SOME SOUTH AFRICAN RED SEAWEED POLYSACCHARIDES

A Thesis

Presented to the University of Cape Town

For the Degree of

Doctor of Philosophy

By

Abraham Lionel Clingman

August 1958

CONTENTS

INTRODUCTION

Algae have been classified by botanists into four large groups: the Chlorophyceae or green algae, the Phaeophyceae or brown algae, the Rhodophyceae or red algae, and the Cyanophyceae or blue-green algae. The polysaccharides which are extracted from marine algae may be differentiated into reserve polysaccharides, analogous to starch in land plants, and into structural polysaccharides, analogous to cellulose in land plants. Laminarin from brown seaweeds and Floridean starch from certain red algae are reserve polysaccharides while alginates (from brown seaweeds) and carrageenin and agar (from red seaweeds) are structural polysaccharides.

The most commonly encountered algal polysaccharides, besides alginic acid, are agar and carrageenin. These are salts of sulphate esters of polysaccharides which contain D-galactose. Agar and carrageenin mucilages are obtained by aqueous extraction from certain red seaweeds of the class Florideae. Agar is extensively used in the meat canning and confectionery trades where it has to a very large extent replaced gelatin. Nearly all the South African production of agar is used in this way. Carrageenin is used in brewing as a clarifying agent, as a stabilising agent in cocoa and in a large number of pharmaceutical products.

AGAR / ...

AGAR

Agar is a gel-forming polysaccharide used for bacteriological culture purposes. It appears to be a linear polysaccharide of number-average molecular weight (for the acetate) between 34,000 and 93,000, with possibly a small component having values of several million[1]. Agar is extracted with hot water from some species of the Florideae such as Gelidium amansii, G. cartilagineum, G. japonicum, G. subcostatum and Gracilaria confervoides[2]. It has been defined as the dried non-nitrogenous extract from Gelidium and other red algae which is the sulphuric acid ester of a linear galactan, and which is insoluble in cold but soluble in hot water[3]. A dilute neutral solution (1-2 per cent) of agar sets upon cooling to a firm gel solidifying at 35-50° and melting at 90-100°.

Early chemical investigations showed that agar is a galactan[4] and that it contains ester sulphate associated with calcium ion[5]. Electrodialysis of agar[6] removes the calcium ions leaving an agar acid which does not set to a gel when cooled, but which after neutralisation with an inorganic or organic base again yields a gel. Percival[7] and Barry and Dillon[8] obtained pure samples of agar from Gracilaria confervoides, Gelidium crinale and Gelidium latifolium which contained only small amounts of sulphate, 1.3, 1.4 and 1.9 per cent respectively.

Agar was acetylated[9] and was converted into the methyl ether, hydrolysis of which gave 2:4:6-tri-O-methyl-D-galactopyranose in 65 per cent yield, based on the methylated agar; it was thus shown that agar contains D-galactopyranose units linked through $C_{(1)}$ and $C_{(3)}$. No 2:3:4:6-tetra-O-methyl-D-galactopyranose was detected in the hydrolysate.

From a mixture obtained by remethylating the hydrolysis products of methylated agar[10,11] a second sugar, methyl 2:4-di-O-methyl-3:6-anhydro-β-L-galactoside was isolated. This demonstrated the presence of 3:6-anhydro-L-galactose units in agar. Pirie[12] had

acetolysed / ...

acetolysed agar and had isolated from the products hepta-O-acetyl-D,L-galactose in 10 to 20 per cent yield. Subsequently it was shown[13] that acetolysis of methyl 3:6-anhydro-β-D-galactopyranoside yields hepta-O-acetyl-D,L-galactose. The hepta-O-acetyl-D,L-galactose obtained from acetolysed agar was therefore probably derived from 3:6-anhydro-L-galactose units in the agar molecule. Forbes and Percival[14] obtained methyl 2:4-di-O-methyl-3:6-anhydro-β-L-galactoside in 10 to 13 per cent yield from methylated agar. These workers also obtained ca. 16 per cent of laevulinic acid from the hydrolysate of methylated agar and they showed that methyl 3:6-anhydrogalactoside, whether methylated or not, was decomposed by dilute acid to laevulinic acid.

Percival and Thomson[15] acetolysed methylated agar and oxidised the reaction products and so obtained a mixture of disaccharied acids, from which after further methylation and hydrolysis they isolated 2:3:4:6-tetra-O-methyl-D-galactose (I) and 2:5-di-O-methyl-3:6-anhydro-L-galactonic acid (II); it was thus shown that the 3:6-anhydro-L-galactose residues were linked through positions $C_{(1)}$ and $C_{(4)}$ in the polysaccharide.

Araki[16] methanolysed methylated agar and obtained 2-O-methyl-3:6-anhydro-L-galactose dimethyl acetal. The presence of a link to position $C_{(4)}$ of the 3:6-anhydro-L-galactose units in the agar molecule was further confirmed by the methylation studies of Jones and Peat[17]. Taking into consideration the then known information about agar and noting the negative specific rotation of agar, Jones and Peat[17] postulated that the agar molecule was built up of a repeating unit of nine 1 ⟶ 3-linked β-D-galactopyranose units terminated by an L-galactopyranose 6-sulphate residue linked through $C_{(4)}$ (III). Since no tetramethylgalactose could be found in the hydrolysate of methylated agar[9,15,17], the proposed structural unit, it was pointed out, must be joined with many other such units in a very long linear chain. Jones and Peat[17] also suggested that as the alkaline hydrolysis

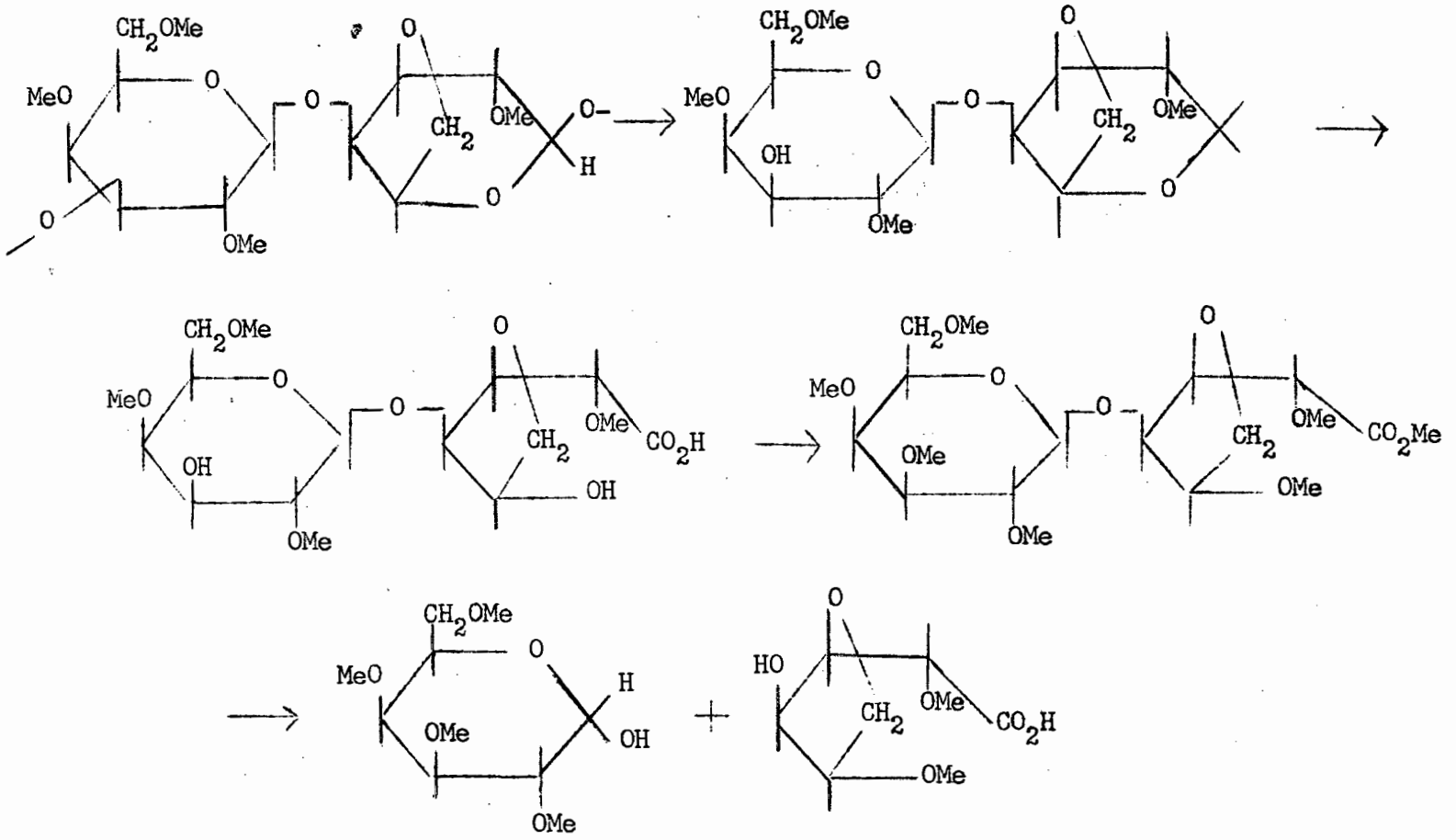
of / ...

of methyl 6-sulphate-D-galactoside readily yields methyl 3:6-anhydro-D-galactoside[18] therefore alkaline methylation of agar should cause the sulphate group to be removed from the L-galactose unit with the formation of a 3:6-anhydro-L-galactose unit.

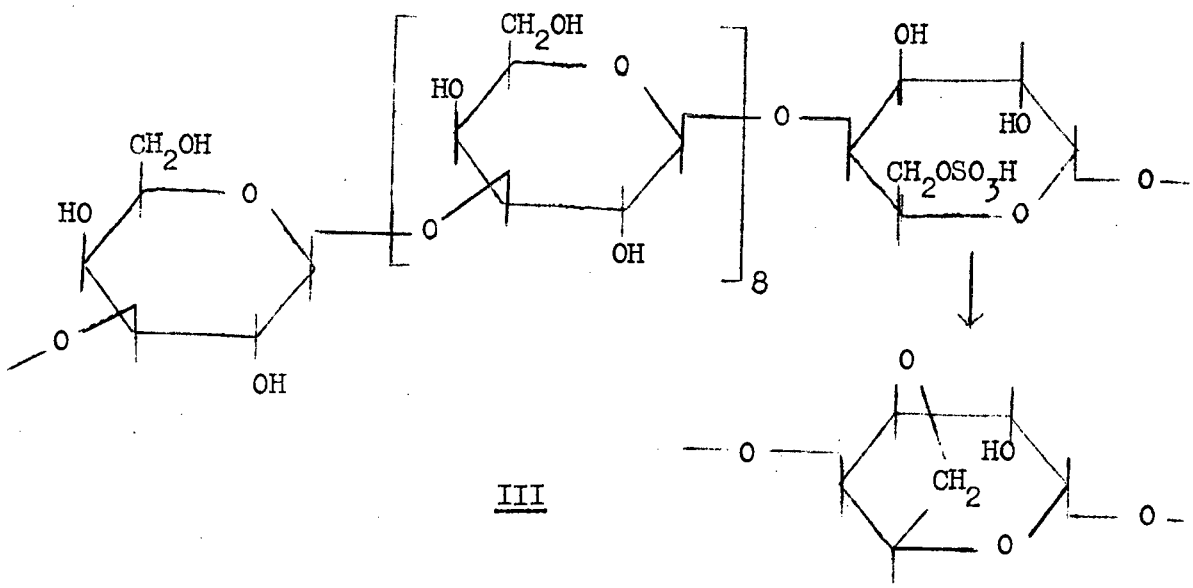
Jones and Peat[17] proposed a scheme whereby D-galactose 1-sulphate (IV) substituted at $C_{(3)}$ could be converted by a simple intramolecular oxidation-reduction change into L-galactose 6-sulphate (V). Consequently they suggested that the starting material in the bio-synthesis of agar might be D-galactose 1-sulphate (IV), from which a chain of 1 $\longrightarrow$ 3-linked D-galactose units would be formed. When ten such D-galactose units had been joined a change in the synthetic mechanism would take place and the tenth unit still bearing its sulphuric acid group would undergo an intramolecular transformation into a L-galactose 6-sulphate unit, now linked at $C_{(4)}$.

It has long been difficult to accept the agar formula (III) proposed by Jones and Peat[17]. The sulphate content of agar[7,8] (1 to 1.5 per cent) is too low to be accounted for by the proposed repeating unit (one L-galactose 6-sulphate residue to nine D-galactose residues) which would require a sulphate content of 5.4 per cent. There is however the possibility, during the life of the seaweed, of a gradual removal of sulphate groups from agar with the resultant formation of 3:6-anhydrogalactose units in the polysaccharide, this followed in turn by the formation of ester sulphates on other galactose residues so that the sulphate content of agar always remains constant[7].

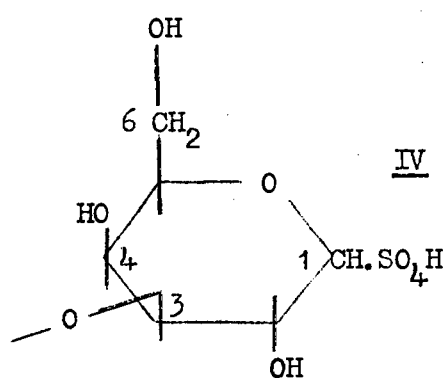
In the proposed formula (III) every L-galactose 6-sulphate unit linked to the chain through $C_{(1)}$ and $C_{(4)}$ would contain a pair of adjacent -CHOH groups. Now agar has been shown to be completely resistant to periodate oxidation[8]. The absence of α-glycollic groups in agar constitutes evidence for the existence of 3:6-anhydro-L-galactose linked through $C_{(4)}$ in the agar molecule though the possible presence of sulphate groups on $C_{(3)}$ of some L-galactose units cannot be entirely eliminated.



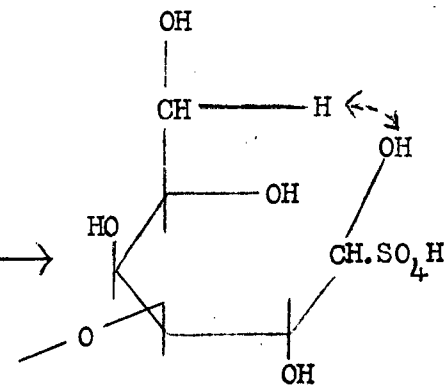
I II

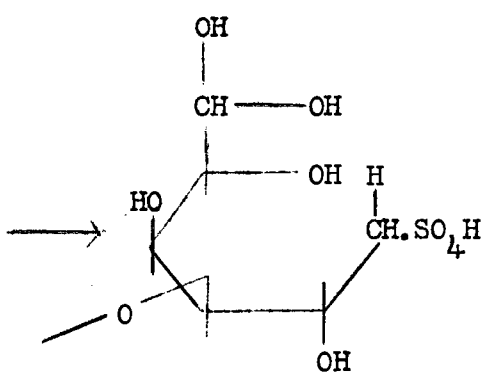


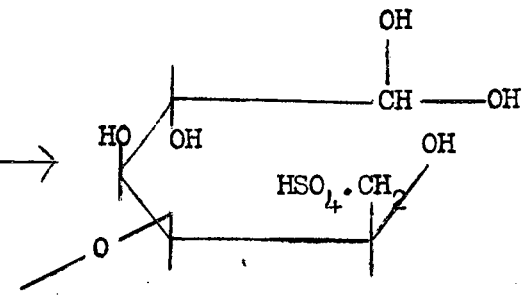
III

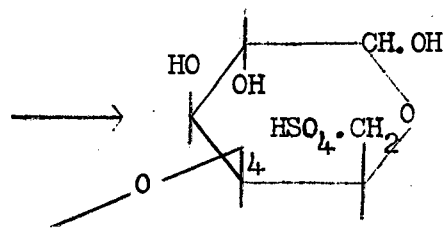


IV









V

Furthermore the proposed formula (III) for agar contains D- and L-galactopyranose units in the ratio of 9 to 1. Percival and Thomson[15], having obtained an 11.5 per cent yield of methyl 2:4-di-O-methyl-3:6-anhydro-β-L-galactoside from hydrolysed methylated agar, found that the ratio of D- to L-galactopyranose units was closer to 15:2 (i.e., 88.5 tri-O-methyl-D-galactose : 11.5 di-O-methyl-3:6-anhydro-L-galactose). Moreover methyl 3:6-anhydrogalactosides undergo drastic decomposition in the presence of dilute acids[14] which fact would suggest an even higher proportion of 3:6-anhydrogalactose in the original agar molecule. Indeed, when agar was completely methanolysed[19] it gave 3:6-anhydro-L-galactose dimethyl acetal in 17-20 per cent yield.

The acetyl and methoxyl values that have been found for acetylated and methylated agar respectively are lower than would be expected for the proposed agar molecule (III). A typical agar acetate sample contained 39.0 per cent acetyl[9], where the formula (III) requires 43.4 per cent acetyl; similarly a methylated agar gave 30.9 per cent methoxyl content as against 43.5 per cent methoxyl calculated from fully methylated (III). Percival and Thomson[15] also found some dimethyl galactose derivatives in the hydrolysate of methylated agar: this may have been due either to incomplete methylation of the agar or else it may indicate some branching in the agar molecule.

