structural subunit. Past work has led to, variously, virtually pure "fucan", glucuronomannan fractions, galactose oligomers and a "galactofucan". Here the nearest approach has been the recovery of most of the

fucose in one fraction (Fraction A) and the isolation of a fraction virtually devoid of fucose and xylose. Though no definitive evidence of the mutual linkage of the various residues can be presented the results point to this conclusion and give no indication of any distinct subunit. The universal appearance of protein in all the polymeric fractions, regardless of their size, suggests several points of linkage to the polysaccharide.

Detailed consideration of how the plant synthesis and modifies such a complex entity is beyond the scope of this study; all the above results indicate that the modifications at least are intricate, in contrast to the relatively simple process involving alginic acid.

CHAPTER 4. REFERENCES

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CHAPTER 5.

EXPERIMENTAL General Methods

5.1. Physical Techniques.

5.1.1. Evaporations were carried out under reduced pressure below 40°C with a Buchi rotary evaporator.

5.1.2. Deionised, distilled water was used in all experiments unless otherwise stated.

5.1.3. Melting points were determined on a Gallenkamp micromelting point apparatus..

5.1.4. Dialysis was performed with Visking Cellophane tubing against distilled water, with either toluene or sodium azide added as a bacteriostat. The tubing was initially rendered free of glycerol by boiling for a few minutes in water.

5.1.5. Specific rotations were measured in a 1 dm polarimeter tube using a Perkin Elmer 141 polarimeter. All measurements were made in aqueous solution using the sodium D-line.

5.1.6. Unless otherwise stated all resins used were either Amberlite or Biodeminrolit.

5.1.7. Lyophilisation (freeze-drying) was carried out on a 'Chem Lab' freeze-drier; the pH of solutions was checked beforehand and adjusted to 7.0 where necessary.

5.1.8. Solutions and hydrolysates were filtered through millipore filters (0.45μ) before quantitative

determinations.

5.1.9. Ultraviolet and visible absorbances were measured using a Pye Unicam SP500 spectrophotometer.

5.1.10. Molecular sieves 3A and 4A (BDH) were activated by heating at 400°C for 4 h.

5.1.11. Samples were dried over P_2O_5 at 40°C in vacuo.

5.2. Purification of solvents.

5.2.1. Methanol was prepared as described by Vogel[1], distilled with the exclusion of moisture, and stored over activated 3A molecular sieve.

5.2.2. Chloroform was distilled from anhydrous sodium sulphate and stored, refrigerated, in brown glass bottles over anhydrous sodium sulphate.

5.2.3. Pyridine was distilled from, and stored over, sodium hydroxide pellets (BDH, Analar).

5.3. Acid Hydrolysis.

5.3.1. Formic acid method

The sample was dissolved in 90% formic acid and solid carbon dioxide added to exclude oxygen. The flask was sealed and heated for 6 h at 100°C. The resultant formyl esters were hydrolysed by addition of water (5 vol.) and heating for 2 h at 100°C. The formic acid was removed by repeated co-distillation with methanol.

5.3.2. 72% Sulphuric acid method.[2]

Cold 72% sulphuric acid was added to the sample and the mixture left at room temperature for 1 h. Water (10 vol.) was added with cooling and

the solution heated for 4 h. at 100°C. After cooling the acid was neutralised with barium carbonate and the solid barium sulphate removed by centrifugation. The solution was deionised by shaking with IR 120 (H^+) resin followed by filtration and concentration.

5.3.3. 2M trifluoroacetic acid method.

The sample was dissolved in 2M trifluroacetic acid and the sealed tube heated for 2h at 120°C. The hydrolysate was cooled and the acid removed by repeated codistillation with water.

5.4. Chromatography

5.4.1. Paper Chromatography

Paper chromatography was carried out by the descending technique using Whatman No.1 paper for qualitative work and 3mm or No.17 papers for preparative work, with maximum loadings of 100 mg and 1000 mg per standard sheet respectively. Preparative papers were washed with distilled water before use.

The following solvent systems were used:

Ethyl acetate:acetic acid:formic acid:water (18:3:1:4)[3] by volume.

N-butanol:pyridine:water (6:4:3) by volume.[4]

N-butanol:ethanol:water (40:11:19) by volume.[5]

N-butanol:ethanol:water (3:1:1) by volume, with 3% w/v cetylpridinium chloride added.[6]

5.4.2. Paper electrophoresis

The Shandon high voltage electrophoresis

apparatus L24 was used with Whatman 3 mm paper and the following electrolytes:

Borate.[7]

0.2M sodium borate in water adjusted to pH 10 with sodium hydroxide. Electrophoresis was carried out for 2h at 2.5kV and 40mA.

Molybdate.

0.2M sodium molybdate in water adjusted to pH5 with hydrochloric acid. Electrophoresis was carried out for 2h at 3.0kV.

Phosphate.

0.05M sodium hydrogen orthophosphate in water adjusted to pH 6.5 with orthophosphoric acid. Electrophoresis was carried out for 2h at 3.0kV.

5.5. Staining reagents.

5.5.1. Silver nitrate dip.[8]

The developed paper was passed sequentially through the following solutions:

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

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

(3) 10% aqueous sodium thiosulphate.

5.5.2. Aniline oxalate spray.[9]

A saturated solution was prepared by stirring aniline oxalate (25 g) in 50% aqueous ethanol (1L) for 24h. The developed paper was sprayed and heated at 105°C for 5 min.

5.5.3. Urea hydrochloride.[10]

Urea (10g) in ethanol (200 ml) and concentrated hydrochloric acid (8 ml) in water (320 ml). A blue colour specific for ketoses appeared after 5 min at 100°C.

5.5.4. Ninhydrin.

A freshly prepared solution of ninhydrin (2g) in ethanol (100 ml). A blue colour specific for amino acids appeared after 5 min at 100°C.

5.5.5. Glucose oxidase.[11]

A Worthington 'Statzyme' kit was made up as directed. A pink colour indicating D-glucose developed after 5 minutes at room temperature.

5.6. Gas liquid chromatography (g.l.c.)

5.6.1. Instrumentation.

A Varian 3700 gas chromatograph fitted with a W.C.O.T. glass capillary column (25 m x 0.3 mm.) with SE 30 stationary phase. The column was operated with helium carrier gas at a split ratio of approximately 30:1, and a flame ionisation detector. Alditol acetates were separated at 170°C.; periodate oxidation fragments were analysed on a temperature programme of 100 to 170°C at 4°C/min followed by 30 minutes at 170°C. Peak areas were integrated by a Varian CDS 111C data system.

A Pye 104 gas chromatograph with nitrogen carrier gas, flame ionisation detector and glass

column (3m x 5mm) was used for methylated alditol acetates. The packing was 3% OV225 coated on 100-120 Gaschrom Q. The operating temperature was 170°C.

5.6.2. Gas chromatography linked to mass spectrometry.[12-14]

A Pye 104 gas chromatograph and OV225 column was coupled to a VG Micromass 12F mass spectrometer with a total ion monitor detector system. Helium carrier gas was used. For EI spectra the 'ion source' was operated at about 200°C, 70eV and 20μA target current under a pressure of 10^{-6} torr. For CI spectra the 'ion source' was operated at about 200°C, 50eV and 1000μA under a pressure of 0.4-0.5 torr with 2-methylpropane as the reactant gas. The column temperature was 170°C.

5.7. Column chromatography.

5.7.1. Preparation of a DE52 ion exchange cellulose column.[15]

The cellulose (200g. Diethylaminoethyl, DEAE, DE52 pre-swollen) was suspended in 0.5M hydrochloric acid (2 l) and deaerated by magnetic stirring under vacuum for 20 minutes. After standing for another 20 minutes the supernatant and fines were discarded, the remaining acid filtered off and the cellulose washed with water until the filtrate was neutral. The wet material was then treated in the same manner with 0.5M sodium hydroxide. These two operations were repeated three times and after the

last alkali treatment the cellulose was transferred to a column and washed to neutrality.

5.7.2. Preparation of anion exchange columns in formate form.[16]

The commercial resin (Cl^- form) was packed into a column and washed with 2M NaOH (~ 10 volumes) until the eluate was free of Cl^-. The column was washed with water until the pH was less than 9 and then washed with 2M formic acid (~ 5 volumes). It was then washed to neutrality with water.

5.7.3. Preparation of Sepharose 4B gel filtration column.[17]

The Sepharose 4B, supplied as a pre-swollen suspension, was degassed under vacuum for about 15 minutes. The dead space of the column outlet was filled with eluant and then the gel slurry was poured carefully into the column (0.6m x 9mm). A funnel was placed on top of the column and filled with slurry. Packing of the column was effected by pumping eluant out of the column until the gel had settled down the full length of the column, eluant being added to the funnel to keep the slurry wet. The funnel was then removed, excess gel wiped away and the column equilibrated by passing through two column volumes of eluant.

5.7.4. Preparation of Sephadex G10, G25 and G100 columns.[17]

The Sephadex gels, supplied as powders, were swollen by suspending in water and heated on a water

bath at 90°C for the periods recommended by the manufacturer. Degassing was thereby effected, and after cooling the gels were packed into columns (1.0m x 16mm i.d.) in the same manner as above {GM 5.7.3.}.

5.7.5. Running and calibration of gel filtration columns.[17]

The Sepharose 4B column was run at a flow rate of 4 ml/h, maintained by peristaltic pump. Fractions (45 drops, 1.3 ml) were collected on a Gilson fraction collector and assayed for carbohydrate {GM 5.10.1} . Sample size was 6-10 mg.

The Sephadex columns were run at a flow rate of 15 ml/h maintained by peristaltic pump. Fractions (166 drops, 5 ml) were collected on a Gilson fraction collector and assayed for carbohydrate {GM 5.10.1}. Sample size was 10-12 mg. In both cases the "bed volume" V_t and "void volume" V_o were determined by elution of glucose (2mg/ml) and Blue Dextran 2000 (2 mg/ml, Pharmacia Fine Chemicals) respectively through the column. Selectivity curves were derived by elution of dextran fractions of known molecular weight and plotting the distribution coefficient K_{AV} against the log of the molecular weight;

K_{AV} is given by the relationship:

$$K_{AV} = \frac{V_e - V_o}{V_t - V_o}$$

where V_e is the elution volume for a particular molecule.