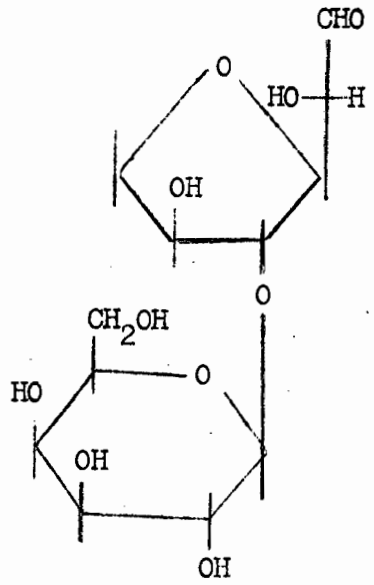
Work on the structure of agar remained at a standstill at this point for a number of years when the matter was taken further by Japanese workers. They were able to obtain a disaccharide, agarobiose (VI), from hydrolysed agar[19]. After methanolysis it yielded methyl D-galactopyranoside and 3:6-anhydro-L-galactose dimethyl acetal[20]. Agarobiose was converted to its dimethyl acetal which was methylated and then methanolysed to give methyl 2:3:4:6-tetra-O-methyl-D-galactoside and 2:5-di-O-methyl-3:6-anhydro-L-galactose dimethyl acetal[20]. Agarobiose was therefore shown to be 3:6-anhydro-4-O-β-D-

galactopyranosyl- / ...

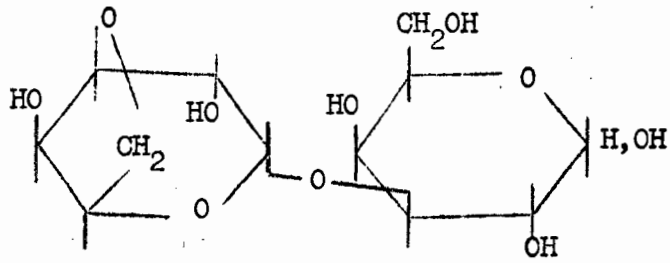
galactopyranosyl-L-galactose. This constitutes further proof that the 3:6-anhydro-L-galactose units are attached in the agar molecule through $C_{(4)}$. A β-linkage was assumed for the disaccharide because of its low specific rotation. Partial methanolysis of agar[21] yielded agarobiose dimethyl acetal together with some 3:6-anhydro-L-galactose dimethyl acetal and methyl D-galactoside; on the basis of the yields obtained of agarobiose and its break-down products, agarobiose (as $C_{12}H_{18}O_9$) was calculated to constitute at least 69 per cent of the reacted agar.

In an earlier series of experiments agar was exhaustively mercaptolysed[22] to yield D-galactose diethylmercaptal (30.2 per cent), 3:6-anhydro-L-galactose diethylmercaptal (24.4 per cent), and D,L-galactose diethylmercaptal (1.0 per cent). Partial mercaptolysis of agar[23] gave agarobiose diethylmercaptal (46.2 per cent of agar starting material).

After the conclusion of the work on agar to be described in this thesis, further information became available from the Japanese workers. An enzymic extract from the agar-digesting bacterium Pseudomonas kyotoensis was used to hydrolyse agar[24], producing a mixture of four reducing oligosaccharides. A crystalline disaccharide, neo-agarobiose (VII), was separated from the mixture in 28 per cent yield of the total hydrolysate. The compound was rapidly hydrolysed by very dilute acid to give D-galactose and 3:6-anhydro-L-galactose. Sodium borohydride reduction of the disaccharide followed by methanolysis gave 3:6-anhydro-L-galactose dimethyl acetal and dulcitol. Neo-agarobiose was shown to be 3-O-3:6-anhydro-α-L-galactopyranosyl-D-galactose by methylation and hydrolysis investigations. Since neo-agarobiose has a positive specific rotation which mutorotates downwards the glycosidic link was assumed to be α. More recently the isolation and identification of neo-agarotetraose obtained from the products of enzymic hydrolysis of agar has been reported[24a].



VI



VII

A1 $\xrightarrow{\alpha}$ 3Ga1 $\xrightarrow{\beta}$ 4A1 $\xrightarrow{\alpha}$ 3 Ga1 $\xrightarrow{\beta}$ 4A1 $\xrightarrow{\alpha}_{n}$ 3Ga

A = 3:6-anhydro-L-galactopyranose

Ga = D-galactopyranose

VIII

In conclusion it has been claimed[25] that agar is really composed of two polysaccharides, agarose (VII), the acetate and methyl ether of which are soluble in chloroform, and agaropectin, having its acetate and methyl ether insoluble in chloroform. Agaropectin is assumed to be a complicated polysaccharide containing uronic acid and sulphate as well as D-galactose and 3:6-anhydro-L-galactose

as / ...

as constituents. Agarose, the principal polysaccharide then consists of alternate 1 → 3-linked β-D-galactopyranose units and 1 → 4-linked 3:6-anhydro-α-L-galactopyranose units. It is believed that the agarose chain is terminated at the non-reducing end by a 3:6-anhydro-L-galactose residue and at the reducing end by a D-galactose residue, since neither D-galactose nor 3:6-anhydro-L-galactose was detected in the enzymic hydrolysate of agar which involved cleavage of β-glycosidic links.

The proposed structure for agarose does fit the recorded facts. Hydrolysis of methylated agarose would yield 60 per cent of 2:4:6-tri-O-methyl-D-galactose (cf. 65 per cent obtained by Percival and Thomson[15]) and no tetra-O-methyl-D-galactose. Agarose would be resistant to periodate oxidation as has been observed for agar[8].

It must be noted that Araki's work has been carried out on unfractionated agar. As yet no uronic residues have been recovered from agar hydrolysates nor have they been detected on paper chromatograms. Possibly agarose is true agar and agaropectin is one (or more) polysaccharide associated with agarose in the algal cell wall.

The excellent work of the Japanese school has done much to elucidate the structure of agar. However all the work done on agar has suffered from a very serious drawback in that agar of unknown origin and purity has been used. There is no real reason why agars from different algal sources should be identical. O'Neill and Stewart[26] however investigated the agar extracted from Gelidium cartilaginium and from a study of the mercaptolysis products and from their analytical results they concluded that this agar was built up of a chain of alternating D-galactose and 3:6-anhydro-L-galactose residues with a half-ester sulphate on every tenth galactose unit.

CARRAGEENIN

Carrageenin, which was first isolated by Schmidt[27], is the dried aqueous extract from the red seaweeds Chondrus crispus[28] and Gigartina stellata[29] of the family Gigartinaceae. The polysaccharide is a sulphate ester[30] containing D-galactose (ca. 35 per cent) and a sugar giving a ketose reaction[31] (ca. 20 per cent). It was long suspected that carrageenin was heterogeneous[30,31], and the cold and hot water extracts were found to have slightly different physical properties, the hot water extract setting to a gel but not the cold water extract.

Hydrolysis of the methylated polysaccharide with dilute oxalic acid[29,31] gave sulphuric acid, 2-O-methyl-D-galactose and 2:6-di-O-methyl-D-galactose. The main structure of carrageenin was thought to consist of a chain of 1 $\longrightarrow$ 3-linked α-D-galactopyranose units with the sulphate group located on $C_{(4)}$ and with some D-galactose units joined through $C_{(6)}$ forming branches[29,31]. The presence of sulphate on $C_{(3)}$ of the D-galactose unit may be discounted since it would be readily hydrolysed with alkali to give 3:6-anhydrogalactose[32]. Confirmation of this structure was obtained when a partially degraded desulphated carrageenin was methylated to give 2:4:6-tri-O-methyl-D-galactose and some 2:6-di-O-methyl-D-galactose on hydrolysis[33,34]. (In addition 2:3:4:6-tetra-O-methyl- and 2:4:6-tri-O-methyl-L-galactose were isolated in a different experiment from the hydrolysate of a methylated carrageenin that had been partially degraded and desulphated[33]. Later work[38] however has shown that carrageenin does not contain any L-galactose units, the L-galactose originating from a contaminant polysaccharide.)

The isolation of a crystalline derivative of 2-keto-D-gluconic acid was reported from a carrageenin hydrolysate[35], but it is now believed to have been an artefact; Dewar and Percival[29] were unable to isolate the compound from their material.

Recent / ...

To account for the precipitation of κ-carrageenin by potassium chloride (as well as by ammonium, rubidium and caesium salts) Bayley[103] has suggested a possible packing arrangement in which two κ-carrageenin molecules are held closely together by electrostatic forces between their nearest sulphate groups and the univalent hydrated cations. Molecules above and below the plane of these two molecules could be displaced so that each sulphate group would be effectively surrounded by cations. It is noted that only the smaller hydrated cations (K^+, NH_4^+) are able to pack κ-carrageenin molecules sufficiently closely to maintain aggregation and to bring about precipitation. In the case of λ-carrageenin the distance between successive sulphate groups would be too small to allow univalent cations of any size to build up the necessary type of structure.

The potassium chloride fractionation method has been applied to the extracts of other red seaweeds[36]: extracts from Gigartina species appeared to contain less and those from Hypnea musciformis more κ-carrageenin than did Chondrus crispus, while Gracilaria confervoides extract contained no potassium-sensitive material.

An extracellular enzyme has been obtained from a marine bacterium which it is claimed[43] is specific for the hydrolysis of κ-carrageenin. The enzymic hydrolysis of various algal extracts was examined and it was concluded that a κ-carrageenin type polysaccharide might be present in a number of red algae. The rate of enzymic hydrolysis of an extract from Hypnea musciformis was very similar to that of κ-carrageenin. Extracts from Chondrus crispus were hydrolysed at the same rate as commercial carrageenin while extracts from an Iridophycus species were hydrolysed at a slower rate.

Iridophycin is the galactan sulphuric acid ester extracted from red algae of Iridophycus spp.[44]. Hassid[45] hydrolysed the methylated polysaccharide and concluded that iridophycin consisted of 1 ⟶ 4-linked β-D-galactopyranose 6-sulphate units. Since all other red seaweed galactan sulphates are known to be 1 ⟶ 3-linked, Jones

and / ...

and Smith[46] re-interpreted Hassid's conclusions and proposed a 1 ⟶ 3 linkage for iridophycin. The presence of 1 ⟶ 3 linkages in the polysaccharide was proved by Mori[47] who isolated 2:4:6-tri-O-methyl-D-galactose from the hydrolysate of a methylated desulphated iridophycin. Mori[48] is of the opinion that the sulphate group is attached to $C_{(6)}$ since the polysaccharide is resistant to tritylation and he also assumes the glycosidic linkages to be α. Mori[2] has classified iridophycin as a carrageenin. Iridophycin probably is a mixture containing some κ-carrageenin since extracts of an Iridophycus species were hydrolysed by an enzyme specific for κ-carrageenin[43].

That κ-carrageenin is widespread among seaweeds of the family Gigartinaceae is also indicated by the recent isolation of 3:6-anhydro-4-O-β-D-galactopyranosyl-D-galactose dimethyl acetal as a syrup (in 68 per cent yield) and of the crystalline methyl glycoside of the disaccharide (in 5 per cent yield) from the partial methanolysate of the mucilage from Chondrus ocellatus[49].

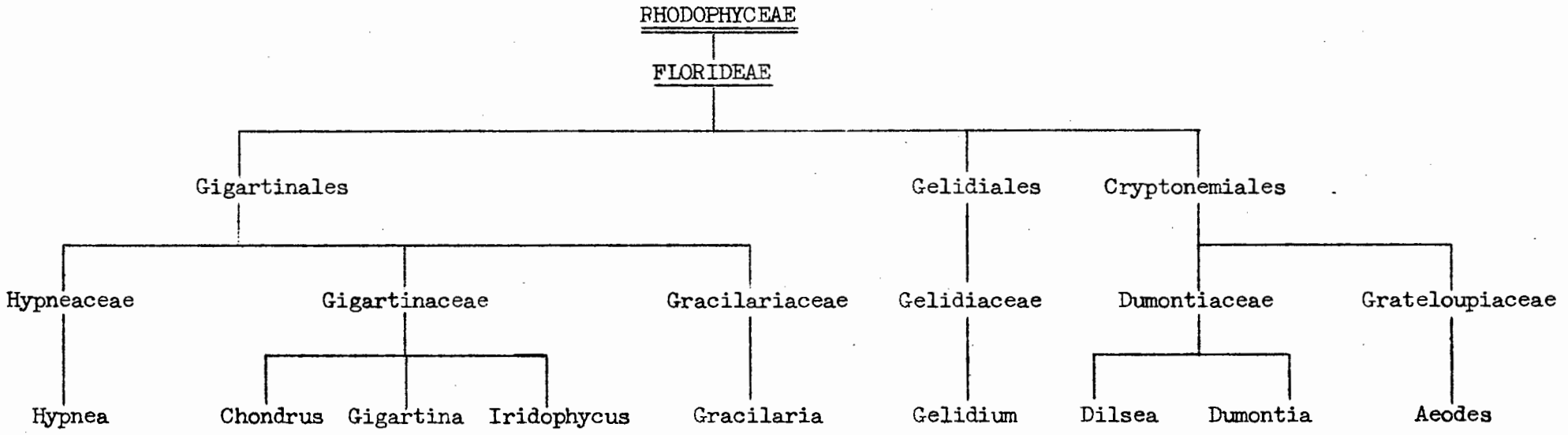
A galactan sulphate similar to carrageenin but with a much lower sulphate content has been isolated from Dilsea edulis[50] (family Dumontaceae). It contains[51] D-galactose 4-sulphate linked through $C_{(1)}$ and $C_{(3)}$. The polysaccharide also contains glucurone residues[52], the ratio of galactose : sulphate : uronic acid being 9:2:1. In addition 3:6-anhydro-D-galactose in small amount and galactose 6-sulphate are present in the polysaccharide[52]. The extract from the related seaweed Dumontia incrassata (family Dumontaceae) contains galactose, sulphate, and uronic acid in the ratio[53] of 9:4:1. Periodate oxidation indicated that most of the galactose linkages were 1 ⟶ 3. Aeodes orbitosa, a South African seaweed of the same order (Cryptonemiales), may well yield a polysaccharide of interest.

The aim of the present research was to investigate the structure of the polysaccharides of some South African red algae of the class Florideae. The object was to collect as much information about these polysaccharides as possible in order to bring some system into a very

confused / ...

confused field. The seaweeds Gracilaria confervoides and Hypnea specifera were selected for study because they are present along South African coasts in relatively large quantities (Gracilaria confervoides is exploited on an industrial scale) and because they are typical examples of algae of the order Florideae.

Phylogeny of Some Floridean Algae



INVESTIGATION AND DISCUSSION

The Hot-Water Soluble Polysaccharide of Gracilaria confervoides

Agar has been extracted from the following South African red seaweeds: Gelidium pristoides, Suhria vittata, and Gracilaria confervoides[54]. The extracts from these different seaweeds have similar physical properties but are not necessarily the same polysaccharide. Gracilaria confervoides the chief source of agar in Ceylon, China, North America and Australia is the most abundant source of South African agar, occurring in considerable amounts at Hout Bay and Langebaan. It grows in calm water and is washed ashore in very considerable amounts after heavy seas so that harvesting is easy and cheap. The South African production of Gracilaria agar has been estimated at 25 - 30 tons annually[117].

The Gracilaria agar used in this work was kindly supplied by Messrs Vitamin Oils (Pty) Ltd, Cape Town. Dried Gracilaria confervoides was suspended in water and heated with steam and subsequently frozen and purified by thawing and then dried at 45°. In order to prepare a very pure sample for analysis the agar was soaked in several changes of water, finally left in water at 37° for a week, and then filtered and washed with ethanol and acetone and dried at 60°/1mm.

Analysis of Gracilaria agar gave 0.78 per cent sulphate. An estimation of galactose by the micro-Somogyi technique[55] after hydrolysis and separation by paper chromatography gave 42 per cent calculated as $C_6H_{10}O_5$. Unfortunately there is no really satisfactory method for the estimation of 3:6-anhydrogalactose but ca. 26.4 per cent 3:6-anhydrogalactose (as $C_6H_8O_4$) was shown to be present in the agar using the method of Smith, O'Neill and Perlin[38].

Gracilaria agar was swollen in formamide[56] and acetylated with a pyridine and acetic anhydride mixture at room temperature to give a

product / ...

product containing 34.4 per cent acetyl. When the acetylation mixture was heated the acetyl content was raised to 36.2 per cent. This acetyl content of supposedly completely acetylated agar is lower than would be expected from the agar formula proposed by Jones and Peat (III)[17], which requires 43.4 per cent acetyl.

When hydrolysed Gracilaria agar was examined on paper chromatograms it gave a spot for galactose and a spot just behind the solvent front, presumably hydroxymethylfurfural; a small spot, possibly xylose from contaminating material, was also detected. Paper chromatograms of partially hydrolysed agar were characterised by pronounced streaking extending from the origin to a position near the solvent front, though the spot for galactose was still fairly well defined. Despite the streaking, several oligosaccharide spots could be detected and it was possible roughly to determine hydrolysis conditions for an optimum yield of disaccharide. The streaking on papers was probably caused by 3:6-anhydro-L-galactose both alone and in oligosaccharide molecules. Lindberg and Theander[57] found that methyl 6-aldehydo-β-D-glucopyranoside on paper chromatograms gave two spots with considerable trailing between them; when either spot was cut out and extracted and re-chromatographed the same result was again obtained. This phenomenon may be caused by the substance occurring in different forms, the equilibrium between which is set up rather slowly. Similarly 3:6-anhydrogalactose is known to exist in the aldehydo-state[63] and it too may streak on paper chromatograms for a like reason.

Several attempts were made to fractionate partial hydrolysates of Gracilaria agar on charcoal[58] using gradient elution[59] with aqueous alcohol. A fractionation of the hydrolysate was also attempted on charcoal pre-treated with stearic acid[59]. Apart from D-galactose no crystalline or pure material could be isolated. However an amorphous substance was obtained which gave a streaky spot of R_{Gal} (relative to galactose) 0.4 on paper and which reduced Fehling's solution in the

cold / ...

cold and gave a positive Seliwanoff test. It was not possible further to purify the substance by fractionation on cellulose columns. Paper chromatograms of the hydrolysed material gave the usual streaking but still showed spots for galactose, an unknown material (R_{Gal} 1.4) and hydroxymethylfurfural. The acetate of the substance could not be crystallised but a crystalline phenylosazone was obtained. A methylated derivative of the substance was prepared which after hydrolysis showed two spots on paper chromatograms, one spot having the same R_F value as tetra-O-methyl galactose; this indicated that galactopyranose occupied the non-reducing end of the molecule. When the substance was oxidised by periodate it yielded two mol. of formic acid and no formaldehyde. The information available about the substance is insufficient to merit any definite conclusion being drawn but the few facts available are consistent with its being 3:6-anhydro-4-O-β-D-galactopyranosyl-aldehydo-L-galactose.

It became evident at this stage of the work that it would be necessary in some way to modify the very labile 3:6-anhydro-aldehydo-L-galactose moieties in the hydrolysate. Reduction to the sugar alcohols seemed a reasonable step. When a partial hydrolysate was reduced with sodium borohydride[60] it gave a series of discrete spots without any streaking on paper chromatograms. A Gracilaria agar partial hydrolysate reduced with sodium borohydride[60] was fractionated on a cellulose column[61] using n-butanol-formic acid-water (45:1:4) as eluting solvent. The products obtained were (a) 1:4-anhydro-D-galactitol (=3:6-anhydro-L-galactitol) a syrup identified by its behaviour on paper chromatograms and by its specific rotation[62], (b) dulcitol, and (c) a crystalline disaccharide glycitol. A further material which could not be identified was obtained in very low yield: on paper it gave a spot of R_{Gal} 0.52, was non-reducing and showed $[\alpha]_D$ -27° in water.

When the disaccharide glycitol was hydrolysed nearly equimolar quantities of D-galactose and 1:4-anhydro-D-galactitol (=3:6-anhydro-L-galactitol) were obtained. The value found for the specific rotation of the hydrolysed material corresponded to the specific rotation of an

equimolar / ...

equimolar mixture of D-galactose and 1:4-anhydro-D-galactitol. Acetylation and benzoylation of the disaccharide glycitol yielded syrups which could not be crystallised. The hepta-O-methyl derivative of the disaccharide glycitol was prepared; from its hydrolysate 2:3:4:6-tetra-O-methyl-D-galactose was isolated and characterised as its aniline derivative thus showing that D-galactopyranose occupied the non-reducing end in the disaccharide glycitol. The linkage between the residues was established by periodate oxidation. A 1 $\longrightarrow$ 5-glycosidic linkage may be eliminated as this would require the 3:6-anhydro-L-galactose units in the original agar molecule to be in the furanose form, while in the galactose series the co-existence of both a furanose and hydrofuranol ring is sterically impossible[63]. Periodate oxidation of the disaccharide glycitol gave 1 mol. formic acid and 1 mol. formaldehyde, and 3 mol. periodate were consumed. The compound must then be 3:6-anhydro-4-O-β-D-galactopyranosyl-L-galactitol. A similar disaccharide glycitol with a 1 $\longrightarrow$ 2-glycosidic linkage would consume 3 mol. periodate but would produce no formaldehyde. The configuration of the linkage is assumed to be β because of the negative rotation of the compound, $[\alpha]_D$ -15° in water.

Methanolysis of agar offered an alternative method for modifying the labile di- or oligosaccharide fragments containing 3:6-anhydro-L-galactose at the reducing end. Paper chromatograms of partially methanolysed Gracilaria agar gave a series of discrete spots and showed no streaking. Furthermore Araki and Hirase[21] claimed to have isolated a disaccharide dimethyl acetal from partially methanolysed agar by a solvent fractionation method. Gracilaria agar was therefore partially methanolysed and fractionated on a cellulose column[61] using n-butanol-water (9:1) as solvent. The following products were obtained: (i) 3:6-anhydro-L-galactose dimethyl acetal, (ii) methyl galactosides, mostly methyl α-D-galactopyranoside and (iii) crystalline agarobiose dimethyl acetal, that is 3:6-anhydro-4-O-β-D-galactopyranosyl-L-galactose dimethyl acetal. No oligosaccharides higher than disaccharide could be

obtained / ...

obtained. Stripping the column with alcohol and water gave material of very low methoxyl value.

The 3:6-anhydro-L-galactose dimethyl acetal, a syrup, was identified by its behaviour on paper chromatograms and by its specific rotation. Hydrolysis of the compound gave a non-crystalline sugar which gave a positive Schiff reaction and which streaked badly on paper chromatograms (R_{Gal} 1.7). The phenylosazone derivative of the free sugar was prepared and two crystalline forms of the product were obtained; the derivative obtained in greater yield had a lower melting point (m.p. 203 - 204.5°) than that previously recorded[64] (m.p. 216°); the other form of phenylosazone derivative gave m.p. 213°. When distilled in vacuum 3:6-anhydro-L-galactose dimethyl acetal was converted into crystalline methyl 3:6-anhydro-α-L-galactoside. Attempts to prepare a crystalline tri-p-nitrobenzoate derivative of the dimethyl acetal[64] were unsuccessful.

The methyl galactosides obtained were mainly methyl α-D-galactopyranoside. No attempt was made to crystallise the methyl galactofuranosides which were recognised by the positions of their spots on paper chromatograms.

The disaccharide dimethyl acetal which was eluted off the column close behind the last of the methyl galactosides gave beautiful hexagonal plates when crystallised from ethanol. The melting point and specific rotation were in agreement with those recorded for agarobiose dimethyl acetal by Araki and Hirase[21]. The hexa-acetate of the agarobiose dimethyl acetal gave the same specific rotation but a higher melting point (138°) than that recorded[21] (88°). Periodate oxidation of the dimethyl acetal yielded one mol. formic acid and consumed two mol. periodate, no formaldehyde being produced. This is in agreement with the accepted structure of agarobiose dimethyl acetal. A 1 ⟶ 5-glycosidic linkage may be eliminated since the 3:6-anhydro-L-galactose unit could not pre-exist in the furanose form in the polysaccharide[63]. A similar disaccharide with a 1 ⟶ 2-glycosidic

linkage / ...

linkage would consume three mol. periodate. Further evidence for the structure of the disaccharide was obtained by Dr. Nunn who reduced the free disaccharide with sodium borohydride to give the disaccharide glycitol identical with the material previously obtained.

The yields of the sugars from methanolysed agar were calculated on reacted agar, that is the weights of undissolved agar (OMe, 4.2 per cent) and of the material which was eluted from the cellulose column with alcohol (OMe, 10.0 per cent) and with water (OMe, 8.4 per cent) were subtracted from the weight of agar starting material. Each sugar was calculated on a parent free anhydro-sugar basis, and the following yields were obtained: 3:6-anhydro-L-galactose (as $C_6H_8O_4$), 12.7; D-galactose (as $C_6H_{10}O_5$), 22.6; and agarobiose (as $C_{12}H_{18}O_9$), 62.0 per cent. When Gracilaria agar was partially methanolysed as before but for only a very short time (10 min.) the yields of sugars obtained from reacted agar, on an anhydro-sugar basis were as follows: 3:6-anhydro-L-galactose (as $C_6H_8O_4$), 5; D-galactose (as $C_6H_{10}O_5$), 19; and agarobiose (as $C_{12}H_{18}O_9$), 76 per cent. The materials stripped from the columns with alcohol and water were further methanolysed to give the same compounds identified on paper chromatograms.

It has thus been shown that at least 76 per cent by weight of the Gracilaria agar molecule is composed of anhydro-agarobiose units. Since during methanolysis some agarobiose dimethyl acetal itself is further degraded to its constituent sugars it follows that the anhydro-agarobiose content of the agar molecule must be even higher, that is at least 86 per cent (cf. 69.5 per cent calculated by Araki and Hirase[21]) if we assume that all the 3:6-anhydrogalactose residues originate from agarobiose units. The ease with which 3:6-anhydro-galactose decomposes probably accounts for the low yields of the sugar as against the yields of D-galactose, though of course it is possible that the original macromolecule may contain some extra D-galactose units. The D-galactose units in the polysaccharide are probably joined

by / ...

by 1 ⟶ 3 linkages in an unbranched chain. This was confirmed by the isolation by Dr. Nunn of 2:4:6-tri-O-methyl-D-galactopyranose from methylated Gracilaria agar. The 3:6-anhydro-L-galactose residues in the macromolecule are assumed to be present in the pyranose form because a furanose form is sterically impossible[63]. Such a structure is consistent with the instability of agar solutions to dilute acids. The 3:6-anhydro-L-galactopyranose units, which contain both a pyranose and a hydrofuranol ring, would be under considerable strain[63]. The resulting highly strained glycosidic bonds involving $C_{(1)}$ of these units would undergo rapid hydrolysis under even mildly acidic conditions. This would result in the formation of 3:6-anhydro-aldehydo-L-galactose, which itself would undergo a series of dehydrations with ring rearrangements to give 5-hydroxymethyl furfural as well as laevulinic acid.

Assuming anhydro-agarobiose to be the repeating unit of Gracilaria agar the acetyl content should be 36.3 per cent of the agar acetate. Typical acetyl values obtained for agar acetate in this work were 34.4, 35.8 and 36.2 per cent. An estimation of galactose by the micro-Somogyi technique[55] in agar acetate after hydrolysis and separation by paper chromatography gave a value of 33.5 compared with a calculated value of 34.2 per cent.

Estimation of sulphate indicates that there is about one sulphate half-ester group to every 40 anhydro-agarobiose units. Possibly these are linked to $C_{(6)}$ of D-galactose but there is no evidence to show where they occur in the individual monosaccharide units or where they are located along the chain.

Throughout this work no galactopyranosylgalactose disaccharide was discovered, thus further discounting any possibility of an agar molecule such as that proposed by Jones and Peat[17].

The / ...

The Hot-Water Soluble Polysaccharide of Hypnea specifera

Hypnea specifera is one of the most widely distributed seaweeds of the South African coastline being however of greater importance ecologically in warmer waters[65]. It is believed to be of possible economic importance[65,66].

The polysaccharide was extracted with boiling water from freshly collected wet Hypnea specifera plants. The hot water extract was filtered through a linen cloth and poured into alcohol to precipitate the polysaccharide. The yield of polysaccharide was 16 per cent based on dry seaweed. For analytical purposes the polysaccharide was further purified by repeated dissolution in hot water followed by precipitation in alcohol; finally it was converted into the sodium salt by means of the sodium form of a cation exchange resin. This material was still contaminated with nitrogen-containing impurities (N, 3.85 per cent) which were assumed to be due to protein and which was allowed for in subsequent analyses. This polysaccharide had a specific rotation of $+65^{\circ}$ in water and contained 36.2 per cent $NaSO_3$. An estimation of galactose by the micro-Somogyi method[55] after hydrolysis and separation on paper chromatograms gave 47.0 per cent (calc. as $C_6H_{10}O_5$). Addition of potassium chloride to an aqueous solution of the sodium salt of the polysaccharide yielded a κ-fraction[36,37] in ca. 75 per cent yield, similar to that given by carrageenin. (There appeared to be very little, if any, λ-fraction present.) This material, regenerated as its sodium salt, contained only a small amount of nitrogen (0.6%), assumed to be due to protein, allowance for which was made in subsequent analyses. It had a specific rotation of $+66.5^{\circ}$ in water and contained 21.2 per cent $NaSO_3$ and 47.4 per cent galactose (calc. as $C_6H_{10}O_5$). A third major component of the polysaccharide, subsequently shown to be 3:6-anhydro-D-galactose, was 31.4 per cent by difference. The molecular ratio of galactose, 3:6-anhydrogalactose and $NaSO_3$ was thus shown to be 1.5 : 1.1 : 1.0.

Paper / ...

Paper chromatograms of hydrolysed Hypnea specifera polysaccharide showed galactose and probably hydroxymethylfurfural together with a very small spot for xylose. Since partial hydrolysates of the polysaccharide streaked badly on paper chromatograms, and since it was thought very likely that the polysaccharide contained 3:6-anhydrogalactose units, it was decided to methanolyse the polysaccharide. When the polysaccharide was partially methanolysed it gave a series of discrete spots on paper chromatograms. The partial methanolysate fractionated on a cellulose column[61] with n-butanol-water (9:1) as developing solvent yielded 3:6-anhydro-D-galactose dimethyl acetal, methyl galactosides, and a disaccharide dimethyl acetal. 3:6-Anhydro-D-galactose dimethyl acetal, a syrup, was identified by its spot on paper chromatograms and by its specific rotation; it was further characterised by the crystalline 1:2-O-isopropylidene derivative of the free sugar[83]. Methyl galactosides were identified by their positions on paper chromatograms. An amorphous substance was obtained next off the column which moved on papers at the same rate as agarobiose dimethyl acetal and which was non-reducing to Fehling's and gave a positive Seliwanoff test. This material, like agarobiose dimethyl acetal, though non-reducing to Fehling's, gave a yellow spot on papers which were sprayed with p-anisidine hydrochloride and heated for fifteen minutes at 110°. It was not possible to obtain a crystalline acetate of this compound. Mild hydrolysis with very dilute oxalic acid yielded a sugar which streaked on paper (R_{Rh} .44) very similarly to agarobiose. This sugar was reduced with sodium borohydride to the disaccharide glycitol which gave a spot on paper chromatograms (R_{Rh} 0.51) together with a small spot (R_{Rh} 0.60), probably dulcitol.

The reduced disaccharide material was fractionated on a cellulose column[61] with n-butanol-formic acid-water (45:1:4) as irrigating solvent. A small amount of dulcitol was obtained. The main fraction gave a crystalline compound from methanol. It had the

same / ...

same melting point (174°) as the agar disaccharide glycitol but a mixed melting point of the two was depressed (161-163°). The specific rotations of the two compounds were equal but opposite in sign. When hydrolysed the compound gave in equimolar proportions D-galactose and a syrup chromatographically identical with 1:4-anhydro-L-galactitol (=3:6-anhydro-D-galactitol) and having the correct specific rotation for that compound[67]. The specific rotation for the hydrolysate mixture corresponded to the specific rotation of an equimolar mixture of D-galactose and 1:4-anhydro-L-galactitol. The compound was thus shown to be a disaccharide glycitol containing D-galactose glycosidically linked to 1:4-anhydro-L-galactitol. Acetylation gave a crystalline hepta-O-acetate indicating the presence of seven free hydroxyl groups. The glycosidic linkage was established by periodate oxidation: the compound consumed three mol. periodate and yielded one mol. formic acid and one mol. formaldehyde. Since 3:6-anhydro-D-galactose units in the parent polysaccharide cannot exist in the furanose form a 1 $\longrightarrow$ 5 glycosidic linkage is impossible[63]. Again a 1 $\longrightarrow$ 2 glycosidic linkage would yield no formaldehyde. The low positive specific rotation of the disaccharide glycitol and its upward mutorotation on acid hydrolysis, as well as the small negative specific rotation of the acetate indicate a β-linkage. The compound must be 3:6-anhydro-4-O-β-D-galactopyranosyl-D-galactitol. The yield of the disaccharide (as $C_{12}H_{18}O_9$) on the original reacted Hypnea specifera polysaccharide was 12 per cent.

The infra-red spectra of the disaccharide glycitol and of the agar disaccharide glycitol are very similar. Optical enantiomorphs of the same crystalline form are believed to show identical infra-red spectra[68] over the frequency range 1790-630 cm^{-1}. These two compounds however are not enantiomorphs but diastereoisomers. The β-configuration for both disaccharide glycitol linkages may be deduced from the presence of absorption[68] at 887 and 890 cm^{-1} respectively and from the absence of absorption at 844 ± 8 cm^{-1}.

Frequencies / ...

Frequencies (cm^{-1}) of absorption peaks												
Disaccharide glycitol												
734	792	860	878	887	900	946	985	-	1040	1070	1138	
Agar disaccharide glycitol												
734	789	870	880	890	895	946	989	1018	1048	1080	1140	

Methylation of the Hypnea specifera polysaccharide proved difficult, no doubt due to the presence of a high percentage of sulphate groups in the molecule[69]. Hydrolysis of the methylated substance gave a syrup which on paper chromatograms gave a spot for 2:6-di-O-methylgalactose, together with small spots for mono-, tri-, and tetra-O-methylgalactoses. Fractionation of the hydrolysate on cellulose[61] using n-butanol-water (9:1) as developing solvent gave as major constituent 2:6-di-O-methyl-D-galactose, identical with 2:6-di-O-methyl-D-galactose prepared synthetically[70]. Retention of the ethereal sulphate during methylation with methyl sulphate and concentrated alkali emphasises the stability of the sulphate grouping to alkali. Were the sulphate attached to $C_{(3)}$ of the D-galactose residues it would be easily hydrolysed with alkali[32]. If it is therefore assumed that $C_{(4)}$ of the D-galactose unit is esterified with sulphate as in carrageenin[33] then besides the 1 $\longrightarrow$ 4-links indicated by the isolation of the disaccharide glycitol, 1 $\longrightarrow$ 3-links are also present in the original molecule.