The eluant for all gel filtration experiments was 0.1$\underline{\underline{M}}$ sodium acetate containing 0.02$\underline{\underline{M}}$ EDTA, adjusted to pH5 with acetic acid.

5.8. Polyacrylamide gel electrophoresis.[18]

5.8.1. The Shandon SAE-2717 analytical polyacrylamide gel electrophoresis apparatus was used. Shandon general instructions were followed for a gel system at pH 8.5, using the following solutions:

Solution 1. Running buffer for small pore gel.

(a) hydrochloric acid (1$\underline{\underline{M}}$, 48 ml)

(b) 2-amino-2-hydroxymethyl-1,3-propanediol (TRIS) added to give pH 8.9

(c) urea (36.0g)

(d) N,N,N',N'-tetramethylethylenediamine (TEMED) (0.46 ml).

(e) water to 100 ml.

Solution 2. Stacking buffer for large pore gel.

(a) hydrochloric acid (1 $\underline{\underline{M}}$, 48 ml)

(b) TRIS added to give pH 7.0

(c) Urea (36.0g).

(d) TEMED (0.46 ml).

(e) water to 100 ml.

Solution 3. Electrode buffer.

(a) TRIS (6.0 g)

(b) glycine (28.8 g).

(c) water to 1 litre.

This solution (pH 8.3) was diluted 1:10 before use.

Solution 4. Small pore gel acrylamide solution.

(a) acrylamide (30.0g) (POISON)

(b) N,N'-methylene bis acrylamide (BIS) (0.735g).

(c) urea (36.0g).

(d) water to 100 ml.

Solution 5. Large pore gel acrylamide solution.

(a) acrylamide (10.0g).

(b) BIS (2.5g).

(c) urea (36.0g)

(d) water to 100 ml.

Solution 6. Ammonium persulphate initiator.

(a) ammonium persulphate (0.028g).

(b) water to 10 ml.

Solution 7. Riboflavin initiator.

(a) riboflavin (0.004g).

(b) water to 100 ml.

Solution 8. Sucrose solution.

(a) sucrose (40.0g).

(b) urea (36.0g).

(c) water to 100 ml.

The above solutions were kept at $2^{o}C$ and replaced every 4 months, with the exception of solution 6 which was freshly prepared for each run.

The working solutions were made up as follows:

A. small pore gel (7.5% acrylamide running gel).

(a) solution 1 (6 ml).

(b) solution 4 (6 ml).

(c) solution 6 (12 ml).

B. large pore gel (2.5% acrylamide stacking gel).

(a) solution 2 (3 ml).

(b) solution 5 (6 ml).

(c) solution 7 (3 ml).

(d) solution 8 (12 ml).

5.8.2. For electrophoresis at pH 6.5 the running buffer, stacking buffer and reservoir buffer (solutions 1,2 and 3) were replaced by the following:

Solution 1a.

(a) hydrochloric acid (1 M, 48 ml)

(b) 2,6-lutidine to pH 6.5

(c) TEMED (0.46 ml)

(d) water to 100 ml.

Solution 2a

(a) hydrochloric acid (1M, 48 ml)

(b) 2,6-lutidine to pH 5.2

(c) TEMED (0.24 ml).

(d) water to 100 ml.

Solution 3a

(a) di-sodium hydrogen orthophosphate (35.8g).

(b) orthophosphoric acid to pH 6.5.

The gels were cast according to the Shandon operating instructions. The stock solutions were de-aerated separately and then mixed in the appropriate proportions for the running gel solution A. The running tubes (75 mm x 5 mm i.d.) were filled with this solution to within 20 mm of the tops of the tubes. A layer of water was

carefully pipetted on to the gel solution surface. After polymerisation was complete (ca. 45 minutes) the water layer was discarded, the tubes rinsed with large pore gel solution and a 10 mm. depth of gel solution applied and polymerised in the same manner as above.

Samples (ca. 100µg in 40µl of stacking buffer, solution 2) were pipetted on to the gel surface and the tubes filled with the reservoir buffer.

Electrophoresis was carried out for 50-80 minutes at 5mA per running tube. The gels were extruded and stained.

5.8.3. Staining methods.

1. 0.1% w/v toluidine blue. Gels were soaked for 10-16 h, then washed repeatedly with water; intense blue bands denoted sulphated polysaccharides and weaker bands polyuronates.

2. Coomassie blue. The solution comprised:

(a) Coomassie blue (1 g)

(b) trichloroacetic acid (60 g)

(c) methanol (150 ml)

(d) water (320 ml)

The gels were soaked overnight and then washed repeatedly with ethanol:acetic acid:water (10:25:65 by volume). Blue bands appeared for proteins.

5.9. Cellulose acetate membrane electrophoresis[19]

Approximately 0.25 µl of sample solution (2-5 mg/ml) was streaked on to a cellulose acetate

membrane (Oxoid Ltd., 150 x 78 mm) and electrophoresis carried out in a Shandon SAE 3555 electrophoresis apparatus using one of the following electrolyte systems:

(a) 0.1M formate buffer, pH 4.0; current = 8mA for 30 min.

(b) 0.05M phosphate buffer, pH 7.2; current = 8mA for 40 mins.

(c) 0.2M $ZnSO_4$ buffer, pH 5.1; current = 8mA for 1h.

After electrophoresis the membrane was stained for 8 min in 1% Alcian Blue-8-GX in 1:1 v/v ethanol: 0.05M acetate buffer (pH 5.8). The excess stain was removed by repeated rinsing and vigorous agitation in an aqueous solution of 5% acetic acid and 10% ethanol (by volume) for 3 min periods until the background was completely white.

5.10. Assays and Analyses.

5.10.1. Carbohydrate content.

Carbohydrate content was assayed by the phenol-sulphuric acid method[20]. 4% aqueous phenol solution (1 ml) was added to water (1 ml) containing 10-100µg of sugar, and concentrated sulphuric acid (5 ml) added rapidly by automatic pipette. The samples were left at room temperature for 30 minutes and the absorbance at 487 nm was measured on a spectrophotometer.

Standard graphs were prepared for different sugars and mixtures of sugars in the ratios corresponding to those of the particular polysaccharide.

Uronic acid content was measured by two different methods.

5.10.2. Modified carbazole reaction.[10]

The samples, containing 10-70µg of uronic acid in 1 ml of water, were reacted at 55° and 100°C. with and without borate solutions.

Reaction without borate (at 55° and 100°C.)

The test solution (1 ml) was cooled in ice. Concentrated sulphuric acid (6 ml) was added carefully and the solution recooled. The sample was mixed and then heated for 20 minutes at the appropriate temperature and again cooled in ice. Carbazole solution (100µl of 0.1% solution in ethanol) was added and the solution mixed and allowed to stand for 3 h for colour development.

Reaction with borate at 100°C.

A 0.1M boric acid solution in concentrated sulphuric acid (6 ml) was cooled in ice and the test solution (0.7 ml) was added carefully. After mixing and recooling the solution was heated at 100°C for 15 minutes and then recooled in ice. Carbazole solution (200 l) was added, the solution mixed and heated for another 10 minutes and finally cooled; colour development was now complete.

Reaction with borate at 55°C.

The same procedure as above was used except heating before the addition of the carbazole reagent was omitted and afterwards the solution was

heated for 30 minutes at 55°C. to give complete colour development.

The absorbance was measured at 530 nm in a spectrophotometer. Standard graphs for different acids under each of the conditions were prepared.

5.10.3. Metahydroxydiphenyl method.[21]

To a sample solution (0.6 ml) containing 2-60µg of uronic acid, a 0.0125M 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°C for 5 minutes. After recooling in ice m-hydroxydiphenyl solution (50µl, 0.15% solution in 0.5% sodium hydroxide) was added. The solutions were mixed and the absorbance measured at 520 nm in a spectrophotometer. Standard graphs were prepared for the different uronic acids.

5.10.4. Nitrogen and protein content.

Nitrogen content was measured by the School of Pharmacy (University of London) and the protein content calculated by multiplying by 6.25.[22]

5.10.5. 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 h at 100°C. The nitric acid was then evaporated off. The residual solid was treated with concentrated hydochloric acid (1 ml) and evaporated to dryness again. After a further treatment with water (1 ml) and evaporation,

the sample was dried at 110°C for 2h. The sulphate content was then determined by the following modification of the Jones and Letham method.[23]

To the sulphate solutions (1 ml) containing 30-100µg of sulphate in microcentrifuge tubes, the reagent 4-chloro-4'-aminodiphenyl (1 ml of 0.19% in 0.1M hydrochloric acid) and a trace of solid hexadecyltrimethylammonium bromide were added. After mixing the solutions, including a blank, were kept for 2h and then centrifuged. Aliquots (0.2 ml) of the supernatants were removed and diluted to 25ml with 0.1M hydrochloric acid. The absorbances were measured at 254 nm on a spectrophotometer. The reduction in absorbance from the blank reading gave the sulphate content by comparison with a standard graph.

5.10.6. Thiobarbituric acid test (TBA).[24]

4,5 unsaturated acids (derived from degradation of uronic acids) were detected by a modified TBA test. The sample solution (0.2 ml) was added to 0.025M $NaIO_4$ in0.125M sulphuric acid (0.25ml). After 20 minutes at room temperature, 2% sodium arsenite in 0.5M hydrochloric acid (0.5 ml) was added with shaking and the solution left to stand for 2 minutes. 0.3% TBA (2 ml., pH2) was added and the mixture heated for 10 minutes at 100°C. After cooling the visible spectrum was scanned by spectrophotometer to detect any absorption maximum at 548 nm.