Kantor and Schubert[71] desulphated chondroitin sulphate by shaking with methanolic hydrogen chloride three times successively.

Hoffman / ...

Hoffman and co-workers[72] used this method to desulphate chondroitin sulphate and obtained in ca. 70 per cent yield a desulphated polysaccharide containing no sulphate. Similarly Johnston and Percival[33] shook the polysaccharide from Gigartina stellata with 1 per cent methanolic hydrogen chloride and found that the greater part of their material dissolved with the production of methyl galactosides and salts of methylsulphuric acid. An insoluble residue (ca. 15 per cent) remained which contained very low sulphate (ca. 1.5 per cent). The resistant fragment gave a methylated derivative which hydrolysed to give among other products 2:3:4:6-tetra-O-methyl- and 2:4:6-tri-O-methyl-L-galactose as well as smaller quantities of the corresponding D-galactose derivatives. It must be remembered that the Gigartina stellata polysaccharide is actually a mixture of κ- and λ-carrageenins together with several other polysaccharides and it has been shown[38] that the L-galactose containing component is separable by fractional precipitation from the D-galactose containing polysaccharides which comprise the bulk of carrageenin. In another experiment Johnston and Percival[33] partially hydrolysed the extract from Chondrus crispus so that the product lost 25 per cent of its originally bound sulphate. Methylation and hydrolysis of this degraded material gave 2:4:6-tri-O-methyl-D-galactose in 18 per cent yield. Thus it was here concluded that the principal D-galactose linkage was through $C_{(1)}$ and $C_{(3)}$ with the sulphate residue on $C_{(4)}$.

When the Hypnea specifera polysaccharide was desulphated with methanolic hydrogen chloride very low yields of product were obtained and sulphate content was ca. 3 to 7 per cent.

Hypnea specifera polysaccharide was treated with sodium borohydride in an unsuccessful attempt to desulphate the material. (Lithium aluminium hydride has been used to de-tosylate the tosyl esters of aldoses[116].) Jones and Perry[73] have treated a polysaccharide with sodium borohydride prior to methylation in order to prevent possible alkaline breakdown during methylation. It may be assumed that such

treatment /

treatment should not radically alter the polysaccharide. Accordingly the Hypnea polysaccharide recovered after sodium borohydride treatment (SO_4^{--}, 19.3 per cent) was methylated with methyl sulphate in the usual way. The methylated product (OMe, 21.4; SO_4^{--}, 15.8 per cent) was treated with methanolic hydrogen chloride partially to methanolyse and to desulphate it. This product (OMe, 32.0; SO_4^{--}, 7.7 per cent) was then twice further methylated with Purdie's reagents. The final product (OMe, 35.8; SO_4^{--}, 7.4 per cent) was hydrolysed and on paper chromatograms gave spots for tri-O-methylgalactose and di- and tetra-O-methylgalactose. The hydrolysate was fractionated on cellulose[61] and 2:3:4:6-tetra-O-methyl- and 2:4:6-tri-O-methyl-D-galactopyranose were isolated and identified as their anilide derivatives; 2:6-di-O-methyl-D-galactose was also obtained.

It may be concluded that the 2:4:6-tri-O-methyl-D-galactose was derived from D-galactopyranose units linked through $C_{(1)}$ and $C_{(3)}$ and carrying a half-ester sulphate group on $C_{(4)}$, since the main product of the hydrolysis of undegraded methylated polysaccharide was 2:6-di-O-methyl-D-galactose.

Since a fairly high yield of disaccharide glycitol was obtained from the polysaccharide and since the principal linkage of the D-galactopyranose residues appears to be 1 $\longrightarrow$ 3 it therefore follows that the polysaccharide is composed to a significant extent of repeating 1 $\longrightarrow$ 3'-α-linked disaccharide units, the α-configuration being assigned because of the positive specific rotation of the polysaccharide. Thus, besides the 1 $\longrightarrow$ 4-links indicated by the isolation of the disaccharide, 1 $\longrightarrow$ 3-links are also present in the original molecule. Furthermore D-galactopyranose units in the polysaccharide are sulphated on $C_{(4)}$.

Most of the Hypnea polysaccharide extract afforded a κ-fraction[36,37,38], similar to that given by carrageenin. (No final λ-fraction was isolated.) This potassium chloride precipitated material gave a specific rotation (+66.5°) in water very similar to

that / ...

that recorded for κ-carrageenin[36,39] (+63°; +60.3°). On analysis it gave D-galactose, 47.4; 3:6-anhydro-D-galactose, 31.4; and $NaSO_3$, 21.2 per cent, the molecular ratio of D-galactose, 3:6-anhydro-D-galactose, and $NaSO_3$ being thus 1.5 : 1.1 : 1.0. Analytical values recorded for κ-carrageenin vary to some extent: sulphate content (as SO_4^{--}) has been given as 24[36], 25[38] and 23[39] per cent. Smith, O'Neill and Perlin[38] report for κ-carrageenin from Chondrus crispus: D-galactose, 38.1, 35.1; 3:6-anhydro-D-galactose, 24.0, 28.1; and $NaSO_3$, 28.2, 28.1 per cent and these authors show that one of their fractions contains D-galactose and 3:6-anhydro-D-galactose residues in a ratio of approximately 1.4:1.0. The general properties of the polysaccharide from Hypnea specifera, and the nature of the degradation products, indicate that it is very closely related to the κ-carrageenin obtained from Chondrus crispus.

It is of interest that when an extract of Hypnea musciformis was fractionated with potassium chloride it was found to contain more κ-material than does Chondrus crispus[36]. Yaphe and Baxter[43] isolated a specific enzyme that hydrolyses the hot-water extract of Hypnea musciformis at nearly the same rate as it hydrolyses κ-carrageenin. It would now appear that the hot-water soluble polysaccharide of the closely related seaweed Hypnea specifera is also very similar in structure to κ-carrageenin.

Many more details of the structure of this and other very similar polysaccharides will still have to be worked out before a more exact comparison can be made or a picture of their structures built up. A study of the products of enzymic hydrolysis of this polysaccharide should prove of interest in further elucidating the constitution of the macromolecule. Meanwhile, Bayley[103] has used the analytical data available, together with measurements of intramolecular spacings from X-ray diffraction studies, to put forward a tentative structure for κ-carrageenin from Chondrus crispus. He has postulated a main chain built up of repeating trisaccharide units,

each / ...

each trisaccharide unit containing two 1 $\longrightarrow$ 3-α-linked sulphated D-galactose residues and one 1 $\longrightarrow$ 4-β-linked 3:6-anhydro-D-galactose residue. Also with every two trisaccharide units there may be a single side residue of 3:6-anhydro-D-galactose attached to the main chain through $C_{(6)}$ of a sulphated D-galactose residue.

In conclusion it may be stated that further studies into other red seaweed polysaccharides may be of considerable phylogenetic importance.

INFRA-RED SPECTRA OF CARBOHYDRATES

In recent years interest has grown in the interpretation of infra-red absorption spectra of carbohydrates. Kuhn[93] has shown that the coincidence of the infra-red spectra of a known and an unknown carbohydrate over a wide frequency range is as reliable for identification as a mixed melting point. However it has been found that infra-red spectra do not differentiate between carbohydrate enantiomorphs if they occur in the same crystal form[94]. In addition a marked movement of peak frequency of up to 20 cm^{-1} has been observed in passing from a crystalline to an amorphous state, such as a syrup[94]: crystalline α-D-glucopyranoside[94] exhibits a characteristic peak at 837 cm^{-1} which is shifted to 849 cm^{-1} in a syrupy α,β mixture. Infra-red spectra may also be used for the identification of functional groups such as acetyl and sulphate which have characteristic absorption bands.

Kuhn[93] and Thompson, Nicholson and Short[95] compared the infra-red curves for a large number of carbohydrates and found that every carbohydrate has its own characteristic set of absorption bands: marked differences were found between the spectra of sugar isomers such as glucose and mannose or even of compounds such as dulcitol and mucic acid. Kuhn[93] showed that the anomeric forms of various glycosides may be readily distinguished by their infra-red curves but he did not assign any particular frequency to the anomeric carbon atom. Whistler and House[96] also reported that the spectra of sugar anomers could be used to differentiate between them.

The infra-red spectrum of a carbohydrate is strongly influenced by the introduction of substituents so that it is not possible to identify a substituted carbohydrate by comparing its spectrum with that of the corresponding unsubstituted carbohydrate[93]. Kuhn[93] has also concluded that it is not possible to correlate the infra-red spectrum of a polysaccharide with the spectra of its constituent mono- and oligosaccharides. Above ca. 1250 cm^{-1} all

sugar / ...

sugar isomers show the same absorption bands some of which have been identified. A very strong band at 3222 cm^{-1} has been attributed to the oxygen-hydrogen stretching frequency of hydrogen-bonded hydroxyl groups[97]. Several closely spaced bands in the 1100 - 1000 cm^{-1} region are probably due to carbon-carbon and carbon-oxygen vibrations[93,95]. A peak at 1645 $\pm$ 5 cm^{-1} has been allocated to water of crystallisation or adsorbed water [98,99].

The infra-red spectra of the following polysaccharides have been investigated: chondroitin sulphuric acid[100], hyaluronic acid[100], alginic acid[95,100], pneumococcal polysaccharides[98], cellulose[95], chitin[101], bacterial dextrans[102] and carrageenin[103]. By examination of these spectra it has been possible to allocate absorption bands to certain functional groups and to distinguish different types of polysaccharides; in the case of pneumococcal polysaccharides spectral similarity was correlated with serological relationship[98].

Absorption at ca. 1612 cm^{-1} is characteristic for the carboxyl ion (i.e., salt form of carboxylic acid), being due to the C=O stretching vibration[99]; for an unionised carboxylic acid group (acid form) an intense absorption band, due to the same vibration appears[98] at 1756 cm^{-1}.

The C=O stretching vibration of an O-acetyl group gives pronounced absorption in the 1725 - 1749 cm^{-1} region. A band at 1250 - 1220 cm^{-1} which frequently accompanies the last band, has been associated with an ester linkage[104] (C-O-C system) and a further band at 1380 cm^{-1} has also been correlated with acetyl, being due to a terminal methyl group[98]. Absorption bands at 1648 cm^{-1} and 1560 - 1508 cm^{-1} are characteristic for molecules containing a monosubstituted amide group: these bands are due to the carbonyl stretching vibration and the N-H deformation mode respectively[101].

The sulphate ester group has been detected in sulphated polysaccharides by a band[100] at ca. 1240 cm^{-1}. This band has been assigned to the S=O stretching vibration and is analogous to the C=O

stretching / ...

stretching vibration present in acetates at ca. 1740 cm^{-1}. Since acetates show an additional absorption at 1250 - 1220 cm^{-1} which has been ascribed[104] to a C-O-C system it follows that there should be a similar mode within the C-O-S system at correspondingly lower frequencies. The spectrum of a polysulphated hyaluronic acid[100] revealed the presence of such a band at 820 cm^{-1}. An attempt was made[100] to correlate the exact position of this band at ca. 820 cm^{-1} with the location of a sulphate group in an equatorial or an axial position on the pyranose ring. One isomer of chondroitin sulphuric acid having bands at 825 and 775 cm^{-1} is believed to contain sulphate bonds in the equatorial position, while another isomer of chondroitin sulphuric acid having three bands of comparable intensity at 928, 855 and 725 cm^{-1} is thought to have its sulphate bonds in the axial configuration[100].

The intensive study of infra-red spectra of the carbohydrates has been confined to the frequency range 730 - 960 cm^{-1} in which region the molecule vibrates as a whole[94]. It is in this region that one would expect stereochemical differences to be evident.

Barker and co-workers[94] have found that D-glucopyranose derivatives (including polyglucosans) which possess the α-configuration absorb at ca. 844 $\pm$ 8 cm^{-1}. This absorption, designated type 2a absorption, has been assigned to the deformation of the equatorial $C_{(1)}$-H bond[94] in D-glucopyranose derivatives of C1 conformation, this being the most stable conformation of the D-glucopyranose ring[105]. β-D-Glucopyranose derivatives do not absorb in the 2a region but give an absorption band, called type 2b, of moderate or strong intensity[94] at 891 $\pm$ 7 cm^{-1}. When α- and β-configurations occur together in D-glucopyranose derivatives both type 2a and type 2b absorptions are present in the spectrum. Type 2b absorption has been assigned to the deformation of an axial $C_{(1)}$-H bond in the most stable conformation of D-glucopyranose[94].

α-D-Galactopyranose / ...

α-D-Galactopyranose and α-D-mannopyranose derivatives, which contain equatorial $C_{(1)}$-H bonds in their most stable chair (C1) conformations[105], give type 2a absorption at 825 ± 11 and 833 ± 8 cm^{-1} respectively[94]. (However, a few β-anomers were found to show a bond in the type 2a region, possibly caused by the presence of contaminants.) Similarly type 2b absorption was shown by derivatives of β-D-galactopyranose (895 ± 9 cm^{-1}) and β-D-mannopyranose (893 ± 6 cm^{-1}), both of which have an axial $C_{(1)}$-H in their most stable chair conformations[105], C1. Recent work by Isbell et al.[106] has shown that care must be taken in the interpretation of type 2a and type 2b absorption: these workers found that both α- and β-D-talopyranose gave both types 2a and 2b absorption, possibly because each anomer exists in both C1 and 1C chair conformations[105] so that each anomer would contain axial and equatorial $C_{(1)}$-H bonds.

Derivatives of D-galactopyranose and D-mannopyranose but not of D-glucopyranose display an absorption peak[94], designated type 2c, at ca. 875 cm^{-1}. Type 2c absorption appears to be due to the deformation of equatorial C-H bonds at $C_{(4)}$ and $C_{(2)}$ in the case of galactopyranose and mannopyranose respectively: these C-H bonds are axial in the case of D-glucopyranose. The stable chair form of the hexahydric inositols and of the pentahydric quercitols, which contain one or more equatorial hydrogen atoms, gave an absorption peak[110] at ca. 875 cm^{-1}.

In the α-D-glucopyranose series of derivatives a peak of moderate to strong intensity has been found at 917 ± 13 cm^{-1} and in the β-D-glucopyranose series a less intense peak at 920 ± 5 cm^{-1} was found[94]. This absorption, type 1 absorption, is characteristic for all pyranose sugars and appears to be analogous to the strong band at 875 cm^{-1} in the spectrum of tetrahydropyran which has been attributed to an antisymmetrical ring vibration in that molecule[107]. Type 1 absorption may be eclipsed by the presence of nearby type 2b absorption (891 ± 7 cm^{-1}) or by absorption due to the C-O stretching mode of acetates or methyl ethers[94].

Tetrahydropyran / ...

Tetrahydropyran has a symmetrical ring breathing frequency[107] at 813 cm^{-1}. By analogy pyranose sugar derivatives should give an absorption peak in this region (type 3) with an intensity very sensitive to changes in sugar configuration. The infra-red spectrum of scyllo-inositol which is a symmetrical molecule showed no type 3 absorption[108]. It is believed that the lower frequency of type 3 absorption in carbohydrates (ca. 770 $\pm$ 14 cm^{-1}) as compared to the symmetrical ring breathing vibration of tetrahydropyran (813 cm^{-1}) is due to the extra weight of constituents involved in the carbohydrate molecule. This absorption (type 3) was found in the case of α-D-glucopyranose derivatives to be of medium intensity and to be at 766 $\pm$ 10 cm^{-1}; a type 3 peak (at 744 $\pm$ 9 cm^{-1}) of weak intensity, which could not always even be detected was observed for β-D-glucopyranose derivatives[94]. The α- and β-anomers of D-galactopyranose, D-mannopyranose as well as of arabinopyranose show strong type 3 absorption. While α-D-xylopyranose gives a type 3 absorption peak at 749 $\pm$ 10 cm^{-1} the β-anomer exhibits no type 3 absorption at all (cf. β-D-glucopyranose). The absence of type 3 absorption in the spectra of β-D-xylopyranose (C1 conformation)[105] and of β-D-glucopyranose (C1 conformation)[105] may be due to the absence of axial C-O bonds in the molecules[99].

Barker et al.[94] have examined a number of polyglucosans (amylose, dextran, nigeran, cellulose, luteose and laminarin) and have found that polyglucosans with different linkages give different type 1 and type 3 absorption.

	Type 1	Type 3
Amylose (α-1 ⟶ 4-linkage)	930 $\pm$ 4	758 $\pm$ 2
Dextrans (α-1 ⟶ 6-linkage)	917 $\pm$ 2	768 $\pm$ 1
Nigerans (α-1 ⟶ 3-linkage and α-1 ⟶ 4-linkage alternately)	-	793 $\pm$ 3

In / ...

In the starch series a systematic movement of the type 1 bands (907 $\longrightarrow$ 930 cm^{-1}) and the type 3 bands (778 $\longrightarrow$ 758 cm^{-1}) was noted in passing from the disaccharide through the oligosaccharides to the polysaccharides.

A peak at 794 cm^{-1} found in the spectra of bacterial dextrans[102] has been attributed to 1 $\longrightarrow$ 3 glucosidic linkages[109].

The infra-red spectra of furanoside sugars and carbohydrates containing a hydrofuranol ring have also been examined[110]. Since the tetrahydrofuran ring is very nearly planar[111] it is to be expected that the C-H bonds in a furanose ring will be in relatively equivalent positions above and below the plane of the ring: there will be no differentiation into axial and equatorial bonds and consequently it will not be possible to differentiate between anomeric furanosides by infra-red spectroscopy.