5.10.7. Degree of polymerisation.[25]

This was determined according to the Timell modification of the method of Peat et al. Three solutions were prepared as follows:

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

2. An aqueous sugar solution (0.5 ml) containing 60-80µg of carbohydrate) and 2% sodium borohydride (0.5ml)

3. A 2M sulphuric acid solution (0.5 ml) containing the same amount of carbohydrate as in 2 and 2% sodium borohydride solution (0.5ml)

These mixtures were left at room temperature for 20 h, after which each was assayed by the phenol-sulphuric acid method. Using solution 1 as a blank the DP is given by the relationship:

$$DP = \frac{(A)N}{(A)N-A(R)}$$

where,

(A)N = absorbance of non-reduced solution 3

(A)R = absorbance of reduced solution 2.

This equation is valid only for homopolymers; for heteropolymers the absorbances must be converted to the weight of carbohydrate read from an appropriate standard graph.

5.11. General reactions and preparations.

5.11.1. Preparation of 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 h and filtered. This was repeated three times, the third time with dry methanol. The resin was

stored under dry methanol.

5.11.2. Reduction of sugars to alditols.

The sample (20-25 mg) was dissolved in water or water/methanol (1:1 v/v) (3 ml) and a few mg of sodium borohydride added. The solution was left for 6h; if it was not alkaline after this time more sodium borohydride was added. After complete reduction (checked with Fehling's solution) the borohydride was destroyed by addition of IR120 (H^+). After filtration, the resultant boric acid was removed by repeated co-distillation with methanol followed by evaporation to dryness.

5.11.3. Acetylation of alditols.[27]

Samples of sugar alditols or partially methylated alditols, dried in a desiccator, were dissolved in pyridine/acetic anhydride (1:1 v/v) and heated at 100°C for 30 minutes. A small amount of toluene was added and the samples were evaporated to dryness at 40°C. The residues were dissolved in chloroform prior to analysis by g.l.c. and g.l.c.-m.s.

5.11.4. Esterification of uronic acids.

The sample was dried in vacuo over P_2O_5 and then refluxed in dry 3% methanolic hydrogen chloride for 18 h. in 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.

5.11.5. Reduction of uronic acids in polysaccharides.[28]

The polysaccharide (10-20 mg) was dissolved in water (10 ml) and the pH adjusted to 4.75 with dilute hydrochloric acid, solid 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (30 mg) was added slowly over a period of 10 min, with maintenance of the pH at 4.75 by addition of dilute hydrochloric acid. When consumption of acid was complete (about 30 min) sodium borohydride (2g) was added slowly with stirring. The solution was heated at 50°C for 2h, thencooled and the borohydride destroyed by dropwise addition of 2M hydrochloric acid. The reaction mixture was dialysed for 3 days and the reduced polymer recovered by freeze drying.

5.11.6. Preparation of dimethylsulphinyl carbanion.[29]

Potassium hydride (0.75g, i.e. 3g of 25% coated with mineral oil) was weighed into a 250 ml three-necked flask and washed with dry n-pentane (5 x 100ml portions). After the final washing the flask was fitted with a condenser and a fine bleed. The system was evacuated while the potassium hydride was stirred with a magnetic follower. Dry nitrogen was flushed into the flask, which was then evacuated again. The process was repeated six times to ensure a dry nitrogen atmosphere.

With dry nitrogen flushing through continuously,

dry DMSO (15 ml) was injected through the seal at the top of the condenser; vigorous evolution of hydrogen took place. The flask was heated on an oil bath at 55°C until the evolution ceased. The carbanion solution was transferred to a sealed flask and stored at 0°C under dry nitrogen.

5.11.7. Methylation of polysaccharides.

The Bjorndal and Lindberg modification[30] of the Hakomori method[31] was used. The polysaccharide (10-20 mg),previously dried over P_2O_5 in vacuo, was dissolved or swelled in dry DMSO (2 ml)in a serum bottle under nitrogen.

Dimethylsulphinyl carbanion (1 ml) was injected and the bottle agitated in an ultrasonic bath for 1h. The solution was then kept at room temperature for 6h.

Iodomethane (0.1ml) was injected dropwise with cooling by water and the bottle agitated in the ultrasonic bath for 20 min. A further 1 ml of carbanion was added and the bottle again agitated for 1h followed by standing at room temperature for 6h.

Iodomethane (1 ml) was added with cooling and the bottle agitated for 20 min. The solution was then poured into water (25 ml) and dialysed for 3 days. The polymer was recovered by freeze drying.

5.12. Periodate Oxidation.

The sample (2-4 mg/ml) was treated with 0.015M

sodium metaperiodate in the dark at 5°C; 5% n-propanol was added to minimise overoxidation.

Periodate consumption was measured by the method of Aspinall and Ferrier.[32] Aliquots (0.1ml) were withdrawn, diluted to 25 ml and the absorption measured at 222.5 nm. Aliquots of 0.015M $NaIO_4$ and 0.015M $NaIO_3$ were treated in the same manner to obtain a calibration curve.

The reaction was terminated by addition of ethane diol (0.1ml) and the derived polyaldehyde reduced overnight with $NaBH_4$ (50 mg). The borohydride was destroyed by adjusting the pH to 5.0 with glacial acetic acid. The pH was adjusted to 7.0 with dilute sodium hydroxide and the solution dialysed for 3-4 days. The resultant polyalcohol was recovered by freeze drying.

5.12.1. Determination of formic acid.[33]

An aliquot of the periodate oxidation solution after addition of ethane diol was diluted to 25 ml and nitrogen bubbled through for 15 min. The solution was titrated to pH 6.25 with 0.001M NaOH with nitrogen bubbling throughout. A blank of unoxidised polysaccharide in sodium iodate was titrated in the same manner.

5.12.2. Determination of acetaldehyde.[34]

Three Pyrex test tubes were connected in series as a gas absorption train. The sample solution (1 - 5 mg acetaldehyde in 5 ml) was placed in the

first tube and alanine (0.2g), M $NaHCO_3$ (4 ml), 0.1M $NaAsO_2$ (10 ml, containing 0.2g $NaHCO_3$) and a drop of Nujol were added. 5ml and 3ml respectively of 2% sodium metabisulphite were placed in the second and third tubes and diluted to 25ml.

Nitrogen was flushed through the tubes at about 1 l/min for 2h. The contents of the second and third tubes were then mixed and the excess bisulphite removed by addition of a slight excess of 0.1M iodine solution. The solution was just decolorised with dilute sodium thiosulphate solution. 10 ml of saturated $NaHCO_3$ solution were added and the liberated bisulphite titrated with 0.001M iodine to a starch end-point. Towards the end of the titration 1 ml of 10% Na_2CO_3 was added to ensure complete dissociation of the aldehyde-bisulphite compound.

5.13. Conversion of calcium alginate to sodium alginate.

The calcium alginate (typically 1.5g) was suspended in 0.5M HCl (250 ml) in a large sintered funnel, the base of which was filled with water. The suspension was stirred occasionally and after 3h the hydrochloric acid was filtered off. The process was repeated until no calcium could be detected in the filtrate. The solid was washed with water until nearly neutral. The solid was then suspended in water under vigorous stirring and titrated with 0.1M NaOH to pH7, when all the

alginic acid had dissolved. The solution was dialysed for two days and then freeze dried.

5.14 ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy.

The sample (50 - 100 mg in H_2O) was shaken with Dowex chelating resin (Sigma Chemical Co.) to remove traces of divalent cations. The resin was filtered off and the sample freeze-dried thrice; once from the H_2O and twice from 99.5% D_2O, to reduce the HOD signal in the proton spectrum.

^{1}H n.m.r. spectra were recorded at 400 MHz with a Bruker WH-400 spectrometer in the Fourier-transform mode with 32k data points. The spectral width was 4800Hz, acquisition time 3.4s and the pulse width 3 or 4 μs. Natural abundance, broad band proton-decoupled ^{13}C n.m.r. spectra were recorded at 100.6 MHz with a Bruker WH-400 spectrometer in the F.t. mode with 32k data points. The spectral width was 23800Hz, acquisition time 0.688s and pulse width 18μs. Sufficient scans were accumulated to obtain a good quality spectrum (usually 20000-80000).

Unless otherwise stated, all spectra were recorded at a probe temperature of 70-80°C to reduce viscosity-broadening of the resonances. Chemical shifts were expressed relative to external tetramethylsilane at room temperature.

5.15. Metal Ion Determination by Atomic Absorption Spectrophotometry.

Sample Preparation.

5.15.1. Acid digestion.

200 mg of dried, finely ground sample was placed in a 100 ml Kjeldahl flask and sulphuric acid (0.5 ml), 60% perchloric acid (1.0 ml) and nitric acid (5 ml) added. The flask was heated slowly until sulphuric acid refluxed down the side of the flask. When destruction of the organic matter was complete (about 15 min) the solution was cooled, transferred to a 50 ml volumetric flask and made up to volume with water.

5.15.2. Dry Ashing.

200 mg of dried, freshly ground sample was placed in a platinum crucible in a muffle furnace and the temperature raised to 450°C. After 3 h the residue was cooled and dissolved in 5M hydrochloric acid (5 ml). A few drops of nitric acid were added and the sample evaporated to dryness. The residue was redissolved in 5M hydrochloric acid (5 ml), warmed, filtered into a 50 ml volumetric flask and made up to volume with water.

5.15.3. Preparation of Standard Solutions.

The following were each made up to 1 litre with water.

(a) Calcium carbonate (2.497g) in M HCl (100 ml).

(b) Magnesium ribbon (1.000g) in 5M HCl (50 ml).

(c) Potassium chloride (dry) (1.905g) in water.

(d) Sodium chloride (dry) (2.542g) in water.

Aliquots of the stock solutions were taken and diluted appropriately to give series of solutions over the following ranges:

Calcium 0 - 20 ppm.

Magnesium 0 - 2.5 ppm.

Potassium 0 - 20 ppm.