Four absorption peaks (types A-D) it is claimed[110] may help to identify a furanose or hydrofuranol ring either alone or fused to a pyranose ring.

Type A absorption at 924 $\pm$ 13 cm^{-1} has been found for compounds containing either a furanose or hydrofuranol ring and may be due to a symmetrical ring breathing frequency. Type D absorption (799 $\pm$ 17 cm^{-1}) may be caused by the deformation of a C-H bond where the hydrogen atom is linked to a carbon atom directly attached to a ring oxygen atom. This peak has been useful in detecting a hydrofuranol ring, particularly when the hydrofuranol ring is fused to a pyranoside ring[110].

Type B absorption (870 $\pm$ 7 cm^{-1}) and type C absorption (838 $\pm$ 16 cm^{-1}) have been reported for compounds containing either furanose or hydrofuranol rings[110].

For the interpretation of infra-red spectra of carbohydrates it is well to bear in mind that assignments below 1350 cm^{-1} are not definite; interactions for instance may well cause frequency shifts.

In order to confirm and perhaps to extend the information already available on the infra-red spectra of carbohydrates certain

carbohydrates / ...

carbohydrates, including some new compounds containing hydrofuranol rings, which became available during the course of the work described in this thesis were examined for their characteristic infra-red spectra. The carbohydrate samples were dispersed in potassium bromide that had been dried at 650° for 4 hours and were then pressed into disks[112]. Spectra were determined with a Perkin-Elmer spectrophotometer with a sodium chloride prism.

The infra-red spectral curves of Gracilaria agar (salt form), Hypnea polysaccharide (salt form), and agar acetate were determined. These substances, being amorphous, tended to give broad badly-defined absorption bands. A band at ca. 1640 cm^{-1} found in each case may be allocated to adsorbed water[98,99]. The pronounced band at 1760 cm^{-1} in the agar acetate spectrum represents O-acetyl[98,99], and the very broad band at ca. 1100 cm^{-1} may be ascribed to C=O stretching and C-O-H bending modes[100]. Both κ- and λ-carrageenin gave infra-red spectra[103] with pronounced absorption at ca. 1240 cm^{-1} (S=O stretching vibrations) and with a single peak showing a strong absorption at ca. 840 cm^{-1} which was associated with sulphate group (C-O-S system) in equatorial position. The spectrum for Hypnea specifera polysaccharide exhibited a very strong band at 1100 - 1160 cm^{-1} probably due to sulphate (S=O stretching vibrations). The peak frequencies (and some tentative assignments) are tabulated in Table I.

Methyl α-D-galactopyranoside and methyl β-D-galactopyranoside obtained from the methanolysates of agar were submitted to infra-red spectral examination. The spectral peaks obtained agreed very well with those reported by Barker et al.[94] (Table II).

The infra-red spectra of 1:4-anhydro-D-galactitol and of 3:6-anhydro-L-galactose dimethyl acetal were obtained and were compared with the reported spectra[110] of 3:6-anhydrosorbitol and 1:4-anhydro-D-mannitol (see Table III). Both 1:4-anhydro-D-galactitol and 3:6-anhydro-L-galactose dimethyl acetal gave type A and type B absorption, 3:6-anhydro-L-galactose dimethyl acetal gave a type D peak, and no type C absorption was observed for either compound.

TABLE I / ...

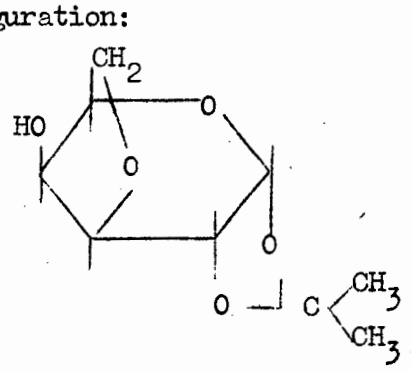
TABLE I

Frequencies (cm^{-1}) Characteristic of the Polysaccharides

Gracilaria Agar	Hypnea Polysaccharide	Agar Acetate
740 vw	-	-
770 w (Type 3)	-	770 w (Type 3)
-	850 w (C-O-S)	860 w
888 vw (Type 2b)	894 vw (Type 2b)	894 m (Type 2b)
930 m (Type 1)	930 w (Type 1)	945 m
965 vw	972 vw	972 vw
1072 m.vb (S=O)	1100-1160 s.vb (S=O)	1090 s.b
-	-	1138 vw
1150 vw.vb	1195 vw	1190 vs
-	-	1380 s
1388 m.vb	1410 vw (carboxyl?)	1444 vw
1650 s (H_2O)	1640 m (H_2O)	1640 m (H_2O)
		1760 vs (Ac)

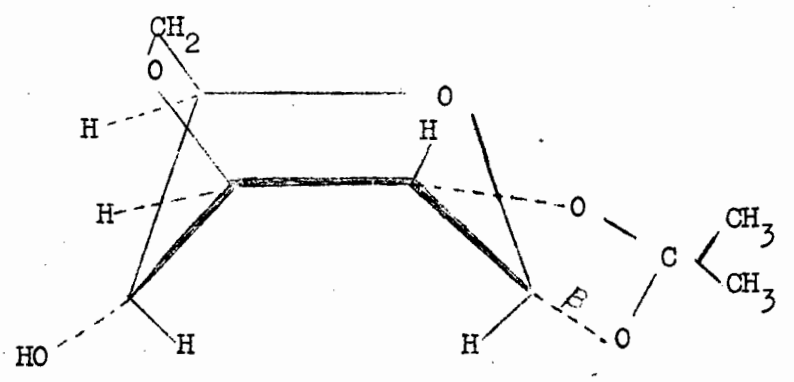
m = medium b = broad
v = very s = strong
w = weak

The spectrum of 1:2-O-isopropylidene-3:6-anhydro-D-galactose, obtained during the course of this work, gave pronounced type 2b absorption, quite distinct from any type A absorption, indicating a β-glycosidic configuration (Table IV). Now if this compound were given the usual planar formula it would appear to possess an α-glycosidic configuration:



However / ...

However, if the compound is visualised in the 1B boat form[113] in which it must exist to form a 1:2-mono-O-isopropylidine derivative (in the 1C form the distance between the $C_{(1)}$-O and $C_{(2)}$-O bonds is too great) it will be seen that a β-glycosidic linkage, involving an axial $C_{(1)}$-H bond is possible.



In the case of the disaccharide compounds obtained in the course of the work previously described, where a D-galactopyranose unit is joined by a β-glycosidic link to a 3:6-anhydrogalactose moeity (open-chain form) it was possible in each case to identify type 2b absorption and to note the absence of type 2a absorption (Table V). It was thus possible to establish that the agar disaccharide glycitol and the disaccharide glycitol obtained from the H. specifera polysaccharide very probably contained β-glycosidic linkages. These two disaccharide glycitols, which are diastereoisomers, gave very similar but not identical spectra in the 730 - 930 cm^{-1} region.

A few sugar enantiomorphs were submitted to spectral analysis: the D- and L-enantiomorphs of 1:4-anhydrogalactitol, methyl 3:6-anhydrogalactoside, and 1:2-O-isopropylidene-3:6-anhydrogalactose in each case gave identical infra-red spectra.

In order to extend the study of the infra-red spectra of carbohydrates containing a hydrofuranol ring it would be of interest to study the spectra of 3:6-anhydrotalose, and 2:5-anhydroarabinose[114] and 2:5-anhydrolyxose.

The / ...

The infra-red analysis of carbohydrates may attain considerable importance. By the correlation of spectra of many related carbohydrate types it may yet become a more highly systematic method of analysis. Attention must also be drawn to proton magnetic resonance spectroscopy which may be of value for configurational and conformational analyses[115].

TABLE II / ...

TABLE III

Infra-red Spectra of Compounds Containing a Hydrofuranol Ring

	Other Peaks	Type A	Type B	Type C	Type 2a	Type D	Other Peaks
3:6-Anhydrosorbitol*	981 s	933 s	886 s	867 s	-	781 m	-
1:4-Anhydro-D-mannitol*	995 vs 949 vs	929 s	880 s	868 s 860 s	-	782 s	-
1:4-Anhydro-D-galactitol**	980 m	909 m	870 s	-	-	-	-
3:6-Anhydro-L-galactose dimethyl acetal**	980 m	940 m	888 m	-	-	815 vw	735 m

* Barker and Stephens[110]

** Syrup

Frequencies of absorption peaks measured in cm^{-1}

m = moderate
s = strong
v = very
w = weak

TABLE IV

Infra-red Spectra of Carbohydrates Containing Fused Hydrofuranol and Pyranose Rings

	C-O-C vibrations, Type 1, etc.		Type A (924 ± 13)	Type 2b (895 ± 9)	Type B (or 2c) (870 ± 7)	Other peaks	Type C (or 2a) (858 ± 7)	Type 2a (844 ± 8)	Type D (799 ± 17)	Type 3 (747 ± 11)
		(917 ± 13)								
Methyl α-D-galacto-pyranoside (C1)	965 s	920 s	-	-	870 s	-	-	824 s	-	790 vs
Methyl 3:6-anhydro-α-D-galactopyrano-side* (1C)	987 m 966 vs		924 vs 903 vs	-	865 w	847 s	835 m	-	768 s	734 vs
Methyl 3:6-anhydro-α-D-galactopyrano-side** (1C)	970 vs		932 vs 909 vs	-	864 w	852 s	837 m	820 vw 786 m	768 s	735 s
1:2-Mono-O-iso-propylidene-3:6-anhydro-D-galacto-pyranoside** (1B)	983 s 970 s		943 vs 907 m	890 vs	859 vs	-	844 w	-	782 s 768 vs	755 s
Methyl-β-D-galacto-pyranoside* (C1)	983 vs	940 m	-	888 s	871 s	-	-	-	-	783 s
Methyl 3:6-anhydro-2:4-di-O-methyl-β-D-galactoside* (1B)	970 vs 928 vs	944 vs	907 vs	898 vs	861 s	811 s	-	-	767 s	733 s

* Barker and Stephens[110] ** Infra-red spectrum reproduced after p.45

Frequencies of absorption peaks measured in cm^{-1}

m = moderate s = strong

v = very w = weak

TABLE V

Infra-red Spectra of Disaccharides Containing a Hydrofuranol Ring

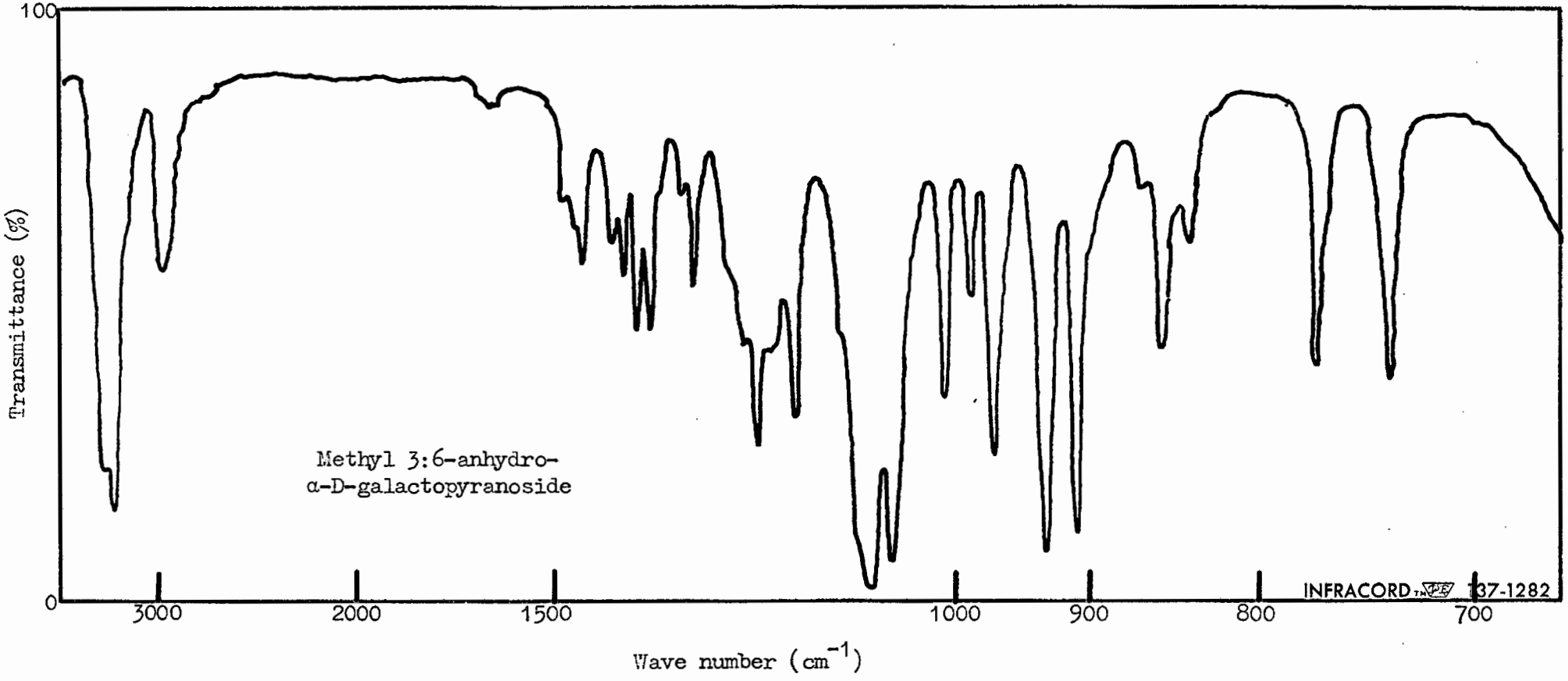
	C-O-C vibrations, Type 1, etc. (917 ± 13)	Type A (924 ± 13)	Type 2b (895 ± 9)	Type B (or 2c) (870 ± 7)	Other peaks	Type C (or 2a) (858 ± 7)	Type 2a (844 ± 8)	Type D (799 ± 17)	Type C (747 ± 11)
Agarobiose dimethyl acetal	996 w 983 m 970 w	933 m 918 w	882 s	861 m	-	-	-	795 w	758 m 730 m
Hexa-acetyl agarobiose dimethyl acetal	996 w 985 m 968 s	934 m 924 m 902 m	880 s	861 m	-	-	-	-	730 s
Agar disaccharide glycitol	989 s	945 m 912 vw 895 vw	891 s	880 m 870 m	-	-	-	790 s	734 s
Hypnea disaccharide glycitol	985 s	946 w 900 s	887 m	863 s 878 m	-	-	-	794 m	734 s
Hepta-acetyl Hypnea disaccharide glycitol	985 w 960 s	935 m 916 vw 910 w	900 vw	859 w 872 s	-	-	-	-	745 m

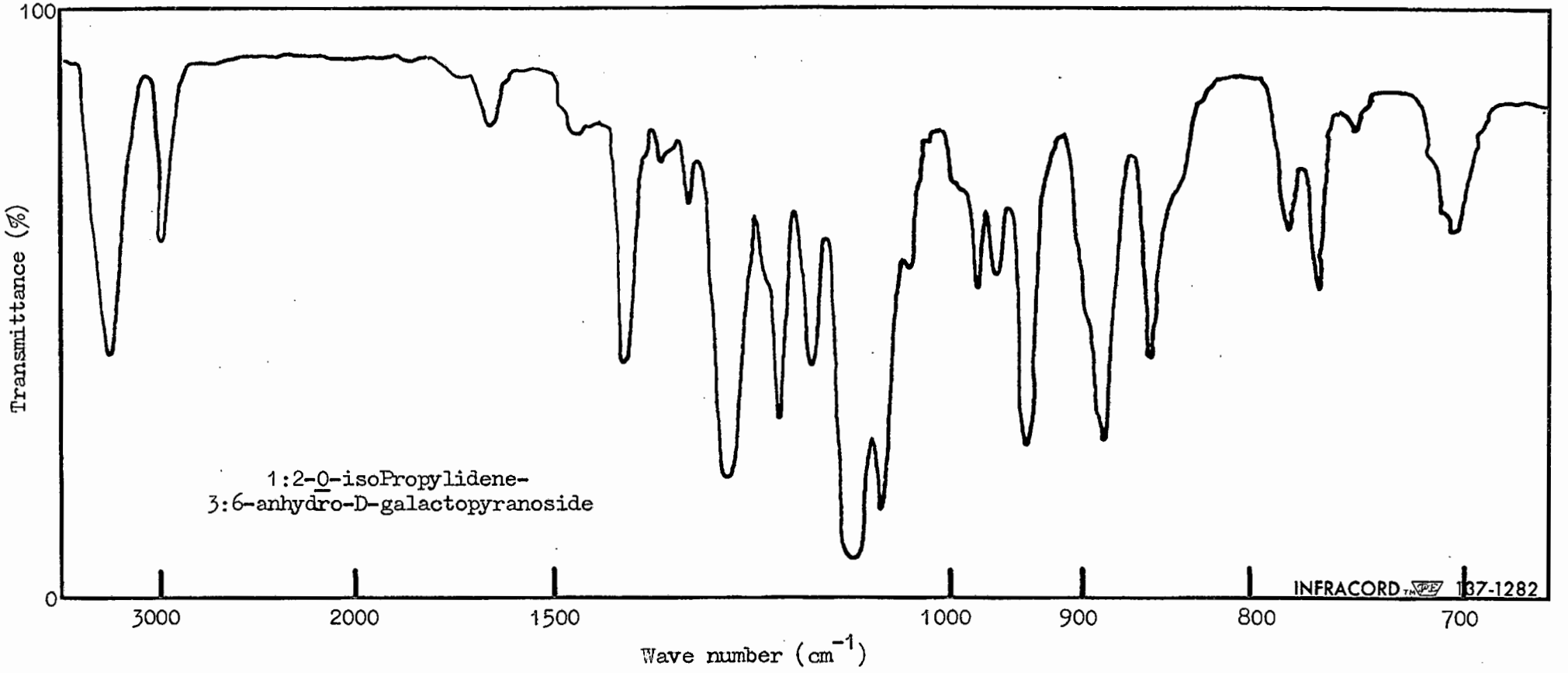
Frequencies of absorption peaks measured in cm^{-1}