Sodium 0 - 10 ppm.

To suppress interference effects, lanthanum (as lanthanum chloride) at 0.5% w/v was added to the calcium and magnesium solutions, and lithium chloride (to a concentration of 0.001M) to the potassium and sodium solutions. The samples to be determined were treated in the same manner.

5.15.4. Spectrophotometry.

Absorbances of blanks, standards and appropriately diluted samples were measured on a Pye Unicam SP90A Series 2 atomic absorption spectophotometer with air/acetylene flame and appropriate hollow cathode lamps at the following wavelengths:

Calcium 422.7 nm.

Magnesium 285.2 nm.

Potassium 766.5 nm.

Sodium 589.0 nm.

The metal ion concentrations of the samples were calculated by comparison with the standard curves. Each ion was determined eight times; two separate measurements on four samples, two prepared by dry ashing and two by acid digestion.

CHAPTER 5

INDIVIDUAL EXPERIMENTS.

First Sequential Extraction of Ascoseira

5.1. Preparation of the Alga

After removal of grit from the holdfast, approximately 400 g of the intact, wet alga were ground to a powder under liquid nitrogen and dried in a current of air. The dried powder (44 g) was soaked in 40% formaldehyde solution for 12 h, and the alga dried again. The dry alga was extracted as outlined in FLOW CHART 1 and described in the following experiments.

5.2. Ethanolic Extraction

The alga was extracted three times with 80% aqueous ethanol. The residue was centrifuged off each time and the combined extracts taken down to small volume and partitioned between toluene and water, with 5% n-butanol added to break the emulsion. The partition was repeated twice to give a pale yellow aqueous layer, and three dark green toluene solutions which were discarded. The aqueous layer was reduced to a small volume (Extract A).

5.3. Cold Aqueous Extraction

The residue was extracted three times with 2% calcium chloride solution at 20°C, the residue being centrifuged off each time. The combined extracts were concentrated and poured into six volumes of ethanol. The precipitate was centrifuged off, dissolved in water, dialysed until

free of chloride and freeze-dried (Extract B). The supernatant was concentrated and freeze-dried (Extract C).

5.4. Hot Aqueous Extraction

The residue was extracted five times with 2% calcium chloride solution at 70°C. The combined extracts were treated as above and the precipitate and supernatant isolated by freeze-drying (Extracts D and E respectively).

5.5. Acidic Extraction

The residue was extracted five times with dilute hydrochloric acid (pH 2.0) at 70°C. The combined extracts were neutralised with sodium hydroxide solution, concentrated and dialysed until free of chloride. The solution was freeze-dried, yielding Extract F. Each batch of dialysis water was checked for carbohydrate {GM 5.10.1}.

5.6. Alkaline Extraction

The residue was extracted seven times with 3% sodium carbonate solution at 70°C., the residue being centrifuged off after each extraction. The combined extracts were concentrated and poured into five volumes of ethanol. After centrifugation the supernatant was dialysed and freeze-dried (Extract G). The precipitate was dissolved in water and dialysed for three days. 2% calcium chloride solution was poured in slowly with constant

stirring until precipitation of calcium alginate was complete. The gelatinous precipitate was separated by centrifugation, washed with water and then dispersed in water and freeze-dried (Extract H). The supernatant was dialysed until free of chloride and freeze-dried (Extract I).

The residue was dried in air.

Investigation of the Extracts

5.7. Ethanolic Extract A

The salts which precipitated during the reduction of volume were filtered off and the remaining salts removed by treatment with Biodeminrolit (carbonate form). Mannitol was precipitated by six sequential additions of cold ethanol, the crystals being filtered off after each addition. The combined crystals were recrystallised from ethanol, dried and weighed, and melting point and mixed melting point measured. The supernatant was examined for carbohydrate {GM 5.10.1}, then concentrated and examined by paper chromatography {GM 5.4.1, 5.5}. An aliquot was converted to the alditol acetates and examined by g.l.c. {GM 5.11.2, 5.11.3, 5.6.1}.

5.8. Aqueous, Acidic and Alkaline Extracts B-G,I.

Extracts B-G and I were analysed for carbohydrate {GM 5.10.1}, uronic acid {GM 5.10.3}, sulphate {GM 5.10.5} and protein {GM 5.10.4}.

5.9. Fractionation of Extracts B,D,F and I.

Portions of the extracts (B-231 mg, D-403 mg, F-500 mg, I-153 mg) were dissolved or suspended separately in water (100 ml) and loaded on to DE52 cellulose columns. The columns were eluted with water and the eluted material freeze-dried in each case (Fractions BN, DN, FN and IN). The columns were eluted with KCl solution on a gradient running from 0 to 1.0M over 2 litres. Fractions (20 ml) were collected and analysed for carbohydrate {GM 5.10.1}. After inspection of the elution profiles, the fractions from extracts B,D and F were pooled into single components, dialysed and freeze-dried to give the acidic fractions BA, DA and FA respectively. The elution profile for I showed three bands, which were separated, dialysed and freeze-dried, yielding fractions IA1, IA2 and IA3.

5.10. Examination of Neutral Fractions

The neutral fractions BN, DN, FN and IN were analysed for carbohydrate {GM 5.10.1} and uronic acid {GM 5.10.3}. Portions of each were hydrolysed {GM 6.3.3.} and the hydrolysates examined by paper chromatography {GM 5.4.1, 5.5} and by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}.

5.11. Examination of Acidic Fractions

The six acidic fractions isolated above were analysed

for carbohydrate {GM 5.10.1}, uronic acid {GM 5.10.3}, sulphate {GM 5.10.5} and protein {GM 5.10.4}. Portions of each were hydrolysed {GM 5.3.3} and the hydrolysates examined by paper chromatography {GM 5.4.1, 5.5.} and by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}. The ^{13}C n.m.r. spectrum of Fraction BA was recorded {GM 5.14}.

5.12. Extracted Residue

A portion of the residue was hydrolysed {GM 5.3.3.}. The solid was filtered off and the filtrate analysed for carbohydrate {GM 5.10.1} and examined by paper chromatography {GM 5.4.1, 5.5.} and by g.l.c. as the alditol acetates{GM 5.11.2, 5.11.3, 5.6.1}.

5.13. Examination of Extract H.

A portion (1 g) was converted to the sodium salt {GM 5.13}. The HCl filtrates and the aqueous washings were checked for carbohydrate {GM 5.10.1}.

A portion of the sodium salt was hydrolysed and examined by paper chromatography {GM 5.4.1, 5.5} and by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}.

Large Scale Sequential Extraction of Ascoseira

5.14. Preparation of Alga

After removal of grit from the holdfast, about 1.5 Kg of the intact alga was ground to a powder under liquid nitrogen. The resultant damp material was soaked overnight in 40% formaldehyde solution, dried in a current of air and finally reground, giving 187 g. of dry powder.

5.15. Ethanolic Extraction

The powdered alga (180 g.) was extracted four times with boiling 80% aqueous ethanol (see FLOW CHART 2.2). The residue was recovered after each extraction by centrifugation. The combined extracts were reduced to a small volume and partitioned between toluene and water, with about 5% n-butanol to break the emulsion. The toluene layer containing chlorophyll and other pigments was discarded and the procedure repeated twice to give a colourless water/ethanol layer (Extract 1).

5.16. Aqueous Extraction

The residue from the ethanolic extraction was again treated with 40% aqueous formaldehyde and air dried. It was then extracted six times with aqueous 2% calcium chloride at 70°C (see FLOW CHART 2.2). The residue was recovered by centrifugation. The combined extracts were reduced to small volume and dialysed until chloride free; at this stage a white precipitate formed inside the dialysis sac. The precipitate was centrifuged off and the precipitate and supernatant freeze-dried separately. (Extracts 2 and 3 respectively).

5.17. Acidic Extraction

The residue was extracted with dilute hydrochloric acid (pH 2.0) at 70°C (see FLOW CHART 2.2). The residue was centrifuged off on each occasion and

the combined extracts reduced to a small volume and dialysed until free of chloride. Ethanol (6 volumes) was poured with stirring into the solution and the precipitate and supernatant recovered by centrifugation and freeze-drying (Extracts 4 and 5 respectively).

5.18. Alkaline Extraction

The residue was extracted seven times with 3% sodium carbonate at 70°C (see FLOW CHART 2.2). The residue was centrifuged off on each occasion, the extracts combined and reduced to a small volume and poured into 5 volumes of ethanol with constant stirring. After centrifugation the supernatant was dialysed and freeze dried (Extract 6). The solid was dissolved in water and dialysed for 3 days. 2% aqueous $CaCl_2$ was poured in slowly with constant stirring until precipitation of calcium alginate was complete. The gelatinous precipitate was separated by centrifugatation, rinsed with water and then dispersed in water and freeze dried (Extract 7). The supernatant was dialysed until chloride free and freeze dried (Extract 8). The residue was dried in air (77 g).

Investigation of the Extracts

5.19. Ethanolic Extract 1

The aqueous extract was reduced to a small volume, precipitating most of the salts which were filtered

off. The remaining salt was removed by treatment with Biodeminrolit(carbonate form). Mannitol was precipitated by six sequential additions of cold ethanol, the crystals being filtered off after each addition. The combined crystals were recrystallised from ethanol, dried and weighed and melting point and mixed melting point measured.

The supernatant was examined for carbohydrate {GM 5.10.1}, then concentrated and examined by paper chromatography {GM 5.4.1} with a number of locating agents {GM 5.5}. An aliquot was examined by g.l.c.{GM 5.6.1} as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}.

An aliquot was separated on 3 mm paper {GM 6.4.1}, slow-moving fragments eluted, hydrolysed {GM 5.3.3.} and examined as above.

5.20. Aqueous Extract 2

The extract was examined for carbohydrate {GM 5.10.1} and uronic acid {GM 5.10.3}. A portion was hydrolysed {GM 5.3.3.} and the hydrolysate was examined by paper chromatography {GM 5.41, 5.5} and by g.l.c. {GM 5.6.1} as the alditol acetates {GM 5.11.2, 5.11.3}.

5.21. Aqueous Extract 3

The carbohydrate content {GM 5.10.1}, uronic acid content {GM 5.10.3 }and protein content {GM 5.10.4} were measured. A portion was hydrolysed {GM 5.3.3.}

and the hydrolysate examined by paper chromatography {GM 5.4.1} and by g.l.c. {GM 5.6.1} as the alditol acetates {GM 5.11.2, 5.11.3}.

5.22 Fractionation of Extract 3

3.8 g of the extract were dissolved in water and fractionated on a large DE52 cellulose column {GM 5.7.1} (2 Kg. cellulose). The column was eluted with water to recover the neutral material 3N, which was isolated by freeze drying. The column was then eluted with 0.5M potassium chloride (2 litres). Fractions (20 ml) were collected and analysed for carbohydrate {GM 5.10.1}. After elution of all the carbohydrate, the fractions were pooled, reduced in volume, dialysed and freeze dried to give the acidic polysaccharide extract 3A. Finally the column was eluted with 250 ml of 2M KCl (with 1% w/v of ethylenediaminetetraacetic acid, sodium salt).

5.23. Neutral Fraction 3N

The carbohydrate {GM 5.10.1} and uronic acid {GM 5.10.3} contents were determined. A portion was hydrolysed {GM 5.3.3} and the hydrolysate examined by paper chromatography {GM 5.4.1, 5.5} and by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}.

^{1}H and ^{13}C n.m.r. spectra of the polymer were recorded {GM 5.14}.

A portion (30 mg) was subjected to periodate oxidation {GM 5.12} in 0.015M $NaIO_4$ (15 ml). On completion of the oxidation the formic acid released was determined by titration {GM 5.12.1}. The recovered polyalcohol was assayed for carbohydrate {GM 5.10.1}; a portion was hydrolysed {GM 5.3.3.} and the hydrolysate examined by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}.

A portion (15 mg) was methylated {GM 5.11.7}, hydrolysed {GM 15.3.1} and the hydrolysate converted to (partially methylated) alditol acetates and examined by g.l.c. {GM 5.11.2, 5.11.3, 5.6.1} and g.l.c./ms. {GM 5.62}.

5.24. Acidic Fraction 3A

The carbohydrate content {GM 5.10.1}, uronic acid content {GM 5.10.2} and sulphate content {GM 5.10.5} were determined.

Portions were hydrolysed {GM 5.3.1, 5.3.2, 5.3.3} and the hydrolysates examined by paper chromatography {GM 5.4.1, 5.5.} and g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}.

Portions were examined by gel electrophoresis {GM 5.8}, gel filtration {GM 5.7.5} and cellulose acetate electrophoresis {GM 5.9}.

^{1}H and ^{13}C n.m.r. spectra were run {GM 5.14}.

400 mg of the extract were subjected to gel filtration

in five separate runs on a G100 column (1.0m x 16 mm) of bed volume 200 ml. The fraction eluting in the void volume was recovered, dialysed and freeze dried giving a "fucan"-rich fraction 3AF. The carbohydrate content {GM 5.10.1}, uronic acid content {GM 5.10.2} and sulphate content {GM 5.10.5} of 3AF were determined. Portions were hydrolysed {GM 5.3.1, 5.3.3} and examined by paper chromatography and by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}.

A portion of 3AF was esterified {GM 5.11.4}, reduced {GM 5.11.2}, and divided into two parts which were hydrolysed {GM 5.3.1, 5.3.3} and examined by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}.

A portion was tested for 4,5 unsaturated acid {GM 5.10.6}.

5.25 Acidic Extract 4

The carbohydrate content {GM 5.10.1}, uronic acid content {GM 5.10.2}, protein content {GM 5.10.4} and sulphate content {GM 5.10.5} were determined.

Portions were hydrolysed {GM 5.3.1, 5.3.3} and examined by paper chromatography {GM 5.3.1, 5.5} and g.l.c. {GM 5.11.2, 5.11.3, 5.6.1}.

Portions were examined by gel electrophoresis {GM 5.8}, cellulose acetate electrophoresis {GM 5.9}

and gel filtration {GM 5.7.5}.

5.26. Acidic Extract 5

The carbohydrate {GM 5.10.1 }and uronic acid {GM 5.10.3 }contents were determined. A portion was hydrolysed {GM 5.3.3.} and the hydrolysate examined by paper chromatography {GM 5.4.1, 5.5} and by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}.

5.27. Alkaline Extract 6

The carbohydrate content was determined {GM 5.10.1}.

5.28. Alkaline Extract 7

A portion (1.5 g) was converted to the sodium salt {GM 5.13}. The HCl filtrates and the washings were combined, neutralised, dialysed and freeze dried (Fraction 7F). The viscosity of a 1% solution of the sodium salt was measured at 25°C in an Ostwald capillary viscometer..

A portion was subjected to gel filtration on Sephadex G100 {GM 5.7.5}.

^{1}H and ^{13}C n.m.r. spectra were recorded {GM 5.14}.

A portion was tested for the presence of 4,5-unsaturated acids {GM 5.10.6}.

5.29. Fraction 7F

The carbohydrate content {GM 5.10.1} and uronic acid content {GM 5.10.3} were measured. A portion was hydrolysed {GM 5.3.3} and examined by paper chromatography {GM 5.4.1, 5.5}, and by g.l.c. as

the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}.

5.30. Alkaline Extract 8

The carbohydrate content {GM 5.10.1}, uronic acid content {GM 5.10.3}, sulphate content {GM 5.10.5} and protein content {GM 5.10.4} were determined.

A portion was hydrolysed {GM 5.3.3} and the hydrolysate examined by paper chromatography {GM 5.4.1, 5.5} and by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}.

2 g of the extract were fractionated on a DE52 cellulose column. The neutral eluate (Fraction 8N) was isolated by freeze drying. The column was then eluted with 0.1M KCl (1 l.), followed by 0.5M KCl (1 l.) and the acidic fractions isolated by dialysis and freeze drying (Fractions 8A1 and 8A2 respectively).

5.31. Neutral Fraction 8N

The carbohydrate content was determined {GM 5.10.1}.
A portion was hydrolysed {GM 5.3.3} and examined by g.l.c. as the alditol acetates {GM 5.11.2,5.11.3, 5.6.1}.

30 mg were oxidised in 0.015M $NaIO_4$ (15 ml) {GM 5.12}.
On completion of the oxidation the formic acid released was determined by titration {GM 5.12.1}.
The recovered polyalcohol was analysed for carbohydrate {GM 5.10.1} and a portion hydrolysed {GM 5.3.3.} and the hydrolysate examined by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}.

A portion (15 mg) was methylated {GM 5.11.7}, hydrolysed {GM 5.3.3.} and the hydrolysate converted to alditol acetates {GM 5.11.2, 5.11.3} and examined by g.l.c./ ms. {GM 5.6.1} .

^{1}H and ^{13}C n.m.r. spectra were recorded {GM 5.14}.

5.32. Acidic Fraction 8A1

The carbohydrate {GM 5.10.1}, uronic acid {GM 5.10.2}, protein {GM 5.10.4} and sulphate {GM 5.10.5} contents were determined. A portion was hydrolysed {GM 5.3.3.} and the hydrolysate examined by g.l.c. {GM 5.11.2, 5.11.3, 5.6.1}.

5.33. Acidic Fraction 8A2

The carbohydrate {GM 5.10.1}, uronic acid {GM 5.10.3}, sulphate {GM 5.10.5} and protein {GM 5.10.4}contents were determined. A portion was hydrolysed {GM 5.3.3} and the hydrolysate examined by paper chromatography {GM 5.4.1, 5.5} and by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}.

Portions were examined by gel electrophoresis {GM 5.8}, cellulose acetate electrophoresis {GM 5.9}and gel filtration {GM 5.7.5}.

A portion (270 mg) was subjected to gel filtration in two separate runs on a Sephadex G100 column {GM 5.7.5}. The fraction eluting in the void volume and the remainder of the eluted material were separated and dialysed and freeze-dried separately (Fractions 8AF

and 8AA respectively). The carbohydrate {GM 5.10.1}, uronic acid {GM 5.10.2}, protein {GM 5.10.4} and sulphate {GM 5.10.5} contents were determined. Portions were hydrolysed {GM 5.3.3} and examined by g.l.c. {GM 5.11.2, 5.11.3, 5.6.1}.

Direct Alkaline Extraction

5.34. Extraction

17.2 g of dried, ground and formaldehyde-treated alga was extracted once with 1% aqueous sodium carbonate (400 ml containing 0.1% sodium hydroxide) for 2 h at 60°C (see FLOW CHART 2.3). The residue was removed by centrifugation and the solution neutralised with dilute hydrochloric acid and diluted to 800 ml. Calcium chloride solution (100 ml) was added with stirring to give a final $CaCl_2$ concentration of 2%. The gelatinous precipitate was centrifuged off, washed with dilute $CaCl_2$ solution and finally suspended in water and freeze dried (Alginate 1). The supernatant was dialysed for three days and then freeze-dried ("Fucan" 1).

The residue was extracted four times with 3% Na_2CO_3 solution at 70°C for 4 h. (see FLOW CHART 2.3). The combined extracts were neutralised and treated as above to give Alginate 2 and "Fucan" 2.

5.35. Examination of Alginate 1

A portion (1 g) of Alginate 1 was converted to the sodium salt {GM 5.13}. The viscosity of a 1%

solution of the sodium salt was measured in an Ostwald viscometer at 25°C. A portion was subjected to gel filtration on Sephadex G100 {GM 5.7.5}. The ^{1}H and ^{13}C n.m.r. spectra were recorded {GM 5.14}. A portion was examined for 4,5-unsaturated acids {GM 5.10.6}.