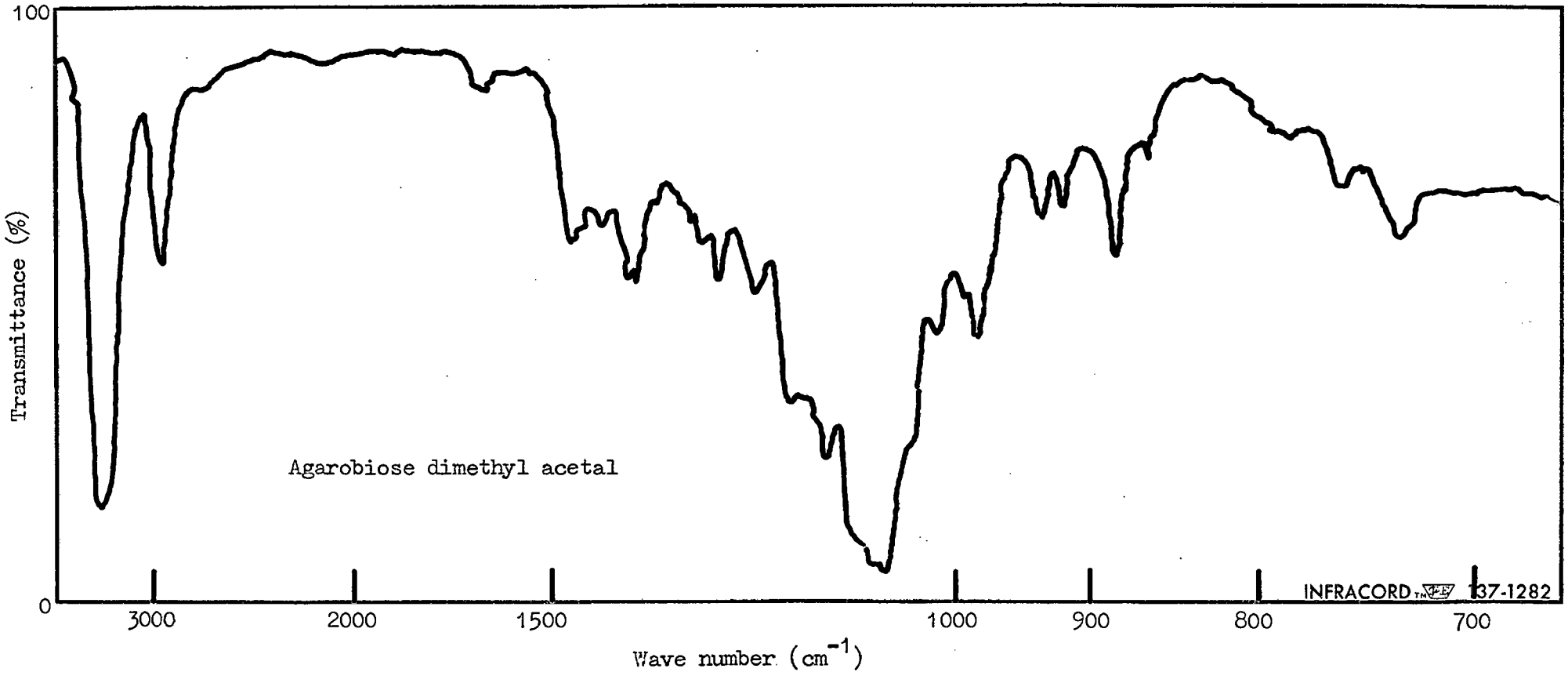
m = moderate s = strong

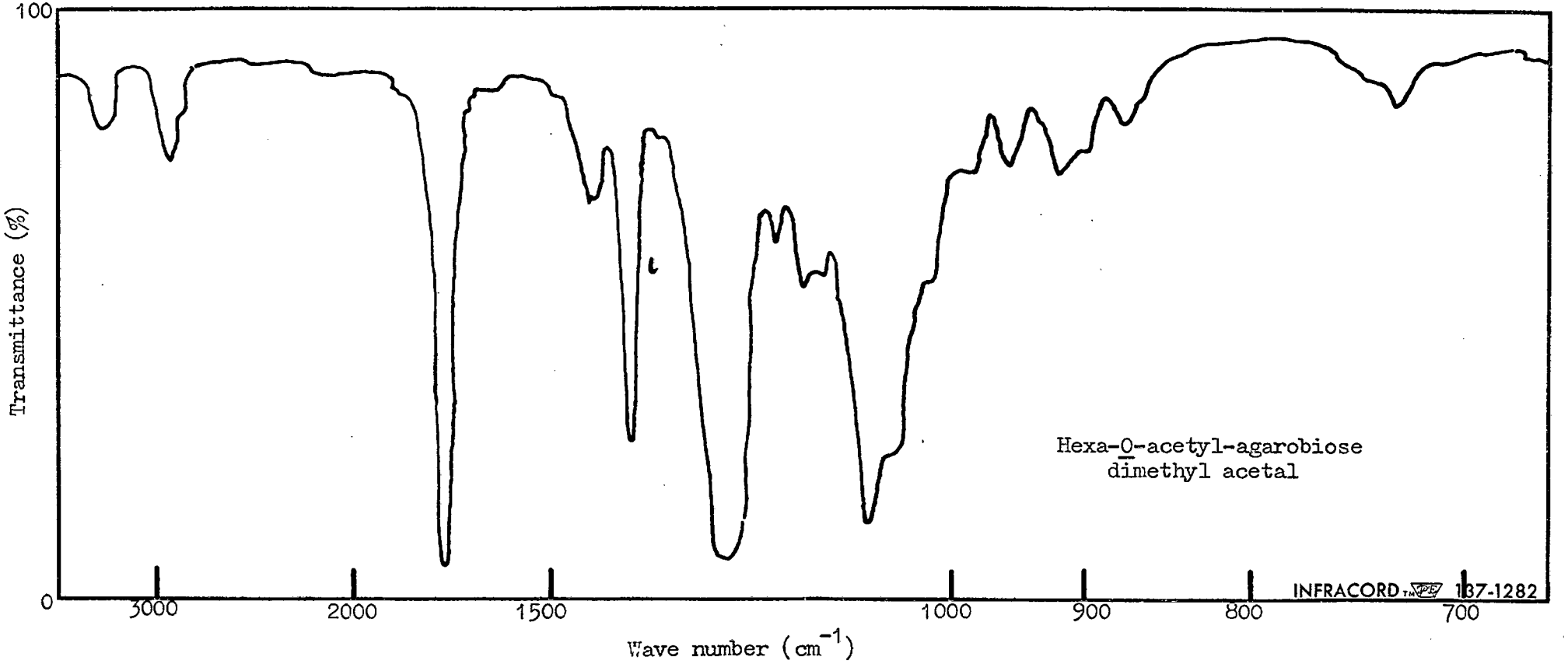
v = very w = weak

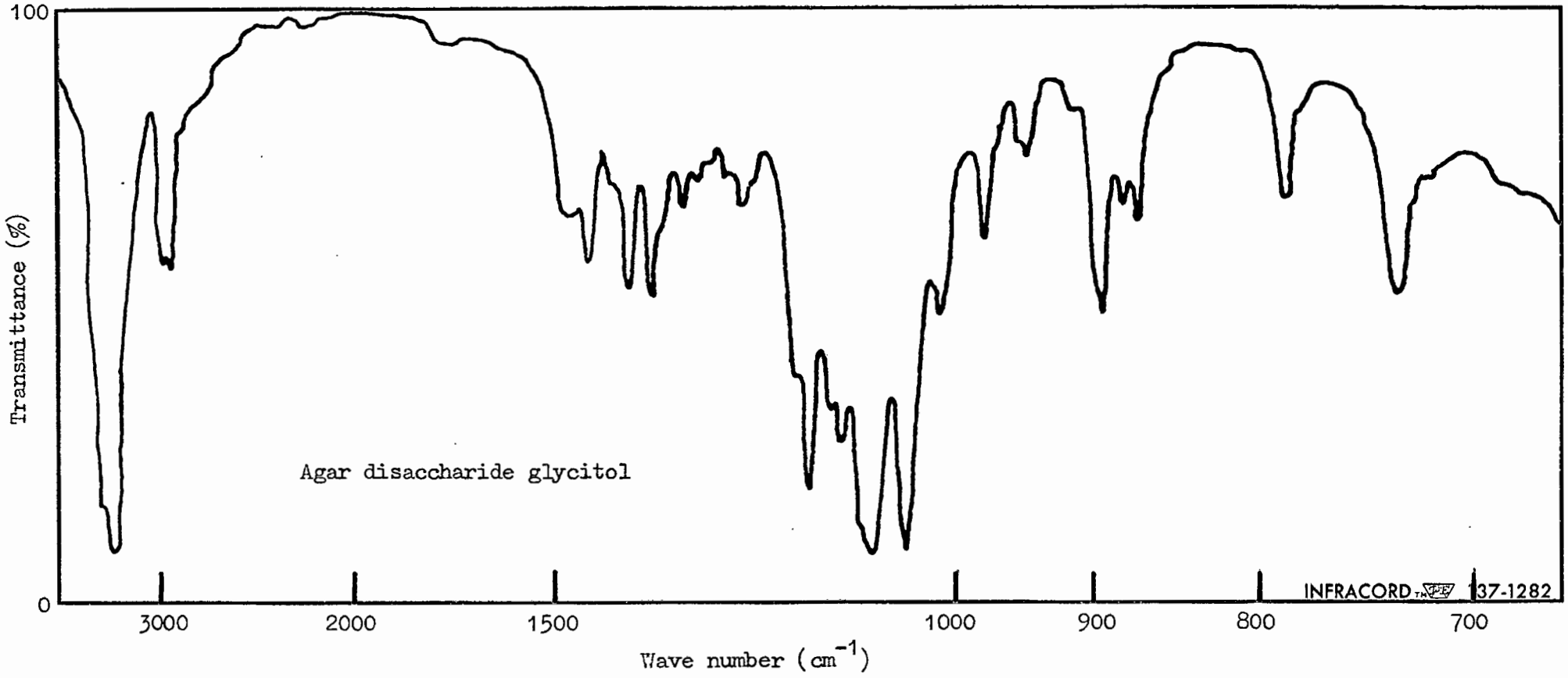
Infra-red spectra of these compounds reproduced on following pages.

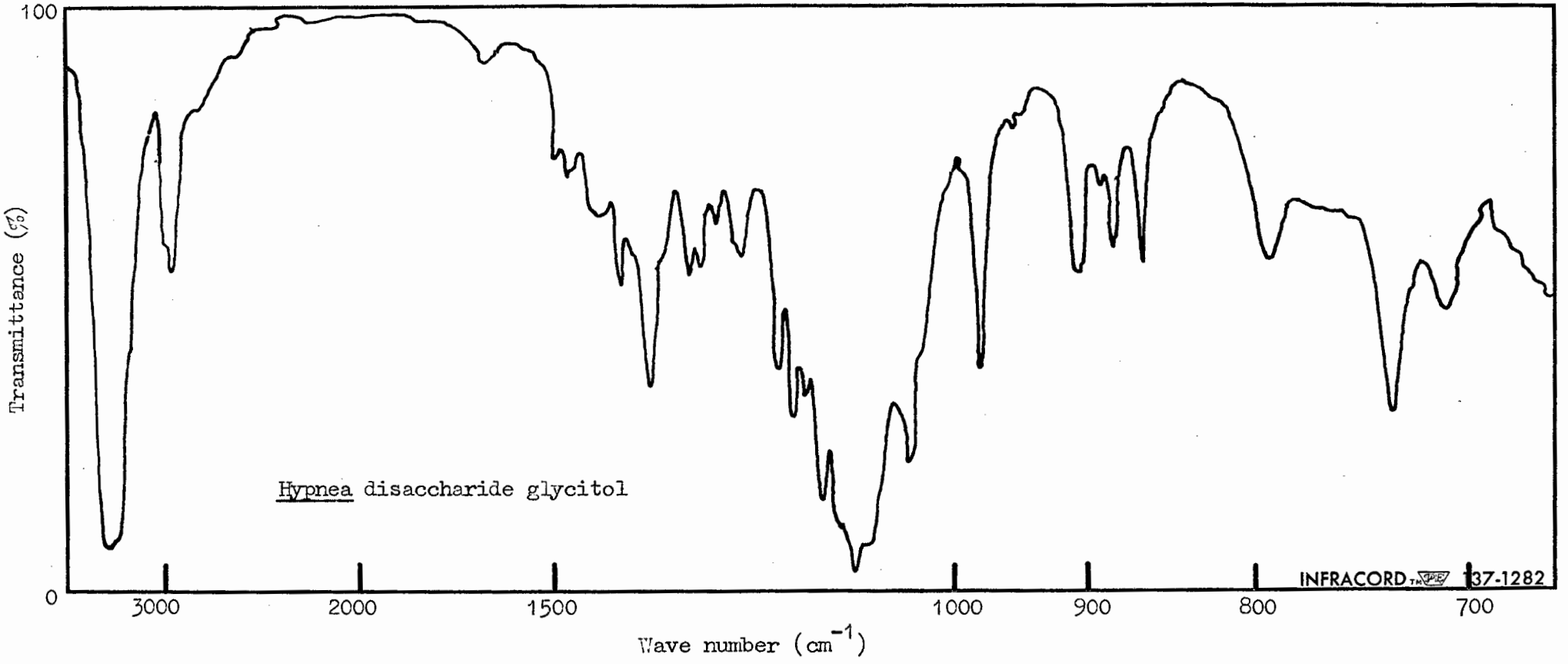

100
0
Transmittance (%)
Methyl 3:6-anhydro-
α-D-galactopyranoside
INFRACORD 137-1282
3000
2000
1500
1000
900
800
700
Wave number (cm-1)

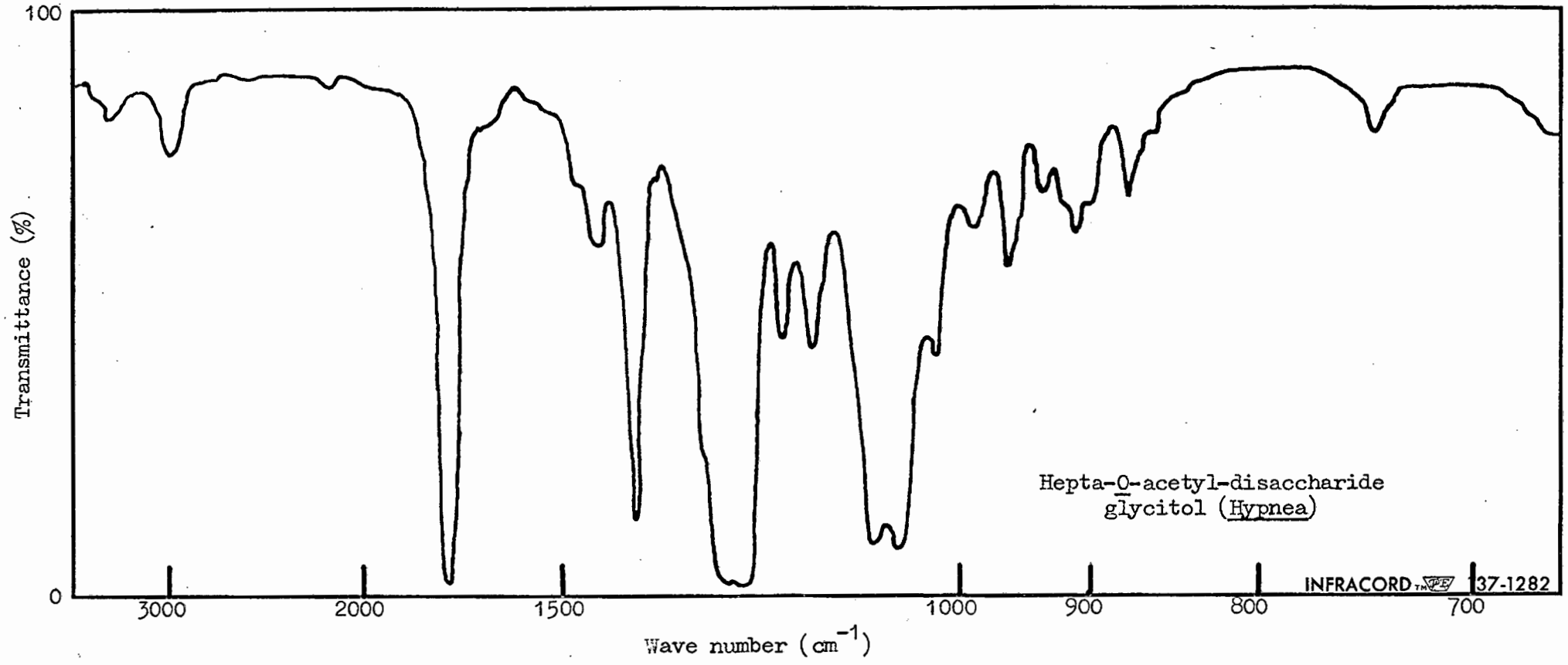

100
0
Transmittance (%)
1:2-O-isoPropylidene-
3:6-anhydro-D-galactopyranoside
INFRACORD 137-1282
3000
2000
1500
1000
900
800
700
Wave number (cm-1)


100
0
Transmittance (%)
Agarobiose dimethyl acetal
INFRACORD
37-1282
3000
2000
1500
1000
900
800
700
Wave number (cm-1)


100
0
Transmittance (%)
3000
2000
1500
1000
900
800
700
Wave number (cm^{-1})
Hexa-O-acetyl-agarobiose
dimethyl acetal
INFRACORD 137-1282


100
0
Transmittance (%)
Agar disaccharide glycitol
INFRACORD 137-1282
3000
2000
1500
1000
900
800
700
Wave number (cm-1)


100
0
Transmittance (%)
Hypnea disaccharide glycitol
INFRACORD
37-1282
3000
2000
1500
1000
900
800
700
Wave number (cm-1)


100
0
Transmittance (%)
3000
2000
1500
1000
900
800
700
Wave number (cm^{-1})
Hepta-O-acetyl-disaccharide
glycitol (Hypnea)
INFRACORD TM 137-1282

EXPERIMENTAL

Solutions were concentrated at 40°/20 mm. Paper chromatograms (Whatman No.1 paper) were run in the following solvents: (a) ethyl acetate-acetic acid-formic acid-water[74] (18:3:1:4), (b) butanol-pyridine-water (9:2:2) or (c) butanol-ethanol-water (20:1:3). p-Anisidine hydrochloride spray was used to detect reducing sugars[75] and a periodate-starch spray[76] was used to detect non-reducing sugars on papers. Melting points are corrected.