5.36. Partial Hydrolysis of Sodium Alginate 1

Sodium alginate 1 (120 mg) was dissolved in water (20 ml) and 2M hydrochloric acid added to a final concentration of 0.3M. The mixture was heated at 100°C for 5 h and then cooled. The solution and solid were separated by centrifugation the solid suspended in water, and both fractions neutralised with 0.1M sodium hydroxide. The uronic acid contents {GM 5.10.2}of the resultant solutions were determined and then the carbohydrate material precipitated from each by addition of ethanol (3 volumes). The precipitates were recovered by centrifugation, freeze-dried and weighed.

^{1}H n.m.r. spectra of both fractions were recorded {GM 5.14}. Both fractions were subjected to gel filtration on a Sephadex G100 column {GM 5.7.5}. The degree of polymerisation {GM 5.10.7} of the soluble fraction was determined.

Investigation of Antarctic Macrocystis Pyrifera Exudate

5.37. Removal of exudate from Macrocystis pyrifera

4 Kg of the intact alga was thawed and the extracellular mucilage allowed to drain from the alga. A total of 1.5 Kg of mucilage was recovered and freeze-dried.

5.38. General composition

1 l of the viscous liquid was freeze-dried, giving a buff solid. The carbohydrate content was determined {GM 5.10.1}.

A sample (200 mg) of the solid was ashed at 450°C to constant weight.

Samples (200 mg) were either acid digested {GM 5.15.1} or dry ashed {GM 5.15.2} and the residues analysed by atomic absorption spectrophotometry {GM 5.15.4}.

5.39. Isolation of "fucan"

25 g of the solid was dissolved in water and dialysed until free of chloride (checked by adding several drops of silver nitrate solution). The dialysis waters were retained, pooled and concentrated. The solution inside the membrane was adjusted to pH7 with dilute sodium hydroxide and freeze dried, giving"fucan" A.

5.40. Low molecular weight carbohydrates.

The carbohydrate content {GM 5.10.1} of the combined dialysis waters was determined. The solution was concentrated and the precipitated inorganic salts removed by filtration; the remaining salt was

removed by treatment with Biodeminrolit. The carbohydrate content was measured again and the solution concentrated to a syrup. Mannitol was precipitated with six sequential additions of cold ethanol, the crystalline mannitol being removed by filtration after each addition. The residual syrup was investigated by paper chromatography {GM 5.4.1, 5.5 } and by g.l.c. of the alditol acetate derivatives {GM 5.11.2, 5.11.3, 5.6.1 }.

5.41. "Fucan" A

The carbohydrate {GM 5.10.1}, uronic acid {GM 5.10.3}, sulphate {GM 5.10.5} and protein {GM 5.10.4} contents were determined. Samples were investigated by gel filtration {GM 5.7.5}, gel electrophoresis {GM 5.8} and cellulose acetate electrophoresis {GM 5.9}.

Samples were acid digested {GM 5.15.1} or dry ashed {GM 5.15.2} and the residues examined by atomic absorption spectrophot ometry {GM 5.15.4}.

5.42. Viscosity measurements

The viscosity of the liquid exudate was measured in an Ostwald capillary visometer at 25°C. The viscosity of the derived "fucan" A was measured as a 1% solution in the same manner.

Investigation of Californian Macrocystis Pyrifera Exudate (lyophilised)

5.43. General composition

A sample (200 mg) was heated to constant weight at 450°C in a platinum crucible.

A sample (200 mg) was dissolved in water (10 ml) and the carbohydrate content determined {GM 5.10.1}.

Samples (200 mg) were ashed and analysed for calcium, magnesium, potassium and sodium ions {GM 5.15.1, 5.15.2, 5.15.4}.

5.44 Isolation of "fucan"

25 g of the solid was dissolved in water and dialysed until free of chloride (checked by addition of silver nitrate solution). Each portion of dialysis water was retained and concentrated. The solution inside the membrane was adjusted to pH7 with dilute sodium hydroxide and freeze dried, giving "fucan" C.

5.45 Low molecular weight carbohydrates

The dialysis waters were pooled and the carbohydrate content determined {GM 5.10.1}. Inorganic salts were removed by concentrating the solution and filtering off the precipitated crystals; final traces were removed by treatment with Biodeminrolit. The carbohydrate content was again measured and the solution concentrated to a syrup. Mannitol was precipitated with six

sequential additions of cold ethanol, the crystalline mannitol being removed by filtration after each addition. The residual syrup was investigated by paper chromatography {GM 5.4.1, 5.5} and by g.l.c. of the alditol acetate derivatives {GM 5.11.2, 5.11.3, 5.6.1}.

5.46. "Fucan" C

The carbohydrate {GM 5.10.1}, uronic acid {GM 5.10.3}, sulphate {GM 5.10.5} and protein {GM 5.10.4} contents were determined.

Samples were investigated by gel filtration {GM 5.7.5}, gel electrophoresis {GM 5.8} and cellulose acetate electrophoresis {GM 5.15.4}.

5.47. Viscosity measurements

1.60 g of the solid were dissolved in 25 ml of water and the viscosity measured in an Ostwald capillary viscometer at 25°C. The viscosity of the derived "fucan" C was measured as a 1% solution in the same manner.

Sequential extraction of Macrocystis pyrifera

5.48. Preparation of alga

0.5 Kg of the alga from which the mucilage had been stripped was ground to a powder under liquid nitrogen. The resultant material was soaked overnight in 40% formaldehyde solution, dried in a current of air and finally reground to give 67 g of dry powder.

5.49. Cold aqueous extraction

The powdered alga (60g) was extracted five times with aqueous 2% $CaCl_2$ at 20°C (see FLOW CHART 4.1). The residue was recovered by centrifugation. The combined extracts were reduced to a small volume and poured into 6 volumes of ethanol. The precipitate was recovered by centrifugation, dissolved in water, dialysed and freeze dried (Extract 1). The supernatant was concentrated (Extract 2).

5.50. Hot aqueous extraction

The residue was extracted six times with aqueous 2% $CaCl_2$ at 70°C (see FLOW CHART 4.1).The residue was recovered by centrifugation. The combined extracts were reduced in volume, dialysed and freeze dried (Extract 3).

5.51. Acidic extraction

The residue was extracted six times with dilute hydrochloric acid (pH2) at 70°C (see FLOW CHART 4.1). The residue was recovered by centrifugation. The combined extracts were reduced in volume, dialysed and freeze dried (Extract 4).

5.52. Alkaline extraction

The residue was extracted six times with 3% sodium carbonate at 70°C (see FLOW CHART 4.1).The residue was centrifuged off on each occasion. The combined extracts were reduced to a small volume and poured

into 5 volumes of ethanol with constant stirring. After centrifugation the supernatant was dialysed and freeze dried (Extract 5). The precipitate was dissolved in water and dialysed for 3 days. 2% aqueous $CaCl_2$ was poured slowly into the solution with constant stirring until precipitation of calcium alginate was complete. The gelatinous precipitate was separated by centrifugation, rinsed with water and then dispersed in water and freeze dried (Extract 6). The supernatant was dialysed and freeze dried (Extract 7).

The residue was dried in air (21 g recovery).

Investigation of the extracts

5.53 Low molecular weight carbohydrates (Extract 2)

Precipitated salts were removed by filtration. The remaining salt was removed by treatment with Biodeminrolit (carbonate form). Mannitol was precipitated by six sequential additions of cold ethanol, the crystals being filtered off after each addition. The combined crystals were recrystallised from ethanol, dried and weighed, and melting point and mixed melting point measured.

The supernatant was examined for carbohydrate {GM 5.10.1}, then concentrated and examined by paper chromatography {GM 5.4.1}, with a number of locating agents {GM 5.5}.

An aliquot was analysed by g.l.c. {GM 5.6.1} as the alditol acetates {GM 5.11.2, 5.11.3}.

5.54. Aqueous Extract 1

The carbohydrate {GM 5.10.1}, uronic acid {GM 5.10.2}, sulphate {GM 5.10.5} and protein {GM 5.10.4} contents were measured.

Portions were hydrolysed {GM 5.3.1, 5.3.3} and the hydrolysates were examined by paper chromatography {GM 5.4.1, 5.5} and by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}.

Portions were examined by gel electrophoresis{GM 5.8}, cellulose acetate electrophoresis {GM 5.9}and gel filtration {GM 5.7.5}.

^{1}H and ^{13}C n.m.r. spectra were recorded {GM 5.14}.

5.55. Aqueous Extract 3

The carbohydate {GM 5.10.1}, uronic acid {GM 5.10.3}, sulphate {GM 5.10.5} and protein {GM 5.10.4} contents were determined.

A portion was hydrolysed {GM 5.3.3} and examined by paper chromatography {GM 5.4.1, 5.5} and by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}.

A portion was examined by cellulose acetate electrophoresis {GM 5.9}.

5.56. Acidic Extract 4

The carbohydrate {GM 5.10.1}, uronic acid {GM 5.10.3}, sulphate {GM 5.10.5} and protein {GM 5.10.4} contents

were determined.

Portions were hydrolysed {GM 5.3.1, 5.3.3} and the hydrolysates examined by paper chromatography {GM 5.4.1, 5.5 }and by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}.

The hydrolyses were repeated, but after removal of acid polymeric material was precipitated by addition of ethanol (6 volumes). The two polymers were hydrolysed again under the same acid conditions as the first hydrolysis, and, along with the supernatants, examined by g.l.c. as above.

Portions of extract 4 were examined by gel electrophoresis {GM 5.8 }, cellulose acetate electrophoresis {GM 5.9} and gel filtration {GM 5.7.5}.

A portion of Extract 4 (30 mg) was eluted from a Sephadex G100 column and, after inspection of the elution profile, the eluate was divided into two portions which were dialysed and freeze dried separately. The carbohydrate content {GM 5.10.1} and uronic acid content {GM 5.10.2} of each fraction were measured. Portions of each fraction were hydrolysed {GM 5.3.3.} and the hydrolysates examined by g.l.c. {GM 5.11.2, 5.11.3, 5.6.1}.