The / ...

The Hot-Water Soluble Polysaccharide of Gracilaria confervoides

Agar extracted from Gracilaria confervoides by Messrs Vitamin Oils (Pty.) Ltd. of Cape Town was used. For analytical purposes agar was left in contact with water which was frequently decanted and renewed. It was then left in water at 37° for a week, filtered, and washed with ethanol and acetone and dried at 60°/1 mm.

Moisture (7.11%) was determined by drying at 70°/1 mm. till constant weight (two days). Sulphated ash was determined in the usual way. Nitrogen was determined by the Kjeldahl method. The sulphate content was obtained by hydrolysing agar (0.8885 g.) in 0.5N-hydrochloric acid (50 c.c.) at 100° for 5 hr. and precipitating the sulphate as barium sulphate; the precipitate (0.0194 g.) was quantitatively filtered and ignited in a tared platinum crucible [Found (for dried agar): sulphated ash, 0.67; SO_4^{--}, 0.78; N, 0.5%].

Acetylation of Agar

Agar (25 g.) was added to formamide (250 c.c.) with shaking[56]. Pyridine (250 c.c.) was added with shaking and then acetic anhydride (300 c.c.) and the mixture left overnight. Acetone (1500 c.c.) was added and the solution was centrifuged. The clear liquors were poured into ice-water (7 l.) with stirring. The precipitate of agar acetate was filtered off and washed with water and alcohol. It was dissolved in acetone (1000 c.c.) and centrifuged, the clear solution being poured into ether (5 l.) with stirring; the precipitate (35 g.) was filtered, washed with ether and dried in a vacuum. The material was dissolved and precipitated in this way once more to give a product (28 g.) having $[\alpha]_D^{20}$ -34° (c, 1.0 in $CHCl_3$) (Found: Ac, 34.4% by the method of Rullen and Pacsu[77]).

Some agar acetate (8 g.) was dissolved in pyridine (75 c.c.), treated with acetic anhydride (75 c.c.) and left at room temp.

for / ...

for 5 days. The product was worked up exactly as described above to give a product (6 g.) having, $[\alpha]_D^{20}$ -34° (c, 1.0 in $CHCl_3$) (Found: Ac, 35.8; N, 0.13; SO_4^{--}, 0.47%).

Another sample of agar acetate (8 g.) was dissolved in pyridine (100 c.c.), treated with acetic anhydride (100 c.c.) and heated at 100° for 6 hr. and then left overnight at room temp. The product was again worked up as before to give a product (6 g.) having, $[\alpha]_D^{20}$ -36° (c, 1.0 in $CHCl_3$) (Found: Ac, 36.2; N, 0.09; SO_4^{--}, 0.30%).

A little agar acetate (4 g.) was de-acetylated with a solution of sodium methylate[78] (15 c.c.). The product (2.5 g.) was washed with methanol and dried (Found: Ac, 5.0%). When the product was dissolved in hot water it formed a gel on cooling.

Estimation of Galactose in Hydrolysed Agar

Dried agar (0.4058 g.) in N-sulphuric acid (5.0 c.c.) was hydrolysed at 100° for 16 hr. A standard 10.0% maltose solution (1.0 c.c.) was added[61] and the solution was neutralised (IR 4B resin) and made up to 25.0 c.c. Some of the solution (0.08 c.c.) was spotted on paper (Whatman No.20) and developed in solvent (a) for 48 hr. Strips of paper containing maltose and galactose (detected by spraying marginal strips) were cut out, macerated in water (20.0 c.c.), filtered, and the sugars estimated by the micro-Somogyi method[55]. A duplicate determination was carried out with agar (0.4036 g.). A blank determination was also performed [Found: galactose (as $C_6H_{10}O_5$), 42.0% of dry agar].

Estimation of Galactose in Hydrolysed Agar Acetate

Dry agar acetate (0.4327 g.) in N-sulphuric acid (3 c.c.) was hydrolysed at 100° for 16 hr. A standard maltose solution (1.2287 g./20 c.c.) was prepared and an aliquot (2.0 c.c.) was added[61] to the hydrolysate and the solution neutralised ($BaCO_3$) and made up to 25.0 c.c. A portion of this solution (0.10 c.c.) was spotted on paper (Whatman No.1) and developed in solvent (a) for 16 hr. The strips of

paper / ...

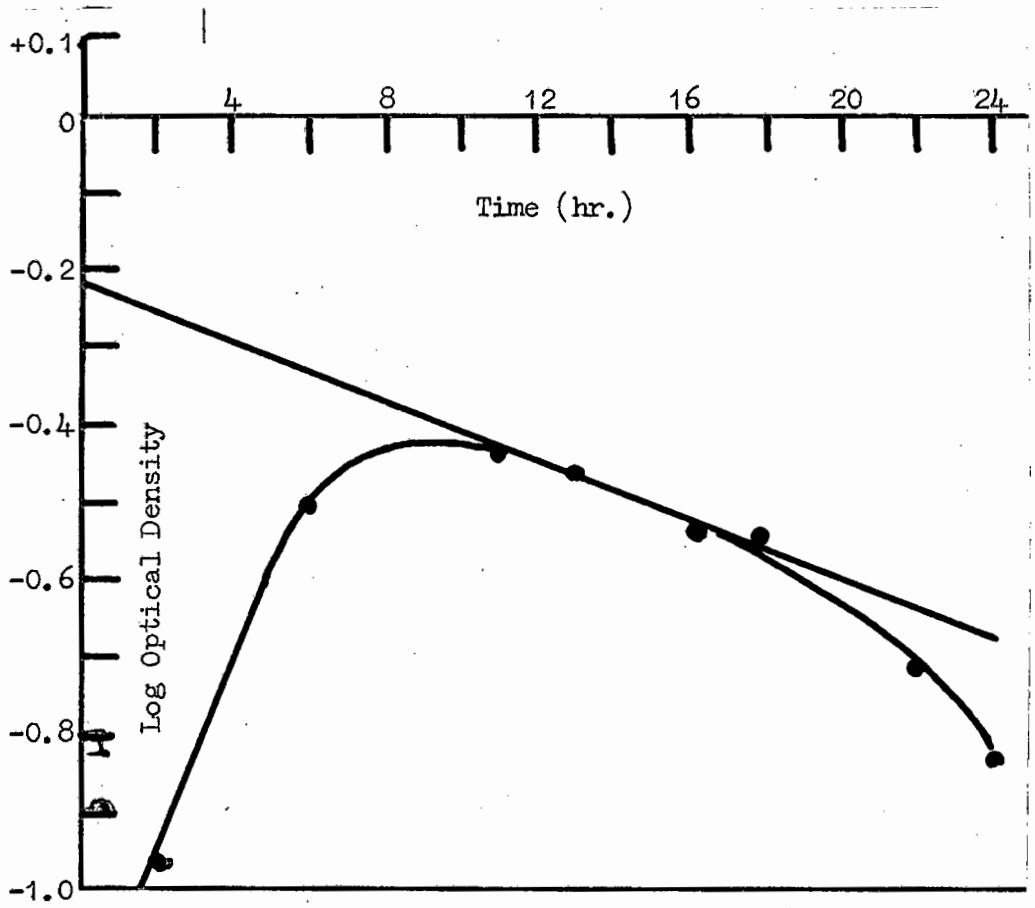
paper containing maltose and galactose were cut out, macerated in water (20.0 c.c.) and aliquots (2.0 c.c.) were used to estimate the sugars by the micro-Somogyi method[55]. A duplicate determination was carried out using agar acetate (0.4593 g.) [Found: galactose (as $C_6H_{10}O_5$) 33.5, 33.8% of agar acetate].

Estimation of 3:6-Anhydrogalactose in Agar[38]

3:6-Anhydrogalactose is degraded during acid hydrolysis to hydroxymethylfurfural. Sealed glass tubes, each containing agar (0.025 g.) and 0.15N-sulphuric acid (2.0 c.c.) were heated at 100°. Tubes were removed at different time intervals up to 24 hr., the contents neutralised ($BaCO_3$) and made up to 25.0 c.c. Aliquots (2.0 c.c.) were diluted to 100 c.c. and measurements of the optical density at 285.0 mμ, the absorption maximum for hydroxymethylfurfural, were made in a Beckman DU spectrophotometer[38]. Since hydroxymethylfurfural decomposes to formic and laevulinic acids during acid hydrolysis[79] the logarithms of the optical densities were plotted against time and the straight line portion of the curve extrapolated to zero time. From the extrapolated value (log optical density -0.22, corresponding to optical density 0.6026) and the molar extinction coefficient of hydroxymethylfurfural (16,500) the amount of hydroxymethylfurfural (23% of agar) was estimated and this was then found to correspond to 26.4% 3:6-anhydrogalactose (as $C_6H_8O_4$).

Time of Hydrolysis	Log Optical Density
2 hr.	-0.9586
6 hr.	-0.4962
8 hr.	-0.4486
11 hr.	-0.4318
13 hr.	-0.4622
16 hr.	-0.5302
18 hr.	-0.5406
22 hr.	-0.7077
24 hr.	-0.8239

Synthesis / ...



Estimation of 3:6-anhydro-L-galactose
in Gracilaria agar

Synthesis of 3:6-Anhydro-D-Galactose

D-Galactose (50 g.) in dry acetone (2000 c.c.) containing conc. sulphuric acid (60 c.c.) was shaken for two days at room temp. The unreacted galactose was filtered off and the solution worked up as described by Freudenberg and Hixon[80] to yield 1:2-3:4-di-O-isopropylidene-D-galactose, a syrup (55 g.).

The syrup (55 g.), dissolved in pyridine (60 c.c.), was treated with p-toluenesulphonylchloride (65 g.) care being taken[80] to keep the reaction temp. below 60°. After 5 hr. at room temp. water was added and a black oil settled which solidified on standing overnight. The solid material (35 g.) was crystallised from 80% aqueous methanol, m.p. 85 - 86°. Freudenberg and Hixon[80] report m.p. 91 - 92° for 1:2-3:4-di-O-isopropylidene-6-p-tosyl-D-galactose.

The material (35 g.) in 3% methanolic hydrogen chloride (350 c.c.) was refluxed for 45 min. and cooled. A crop of crystals was obtained, filtered off, and the filtrate, together with anhydrous methanolic washings, was concentrated to small volume in vacuum, and again filtered to remove crystalline material. This process was repeated several times. The combined crystal crops (21 g.) gave m.p. 162 - 163.5°. Bell and Williamson[81] record m.p. 170° for methyl 6-O-p-tosyl-α-D-galactoside.

The crystals (21 g.) were dissolved in boiling 96% ethanol (1500 c.c.) which contained N-sodium hydroxide (13 c.c.) and a few drops of phenolphthalein indicator solution[82]. Sufficient N-sodium hydroxide (ca. 20 c.c.) was added to neutralise the boiling solution. The solution was concentrated to dryness and the residue extracted with hot ethyl acetate, filtered, and concentrated to small volume when crystals (8 g.) were obtained m.p. 136 - 137°, $[\alpha]_D^{20}$ +75° (c, 1.0 in H_2O). Ohle and Thiel[82] report m.p. 139°, $[\alpha]_D^{20}$ +83.9° (H_2O) for methyl 3:6-anhydro-α-D-galactopyranoside.

1:4-Anhydro-L-Galactitol / ...

1:4-Anhydro-L-Galactitol

Methyl 3:6-anhydro-α-D-galactose (0.2 g.) in 0.1N-sulphuric acid (20 c.c.) was left at room temp. for two days, $[\alpha]_D^{18}$ +70° ⟶ 25.4° (constant value). The solution was neutralised ($BaCO_3$) and concentrated to a syrup (0.18 g.) which reduced Fehling's solution in the cold. The syrup (0.18 g.), in water (3 c.c.), was treated with sodium borohydride (0.02 g.) in water (0.5 c.c.) and left overnight. The solution was neutralised (Amberlite IR 120 resin) and concentrated. The product was spotted on paper and developed overnight in solvent (a). The strips of paper containing the product (R_{Gal} 3.1) were cut out, macerated in water, filtered and concentrated to a syrup (0.15 g.), $[\alpha]_D^{18}$ +13° (c, 1.0 in H_2O). Hocket, Conley, Yusem, and Mason[67] report $[\alpha]_D$ +16.1° (H_2O) for 1:4-anhydro-L-galactitol (=3:6-anhydro-D-galactitol).

1:2-O-iso-Propylidene-3:6-Anhydro-D-Galactose

Methyl 3:6-anhydro-α-D-galactoside (1.5 g.) in N-sulphuric acid (150 c.c.) was left at room temp. for 48 hr., neutralised ($BaCO_3$) and concentrated. The dry syrup was dissolved in anhydrous acetone (45 c.c.) containing conc. sulphuric acid (0.75 c.c.) and anhydrous copper sulphate (5 g.) and left at room temp. for 5 hr. Anhydrous potassium carbonate was added to neutralise the reaction mixture which was filtered and concentrated. The isopropylidene derivative crystallised slowly on standing. The material was recrystallised from petroleum ether (b.p. 60 - 80°), m.p. 90 - 91°, $[\alpha]_D^{18}$ +20° (c, 1.0 in $CHCl_3$) (Found: C, 53.4; H, 7.2. Calc. for $C_9H_{14}O_5$: C, 53.5; H, 7.0%). Araki and Arai[83] report m.p. 92° for 1:2-O-isopropylidene-3:6-anhydro-D-galactose.

Hydrolysis of Agar

Agar (2 g.) in 0.1N-sulphuric acid (50 c.c.) was hydrolysed at 100°. Samples (2 c.c.) were withdrawn at regular time intervals, neutralised ($BaCO_3$), concentrated and spotted on papers developed in

solvents / ...

solvents (a) and (b). All the paper chromatograms were characterised by pronounced streaking but the hydrolysates of 3 to 3.5 hr. appeared to give a maximum concentration of disaccharide; a series of spots was obtained of R_{Gal} (relative to galactose) 0.2, 0.3, 0.35, 0.5, 0.6, 0.75 and 1.0.

Fractionation of Agar Hydrolysate on Charcoal

Agar (20 g.) in 0.1N-sulphuric acid (500 c.c.) was hydrolysed at 100° for 3.5 hr. The hydrolysate was neutralised ($BaCO_3$) and concentrated. It was then deionised (Amberlite IR 120 and IR 4B resins); the deionised solution, being acid, was neutralised (0.1N-sodium hydroxide) and concentrated to a syrup. The syrup was fractionated on a charcoal-Celite column[58] (43 x 3.5 cm.). The column was eluted first with water (350 c.c.) and then with aqueous ethanol of increasing ethanol concentration, using the method of gradient elution[59]: aqueous ethanol (40% v/v) was continuously fed into a reservoir, initially containing only water (1 l.) which was magnetically stirred; solvent from the reservoir was siphoned on to the top of the column at the same rate as the 40% aqueous ethanol entered the reservoir. Fractions, collected from the column, were examined on papers developed in solvent (a). The results of this experiment are tabulated below.

Fraction	Volume c.c.	Wt. of Residue g.	R_{Gal} (relative to galactose) in solvent (a)
1	350	2.8	1.0, 1.2, 1.4
2	180		0.4, 1.0, 1.4
3	150	3.4	0.4, 1.0, 1.5
4	200		0.4, 1.0, 1.5
5	220	1.7	0.4, 0.9
6	170	1.5	0.2, 0.4
7	90		0.2, 0.4
8	250		0.2, 0.4
9	90	1.0	0.1, 0.4
10	90		0.1
11	250	0.2	-
12	500	0.4	-

Fraction / ...