5.57. Alkaline extract 5

The carbohydrate content was measured {GM 5.10.1}.

5.58. Alkaline extract 6

A portion (1.5g) was converted to the sodium salt {GM 5.13}. The HCl filtrates and the washings were combined, neutralised, dialysed and freeze dried (Fraction 6F).

The viscosity of a 1% solution of the sodium salt was measured at 25°C in an Ostwald capillary viscometer. A portion was subjected to gel filtration {GM 5.7.5}.

The carbohydrate content {GM 5.10.1} of Fraction 6F was measured.

5.59 Alkaline extract 7

The carbohydrate {GM 5.10.1}, uronic acid {GM 5.10.2}, sulphate {GM 5.10.5} and protein {GM 5.10.4} contents were determined.

Portions were hydrolysed {GM 5.3.1, 5.3.3} and the hydrolysates examined by paper chromatography {GM 5.4.1, 5.5} and by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}.

Portions were examined by gel electrophoresis {GM 5.8}, cellulose acetate electrophoresis {GM 5.9} and gel filtration {GM 5.7.5}.

^{1}H and ^{13}C n.m.r. spectra were recorded {GM 5.14}.

5.60. Mild Acid Treatment of Extract 1

Extract 1 (750 mg) was dissolved in 0.02M hydrochloric acid (80 ml) and heated at 80°C for 1 h. The solution was cooled, neutralised with dilute sodium hydroxide solution and dialysed for 4 days, the dialysis water being checked for carbohydrate {GM 5.10.1}. Precipitated material was recovered by centrifugation and freeze-dried (Fraction C), and the solution freeze-dried. The latter material was eluted through a Sephadex G100 column {GM 5.7.5} in 3 portions. After inspection of the elution profile the combined eluates were separated into high molecular weight and low molecular weight portions (Fractions A and B), which were dialysed and freeze-dried. A portion of Fraction A (25 mg) was eluted through a Sepharose 4B column {GM 5.7.5} and, after inspection of the elution profile the eluate was separated into 3 portions which were dialysed and freeze-dried (Fractions A1, A2 and A3).

The five fractions were analysed for carbohydrate {GM 5.10.1}, uronic acid {GM 5.10.3} and protein {GM 5.10.4}. Portions of each were hydrolysed {GM 5.3.3} and examined by g.l.c. {GM 5.11.2, 5.11.3, 5.6.1}.

5.61 Alkaline Degradation of Extract 1

Extract 1 (300 mg) was dissolved in 2% aqueous sodium borohydride solution (50 ml) and left at

20°C for 20 h. Sodium hydroxide (2.4g) and sodium borohydride (1g) in 50 ml of water were added and the solution heated at 80°C for 8 h. The solution was cooled, neutralised and the carbohydrate content { GM 5.10.1 } measured. The solution was dialysed for 3 days, each change of water being checked for carbohydrate and uronic acid { GM 5.10.3 }. The polymer was recovered by freeze-drying and examined for carbohydrate { GM 5.10.1 }, uronic acid { GM 5.10.3 }, sulphate { GM 5.10.5 } and protein { GM 5.10.4} . A portion of the polymer was hydrolysed { GM 5.3.3 } and examined by paper chromatography { GM 5.4.1, 5.5 } and g.l.c. { GM 5.11.2, 5.11.3, 5.6.1 } . Portions were examined by gel electrophoresis { GM 5.9 } and gel filtration { GM 5.7.5} .

Direct Alkaline Extraction

5.62 Extraction

35 g of dried, ground and formaldehyde-treated alga was extracted once with 1% aqueous sodium carbonate (400 ml containing 0.1% sodium hydroxide) for 2 h at 60°C. (see FLOW CHART 4.2). The residue was removed by centrifugation and the solution neutralised with dilute hydrochloric acid and diluted to 800 ml. Calcium chloride solution (100 ml) was added with stirring to give a final $CaCl_2$ concentration of 2%. The gelatinous precipitate was centrifuged off, washed with dilute $CaCl_2$ solution and finally

suspended in water and freeze-dried (Alginate A). The supernatant was dialysed for three days and then freeze dried ("Fucan" A).

The residue was extracted four times with 3% Na_2CO_3 solution at 70°C for 4 h (see FLOW CHART 4.2). The combined extracts were neutralised and treated as above to give Alginate B and "Fucan" B.

5.63. Examination of Alginate A

A portion (1g) of Alginate A was converted to the sodium salt {GM 5.13}. The viscosity of a 1% solution of the sodium salt was measured in a Ostwald viscometer at 25°C. A portion was subjected to gel filtration {GM 5.7.5}. The ^{1}H and ^{13}C n.m.r. spectra were recorded {GM 5.14}.

5.64. Partial Hydrolysis of Sodium Alginate A

Sodium alginate A (127 mg) was dissolved in water (20 ml) and 2$\underline{\underline{M}}$ hydrochloric acid added to a final concentration of 0.3$\underline{\underline{M}}$. The mixture was heated at 100°C for 5 h and then cooled. The solution and solid were separated by centrifugation, the solid suspended in water and both fractions neutralised with 0.1$\underline{\underline{M}}$ sodium hydroxide. The uronic acid contents {GM 5.10.2} of the resultant solutions were determined and then the carbohydrate material precipitated from each by addition of ethanol (3 volumes). The precipitates

were recovered by centrifugation and separately freeze-dried and weighed. ^{1}H n.m.r. spectra of both fractions were recorded {GM 5.14}. Both fractions were subjected to gel filtration on a Sephadex G100 column {GM 5.7.5}. The degree of polymerisation {GM 5.10.7} of the soluble fraction was determined.

Large Scale Aqueous Extraction of Macrocystis Pyrifera

5.65. Extraction

Approximately 2 Kg of the alga (from which the mucilage had been removed) were soaked overnight in 40% formaldehyde solution and dried in a current of air. The alga was extracted 8 times with 2% calcium chloride (5 l); the extracts were strained through muslin, combined, reduced in volume, dialysed and freeze dried, giving 27g of crude "fucan". The carbohydrate {GM 5.10.1}, uronic acid {GM 5.10.3}, sulphate {GM 5.10.5} and protein {GM 5.10.4} contents were determined.

Portions of the fucan were examined by gel filtration {GM 5.7.5} and cellulose acetate electrophoresis {GM 5.9}. A portion was hydrolysed {GM 5.3.3} and the hydrolysate examined by paper chromatography {GM 5.4.1, 5.5} and by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}.

1 g of the extract was dissolved in water and fractionated on a DE52 cellulose column {GM 5.7.11}. The column was eluted with water (1 l) to remove the neutral material, which was recovered by freeze drying (Faction 1N).

The column was then eluted with $\underline{\underline{M}}$ KCl (2 l) and the polymer recovered by dialysis and freeze drying (Fraction 1A).

5.66. Neutral Fraction 5N

The carbohydrate content was measured {GM 5.10.1}. A portion was hydrolysed {GM 5.3.3} and the hydrolysate examined by g.l.c. {GM 5.6.1} as the alditol acetates {GM 5.11.2, 5.11.3}.

5.67. Acidic Fraction 5A

The carbohydrate {GM 5.10.1}, uronic acid {GM 5.10.3}, sulphate {GM 5.10.5} and protein {GM 5.10.4} contents were measured. A portion was hydrolysed {GM 5.3.3} and the hydrolysate examined by paper chromatography {GM 5.4.1, 5.5} and by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}.

Portions were examined by gel electrophoresis {GM 5.8} , cellulose acetate electrophoresis {GM 5.9} and gel filtration {GM 5.7.5} .

^{1}H and ^{13}C n.m.r. spectra were recorded {GM 5.14}.

5.68. Periodate Oxidation of Extract 5A

A portion (284 mg) was oxidised with 0.005 M $NaIO_4$ (100 ml) {GM 5.12} at 5°C in the dark. After 4 days the reaction was terminated and the polyaldehyde reduced, dialysed and freeze-dried. The dialysis water was checked for carbohydrate {GM 5.10.1}. The recovered polyalcohol was analysed for carbohydrate {GM 5.10.1 }, uronic acid { GM 5.10.3} , and sulphate {GM 5.10.5}. A portion was hydrolysed {GM 5.3.3.}and examined by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}. A portion was examined by gel filtration {GM 5.7.5}.

The remainder of the polyalcohol (175 mg) was treated with 0.5M trifluoroacetic acid at 20°C for 12 h. The acid was removed by co-distillation with methanol. The polymeric material was recovered by precipitation with 10 volumes of ethanol, followed by freeze-drying.

The supernatant was analysed for carbohydrate {GM 5.10.1} and uronic acid {GM 5.10.3}. A portion was evaporated to dryness and hydrolysed {GM 5.3.3}; the hydrolysate was examined by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}. A portion was examined by paper chromatography {GM 5.4.1, 5.5}.

The polymer was analysed for carbohydrate, uronic acid and monosaccharide composition as above. A portion was examined by gel filtration {GM 5.7.5}.

Partial Hydrolysis of "Fucan"

5.69. Hydrolysis

20 g of the fucan was hydrolysed three times with 0.04 $\underline{\underline{M}}$ oxalic acid (500 ml) for 20 min. at 100°C. After each hydrolysis the solution was dialysed against water (3 x 2 l) and each change of water was neutralised with $CaCO_3$. The neutralised dialysis water was centrifuged to remove calcium oxalate and then concentrated (Fraction A). The solution inside the dialysis membrane was dialysed exhaustively, centrifuged and the solid (Fraction B) and the supernatant (Faction C) were freeze-dried separately.

5.70. Fraction A. (Dialysate Fragments).

The carbohydrate content of the solution was determined {GM 5.10.1}.

The solution was loaded on to an anion exchange column {GM 5.7.2} and the column eluted firstly with water (1 l), then with 2$\underline{\underline{M}}$ formic acid (500 ml) and finally with a gradient of sulphuric acid running from 0 to 1$\underline{\underline{M}}$ over 2 l.

5.71 Neutral eluate

The carbohydrate content {GM 5.10.1} was determined. A portion was concentrated to a syrup and examined by paper chromatography {GM 5.4.1, 5.5} , and by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}. A portion of the syrup was hydrolysed {GM 5.3.3} and examined by g.l.c. as the alditol

acetates {GM 5.11.2, 5.11.3, 5.6.1}.

Approximately half the syrup was streaked on to a sheet of Whatman No 17 paper and the paper run in solvent 18:3:1:4 {GM 5.4.1} for 20 h. The eluate running off the bottom of the paper was collected and the solvent removed (Fraction 10). Nine bands were cut from the paper and eluted with water. The carbohydrate content {GM 5.10.1} of all ten fractions was measured. Portions of fractions 6 - 9 (containing only monosaccharides) were converted to alditol acetates {GM 5.11.2, 5.11.3}; portions of fractions 1 - 5 were first hydrolysed {GM 5.3.3} before conversion. All ten fractions were analysed by g.l.c. {GM 5.6.1}. The degrees of polymerisation of fractions 2 - 4 were determined {GM 5.10.7}.

5.72 Formic acid eluate

The carbohydrate content was determined {GM 5.10.1}. A portion was concentrated to a syrup (after removal of formic acid by co-distillation with methanol) and examined by paper chromatography {GM 5.4.1, 5.5}.

The remainder was fractionated on No.17 paper in solvent system 18:3:1:4. for 24 h. The monosaccharides were located and removed as one band and the carbohydrate content determined {GM 5.10.1}. Two dominant oligomeric bands were located and

eluted separately with water. The carbohydrate content of each was determined {GM 5.10.1}. Two portions of each were taken; one portion was esterified {GM 5.11.4}, reduced {GM 5.11.2} and hydrolysed {GM 5.3.3} in each case, and the other portion directly hydrolysed {GM 5.3.3}. The four hydrolysates were examined by g.l.c. {GM 5.6.1} as the alditol acetates {GM 5.11.2, 5.11.3}. The degrees of polymerisation of the two oligomeric bands were determined {GM 5.10.7}.

5.73. Sulphuric acid eluate

Fractions (20 ml) were collected from the column and analysed for carbohydrate {GM 5.10.1}. Inspection of the elution profile indicated two broad, overlapping bands. The fractions containing the two peaks were separately pooled, neutralised with barium hydroxide and centrifuged to remove barium sulphate. The two peaks (A1 and A2, in order of elution) were converted to sodium salts by passage through a column of IR120 (Na^+) and recovered as solids by freeze-drying.

The carbohydrate content of A1 and A2 was determined {GM 5.10.1}. Portions of each were hydrolysed {GM 5.3.3}and the hydrolysates examined by paper chromatography {GM 5.4.1, 5.5} and by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}.

Portions of each were examined by paper chromatography { GM 5.4.1, 5.5 } , paper electrophoresis {GM 5.4.2}and gel filtration { GM 5.7.5 }.

The degree of polymerisation of each was determined { GM 5.10.7 }.

Portions of both were subjected to periodate oxidation { GM 5.12 }. On completion of the oxidation, the formic acid { GM 5.12.1 } and acetaldehyde released { GM 5.12.2 } were determined.

^{1}H and ^{13}C n.m.r. spectra of A1 and A2 were recorded { GM 5.14 } .

5.74. Fraction B (Insoluble Residue)

The carbohydrate { GM 5.10.1 } , uronic acid {GM 5.10.3} , sulphate {GM 5.10.5} and protein {GM 5.10.4} contents were determined.

A portion was hydrolysed {GM 5.3.3} and the hydrolysate examined for uronic acid {GM 5.10.3} and then examined by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}.

The ash content was determined by combustion to constant weight in a muffle furnace at 450°C.

5.75. Fraction C (Soluble Residue)

The carbohydrate {GM 5.10.1}, uronic acid {GM 5.10.3}, sulphate {GM 5.10.5} and protein {GM 5.10.4} contents were determined.

A portion was hydrolysed {GM 5.3.3} and the hydrolysate

examined by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}.

Portions were examined by gel electrophoresis {GM 5.8}, cellulose acetate electrophoresis {GM 5.9} and gel filtration {GM 5.7.5}.

A portion of Fraction C (40mg) was eluted from a Sephadex G100 column and, after inspection of the elution profile, the eluate was divided into three portions which were dialysed and freeze dried separately. Portions of each sub-fraction were hydrolysed {GM 5.3.3} and the hydrolysates examined by g.l.c. {GM 5.11.2, 5.11.3, 5.6.1}.

Second Partial Hydrolysis

5.76. Hydrolysis

Fraction C (1.5g) was hydrolysed with 0.1M oxalic acid (100 ml) for 1 h at 100°C. The hydrolysate was dialysed against water (3 x 2 l) and each change of water was neutralised with $CaCO_3$. The neutralised dialysis water was centrifuged to remove calcium oxalate and concentrated (Fraction D). The contents of the dialysis sac were dialysed exhaustively, centrifuged and the solid (Fraction E) and the supernatant (Fraction F) were freeze dried separately.

5.77. Fraction D (Dialysate Fragments)

The carbohydrate {GM 5.10.1} and uronic acid {GM 5.10.3} contents were determined.

A portion was examined by paper chromatography {GM 5.4.1, 5.5}.

A portion was hydrolysed {GM 5.3.3} and examined by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}.

The remainder was fractionated on an anion exchange column (formate form). The column was eluted successively with water (1 l), 2$\underline{\underline{M}}$ formic acid (500 ml) and 0.5$\underline{\underline{M}}$ sulphuric acid (500 ml).

5.78. Neutral Eluate

The carbohydrate content {GM 5.10.1} was determined.

A portion was concentrated to a syrup and examined by paper chromatography {GM 5.4.1, 5.5}. The remainder of the syrup was hydrolysed {GM 5.3.3} and examined by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}.

Half the eluate was concentrated and fractionated on a sheet of Whatman No.17 paper in solvent system 6:4:3 {GM 5.4.1} for 36 h. Six bands were cut and eluted with water and the carbohydrate content {GM 5.10.1} and degree of polymerisation {GM 5.10.7} of each fraction (1 - 6) determined. Portions of fractions 1 - 4 (the slowest four bands) were hydrolysed {GM 5.3.3} and examined by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}. Portions of fractions 1 - 4 were

methylated {GM 5.11.8}, hydrolysed {GM 5.33}, converted to alditol acetates {GM 5.11.2, 5.11.3} and examined by g.l.c. {GM 5.6.1}.

5.79. Formic acid eluate

The carbohydrate content {GM 5.10.1} and uronic acid content {GM 5.10.3} were determined.

A portion was hydrolysed {GM 5.3.3} and the hydrolysate examined by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}.

A portion was esterified {GM 5.11.4}, reduced {GM 5.11.2}, hydrolysed {GM 5.3.3} and examined by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}.

5.80. Sulphuric acid Eluate

The carbohydrate {GM 5.10.1} and uronic acid {GM 5.10.3} contents were determined. The eluate was neutralised with barium hydroxide, centrifuged to remove barium sulphate, and concentrated. A portion was hydrolysed {GM 5.3.3} and examined by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3, 5.6.1}.

5.81. Fraction E (Insoluble Residue)

The carbohydrate {GM 5.10.1}, uronic acid {GM 5.10.3}, sulphate {GM 5.10.5} and protein {GM 5.10.4} contents were determined.

A portion was hydrolysed {GM 5.3.3} and examined by g.l.c. as the alditol acetates {GM 5.11.2, 5.11.3,5.6.1}.

A portion was esterified { GM 5.11.4}, reduced { GM 5.11.2}, hydrolysed { GM 5.3.3 } and examined by g.l.c. as the alditol acetates { GM 5.11.2, 5.11.3, 5.6.1}.

5.82 Fraction F (Soluble Residue)

The carbohydrate { GM 5.10.1 }, uronic acid { GM 5.10.3 }, sulphate { GM 5.10.5 } and protein { GM 5.10.4} contents were determined.

A portion was hydrolysed { GM 5.3.3} and the hydrolysate examined by g.l.c. as the alditol acetates { GM 5.11.2, 5.11.3, 5.6.1} .

Portions were examined by gel electrophoresis { GM 5.8 }, cellulose acetate electrophoresis { GM 5.9} and gel filtration { GM 5.7.5} .

^{1}H and ^{13}C n.m.r. spectra of F were recorded { GM 5.14} .

A portion was esterified {GM 5.11.5}, reduced {GM 5.11.2}, methylated {GM 5.11.7}, hydrolysed {GM 5.3.1}, converted to alditol acetates {GM 5.11.2, 5.11.3} and examined by g.l.c. {GM 5.6.1} and g.l.c./m.s. {GM 5.6.2}.

A portion (191 mg) was subjected to periodate oxidation at 5°C {GM 5.12} and the periodate consumption measured. After 96 h the oxidation was terminated and the polyalcohol recovered by

dialysis and freeze-drying. The carbohydrate {GM 5.10.1} and uronic acid {GM 5.10.3} contents of the polyalcohol were determined. A portion was hydrolysed {GM 5.3.3} and examined by g.l.c. {GM 5.11.2, 5.11.3, 5.6.1}.

A portion (108 mg) of the polyalcohol was subjected to a Smith Degradation in 0.05 $\underline{\underline{M}}$ H_2SO_4 at 20°C for 20 h. The resultant solution was neutralised with dilute sodium hydroxide and dialysed. The carbohydrate {GM 5.10.1} and uronic acid {GM 5.10.3} contents of the dialysis waters were checked. The first dialysis water was concentrated, deionised and examined by g.l.c. {GM 5.11.2, 5.11.3, 5.6.1}. The contents of the dialysis membrane were freeze dried and examined for carbohydrate {GM 5.10.1} and uronic acid {GM 5.10.3} . A portion was hydrolysed and examined by g.l.c. {GM 5.11.2, 5.11,3, 5.6.1}.

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