Fraction 1 which contained mainly galactose (R_{Gal} 1.0) was discarded.

Fractions 2, 3 and 4 spotted on papers [solvents (a) and (b)] were shown to contain disaccharide material (R_{Gal} 0.4) but pronounced streaking was evident, and it was obvious that the material was not pure. The fractions were combined and concentrated to a white foam (3.4 g.) which reduced Fehling's solution and gave a positive Seliwanoff test, $[\alpha]_D^{17}$ -20° (c, 4.7 in H_2O) (Found: C, 43.35; H, 6.4. Calc. for $C_{12}H_{22}O_{11}$: C, 42.0; H, 6.4; Calc. for $C_{12}H_{20}O_{10}$: C, 44.4; H, 6.3%). The phenylosazone was prepared by reacting the material (0.2 g.) in water (1 c.c.) with a solution of phenylhydrazine (0.5 c.c.) in acetic acid (2 c.c.) and water (2 c.c.) and heating on a water bath for 0.5 hr. The phenylosazone (0.053 g.) crystallised on cooling, m.p. 174 - 176°. Two recrystallisations from water gave m.p. 194 - 195° (Found: N, 10.9. Calc. for $C_{24}H_{30}O_8N_4$: 11.0%).

Some of the material (0.27 g.) in acetic anhydride (4 c.c.) and sodium acetate (0.27 g.) was heated at 100° for one hour and then poured into ice-water (100 c.c.). The mixture was extracted with chloroform and the chloroform layer washed with dilute sodium bicarbonate solution and water. The chloroform extract was dried (K_2CO_3) and evaporated, leaving a brown syrup which could not be crystallised.

Disaccharide material (0.5 g.) in N-sulphuric acid (5 c.c.) was hydrolysed at 100° for 1.5 hr. (specific rotations of the hydrolysate could not be read due to the rapid darkening of the solution), neutralised ($BaCO_3$) and concentrated. Paper chromatograms [solvent (a)] showed the presence of four substances R_{Gal} 0.4 (starting material), 1.0 (galactose), 1.4 (pentose?), and 2.4 (probably hydroxymethylfurfural).

Some disaccharide material (0.02 g.) in a little water was spotted along the starting line of a paper, which was developed in solvent (a). Marginal strips were cut out and sprayed to detect the whereabouts of the disaccharide on the paper. The strip of paper containing the disaccharide was cut out, macerated in water (10 c.c.),

filtered / ...

filtered, and concentrated. The concentrate in N-sulphuric acid (2 c.c.) was hydrolysed at 100° for 3 hr., neutralised ($BaCO_3$), concentrated and spotted on a paper developed in solvent (a); identical spots as were obtained for hydrolysed original disaccharide material were obtained: R_{Gal} 0.4, 1.0, 1.4 and 2.4.

Disaccharide material (0.74 g.) in water (10 c.c.) was methylated with methyl sulphate (12 c.c.) and 30% sodium hydroxide solution (25 c.c.) in the usual way, care being taken not to add excess sodium hydroxide until glycoside formation had taken place. When all the reagents had been added after 6 hr. the mixture was heated on a boiling water bath for 30 min., and after cooling, carefully neutralised with 50% sulphuric acid. The solution was concentrated to a workable volume. Methyl sulphate (10 c.c.) and 30% sodium hydroxide solution (20 c.c.) were added slowly to the vigorously stirred mixture at room temperature over 5 hr. The mixture was heated on a boiling water bath for 30 min., cooled, neutralised (50% H_2SO_4), and concentrated to half its volume. The precipitated salts were filtered off and washed with a little water. The combined filtrate and washings was extracted with chloroform six times; the chloroform extract was washed with water, dried (Na_2SO_4), and concentrated to a syrup (0.5 g.). The syrup (0.5 g.) was refluxed with methyl iodide (8 c.c.), and silver oxide (2 g.) added in portions over 6 hr. The solution was filtered and the precipitate washed with hot methanol. The combined filtrate and washings was concentrated to a syrup (0.4 g.) which was hydrolysed in N-sulphuric acid (5 c.c.) at 100° for 3 hr., neutralised ($BaCO_3$) and concentrated. The hydrolysed product was spotted on paper developed in solvent (c) and sprayed with p-ansidine hydrochloride to show two carmine spots R_{TMGal} (relative to tetramethylgalactose) 1.0 and 0.86.

Fraction 5 (1.7 g.), $[\alpha]_D^{18}$ -19° (c, 1 in H_2O) was fractionated on a cellulose column (29 x 2.5 cm.) using n-butanol-water (9:1) as eluting solvent; fractions, collected in an automatic fraction cutter, were spotted on paper chromatograms, which were developed in solvents (a)

and / ...

and (b). All papers showed the usual streakings, and it was not possible to differentiate between fractions. Some of the material obtained from the column was hydrolysed with N-sulphuric acid at 100° for 4 hr., neutralised ($BaCO_3$), and spotted on papers [solvent (a)] to reveal spots of R_{Gal} 1.0 (galactose), 1.3 (pentose?) and 2.5 (hydroxymethylfurfural?).

Fraction 5 was again worked up and an attempted fractionation on a cellulose column (29 x 2.5 cm.) using n-butanol-pyridine-water (6:4:3) gave no better results.

Fraction 5 (0.1163 g.) in water (110.0 c.c.) containing potassium chloride (5 g.) was treated with 0.25M-sodium metaperiodate (11.0 c.c.), and after being shaken was left in the dark. A blank was also prepared[84]. Aliquots (10.0 c.c.) were taken at time intervals, ethylene glycol (0.2 c.c.) was added, and the solution titrated against 0.02134N-sodium hydroxide solution using methyl red indicator to estimate formic acid produced.

Time of Reaction	Formic Acid Produced
hr.	mol.
72	1.85
96	1.90
238	2.06
264	2.07
300	2.07

No formaldehyde could be detected in the reaction product[85].

Fractionation of Agar Hydrolysate on Stearic Acid Treated Charcoal

Agar (20 g.) in 0.1N-sulphuric acid (500 c.c.) was hydrolysed at 100° for 3.5 hr., neutralised ($BaCO_3$) and concentrated to a syrup.

A charcoal-Celite column[58] was prepared (47 x 3.5 cm.). A saturated solution of stearic acid in ethanol (1000 c.c.) was washed through the column. The packing was then extruded from the column and filtered under suction, being successively washed with 50% aqueous

ethanol / ...

ethanol (1000 c.c.), 20% aqueous ethanol (1000 c.c.), and 5% aqueous ethanol (1000 c.c.), and then sucked dry. The charcoal-Celite was then packed into the column ready for use[59].

The syrup was fractionated on the column, first by elution with water (150 c.c.), and thereafter with aqueous ethanol, the method of gradient elution[59] being used; 40% aqueous ethanol was fed into a reservoir of water (1000 c.c.) with stirring, and the resulting solution was siphoned over to the top of the column at the same rate as the aqueous ethanol entered it. Fractions (50 c.c.) were collected with the aid of an automatic fraction-cutter, and were examined on paper chromatograms developed in solvents (a) and (c). After monosaccharides had been eluted from the column (R_{Gal} 1.0 and 1.3) the fractions could not be satisfactorily differentiated; all these fractions exhibited pronounced streaking on papers but three spots of R_{Gal} 0.3, 0.7 and 1.0 were tentatively noted.

Reduction of Partially Hydrolysed Agar

Agar (15 g.) in 0.1N-sulphuric acid (250 c.c.) was hydrolysed at 100° for 3.5 hr., neutralised ($BaCO_3$) and concentrated to a syrup (12.5 g.). The syrup in water (100 c.c.) was treated with sodium borohydride (1 g.) in water (20 c.c.) with stirring. It was left overnight when the solution was non-reducing (Fehling's). It was neutralised (IR 120 resin) and concentrated. Chromatograms run in solvent (a) and sprayed with periodate-benzidine[86] showed a series of discrete spots, R_{Gal} 3.1, 1.2, 0.8, 0.3 and 0.16. This mixture was fractionated on a cellulose column (36 x 5 cm.), using n-butanol-formic acid-water (45:1:4) as solvent. The first fraction obtained was a syrup (0.4 g.) chromatographically identical with 1:4-anhydro-D-galactitol, R_{Gal} 3.1. This was followed by dulcitol (1 g.) R_{Gal} 1.2, m.p. and mixed m.p. 185 - 187°, then by a disaccharide glycitol (3 g.), a mixture of disaccharide glycitol and another glycitol (0.9 g.) R_{Gal} 0.52 followed by the other glycitol (0.5 g.) R_{Gal} 0.52 $[\alpha]_D^{17}$ -27°

(c, 1.0 / ...

(c, 1.0 in H_2O). No further material could be eluted from the column with the solvent used but elution with water yielded an amorphous mixture (2.6 g.) of oligosaccharide glycitols.

The Disaccharide Glycitol

The substance crystallised in large colourless needles from methanol, $[\alpha]_D^{22}$ -15° (c, 1.2 in H_2O), m.p. 174° (Found: C, 44.4; H, 7.05. $C_{12}H_{21}O_{10}$ requires C, 44.2; H, 6.8%). It (0.69 g.) was hydrolysed in N-sulphuric acid (15 c.c.) at 100° for 10 hr., $[\alpha]_D^{21}$ -15° → +31°. The hydrolysate was neutralised ($BaCO_3$) and concentrated, the residue being dissolved in methanol. Crystals of D-galactose were deposited (0.272 g.), m.p. and mixed m.p. 165 - 166°, which were filtered off, and the syrup obtained by evaporation of the filtrate was spotted on large sheets of paper. The chromatograms were run overnight in solvent (a) and the strips of paper containing carbohydrate material (detected by spraying marginal strips) were cut out and extracted by being macerated in water in the usual way. A further crop of D-galactose (0.057 g., total yield 87%) was obtained, and also a syrup (0.276 g., 79%) $[\alpha]_D^{18}$ -18° (c, 0.86 in H_2O), chromatographically identical with 1:4-anhydro-D-galactitol. Ness, Fletcher and Hudson[62] reported $[\alpha]_D^{20}$ -18° (H_2O) for 1:4-anhydro-D-galactitol (=3:6-anhydro-L-galactitol).

Acetylation and benzoylation of the disaccharide glycitol yielded syrups, $[\alpha]_D^{23}$ 0° (c, 1.1 in MeOH) and $[\alpha]_D^{16}$ +64° (c, 1.4 in $CHCl_3$) respectively.

Methylated Disaccharide Glycitol

The disaccharide glycitol (0.8 g.) in 30% (w/w) sodium hydroxide solution (23 c.c.) was treated with methyl sulphate (10 c.c.) with stirring at 0° for 3 hr. The mixture was heated on a boiling water bath for 30 min., and again methylated as before. The mixture was neutralised (50% H_2SO_4, cold) and extracted several times with chloroform. The chloroform layer was washed, and dried (K_2CO_3) and

evaporated / ...

evaporated, leaving a syrup (0.4 g.) which was methylated twice with methyl iodide (5 c.c.) and silver oxide (2.5 g.) to give a syrup (0.15 g.), $[\alpha]_D^{22}$ -36° (c, 0.55 in MeOH) (Found: C, 54.0; H, 8.5; OMe, 48.0. $C_{19}H_{36}O_{10}$ requires C, 53.8; H, 8.6; OMe, 51.2%).

The methylated product (0.075 g.) was hydrolysed in N-sulphuric acid (5 c.c.) at 100° for 5 hr., $[\alpha]_D^{25}$ -15° ⟶ +27°, neutralised ($BaCO_3$), and evaporated to leave a syrup. Chromatograms of this syrup showed a spot corresponding to tetra-O-methyl-D-galactose. The syrup was separated on large papers in solvent (c) and the strips corresponding to tetra-O-methyl-D-galactose were cut out and eluted with water and concentrated. The material (0.021 g.) was refluxed with aniline (7.1 mg.) in ethanol (3 c.c.) for 30 min. After standing at room temp. for some time the crystals which had formed were filtered off and recrystallised from ethanol, m.p. and mixed m.p. 189 - 190° with authentic 2:3:4:6-tetra-O-methyl-N-phenyl-D-galactosylamine.

Periodate Oxidation of Disaccharide Glycitol

The disaccharide-glycitol (0.1101 g.) in water (25 c.c.) was treated with 0.4M sodium metaperiodate (5.0 c.c.) and the mixture made up to 50 c.c. The progress of the reaction was followed by titrating aliquots (5.0 c.c.), after excess periodate had been destroyed with ethylene glycol (0.25 c.c.), against 0.02N-sodium hydroxide at time intervals using bromocresol purple as indicator[87].

Time of Reaction hr.	Formic Acid Produced mol.
3	0.85
20	0.91
25	0.94
48	0.95

The periodate consumed was determined in aliquots (2 c.c.) which were treated with excess 0.1N-sodium arsenite (5.0 c.c.) in the presence of sodium bicarbonate, potassium iodide, and starch, and then

back / ...

back-titrated with 0.1N-iodine. A blank determination was also carried out.

Formaldehyde was estimated in an aliquot (5.0 c.c.) as its dimedone derivative[85] m.p. and mixed m.p. 188 - 189° (Found: 0.95 mol. formic acid; 1.25 mol. formaldehyde produced; 3.10 mol. periodate consumed).

The Methanolysis of Agar

Agar (25 g.) that had been dried at 60°/1 mm. for 24 hr., was refluxed in 1% methanolic hydrogen chloride (250 c.c.) for 1 hr. The product was cooled, filtered free of undissolved material (4 g.) (Found: C, 43.0; H, 6.8; OMe, 4.2%), and the filtrate neutralised (Ag_2CO_3), and concentrated. The syrup (25 g.) obtained was fractionated on a cellulose column[61] (43 x 8 cm.) using n-butanol-water (9:1) as irrigating solvent. Fractions (100 c.c.) were collected using an automatic fraction cutter. Fractions were spotted on papers (solvent c) and similar fractions were combined and concentrated, to give four main fractions, after which no more material could be eluted off the column with this solvent. The column was subsequently stripped with ethanol and water to yield material (6.6 g.) of very low methoxyl value (Found: C, 43.5; H, 7.3; OMe, 8.4%). The weight of this material (6.6 g.) together with that of the initially undissolved agar (4 g.) was subtracted from the original weight of agar for the purpose of calculating percentages of methanolysate products.

Fraction 1 was a syrup [2.8 g.; 12.7% (as $C_6H_8O_4$) of reacted agar] $[\alpha]_D^{20}$ -26° (c, 1.0 in H_2O), R_{Rh} 1.8 (relative to rhamnose) in solvent (c) (Found: C, 46.7; H, 7.5; OMe, 24.2. Calc. for $C_8H_{16}O_6$: C, 46.3; H, 7.8; OMe, 29.8%). 3:6-Anhydro-L-galactose dimethyl acetal of $[\alpha]_D^{10}$ -28.7° (H_2O) is reported by Araki and Hirase[21]. The syrup (0.37 g.) in N-sulphuric acid (37 c.c.) was left at room temp. for 36 hr., $[\alpha]_D^{20}$ -26° ⟶ -20° (constant value). The solution was neutralised ($BaCO_3$), concentrated to a clear syrup which reduced

Fehling's / ...

Fehling's solution in the cold and gave positive Seliwanoff and Schiff reactions. The syrup was dissolved in water (2 c.c.) and the phenylosazone derivative was prepared. A yellow crystalline product was obtained which after recrystallisation from methanol gave m.p. 203 - 204.5°, and mixed m.p. with 3:6-anhydro-L-galactosazone derived from Porphyra capensis polysaccharide[88] 203 - 204.5° (Found: N, 16.5. Calc. for $C_{18}H_{20}O_3N_4$: N, 16.5%). The combined filtrate and washings from the first crop of crystals was left at room temp. for several days when a further crop of crystals was deposited, m.p. 212 - 213° (Found: N, 16.45. Calc. for $C_{18}H_{20}O_3N_4$: 16.5%). Haworth, Jackson and Smith[64] and O'Neill[41] report m.p. 216° for 3:6-anhydro-D-galactosazone.

The remainder of fraction 1 (2.4 g.) was distilled to give a light yellow syrup (1.4 g.) b.p. 120 - 160°/0.03 mm. (bath tem.). Trituration with ethyl acetate resulted in crystallisation of the material. Five recrystallisations from ethyl acetate yielded colourless needle-like crystals, m.p. and mixed m.p. 139 - 139.5° with methyl 3:6-anhydro-α-L-galactoside, $[\alpha]_D^{20}$ -85° (c, 1.0 in H_2O) (Found: C, 47.7; H, 6.9; OMe, 16.7. Calc. for $C_7H_{12}O_5$: C, 47.7; H, 6.8; OMe, 17.6%). Haworth, Jackson and Smith[64] report $[\alpha]_D^{18}$ +80° (c, 1.0 in H_2O) for methyl 3:6-anhydro-α-D-galactoside.

Two attempts to prepare a tri-p-nitrobenzoate derivative[64] were unsuccessful.

Fraction 2 was a syrup (1.77 g.) assumed to be a mixture of the methyl α- and β-galactofuranosides, R_{Rh} 1.3 and 1.1 in solvent (c). Fraction 3 (2.34 g.) R_{Rh} 0.87 in solvent (c) crystallised from ethyl acetate, m.p. 108 - 109°, $[\alpha]_D^{20}$ +180° (c, 0.5 in H_2O), lit. reports m.p. 112°, $[\alpha]_D^{20}$ +179° (H_2O) for methyl α-D-galactopyranoside. The residue from the filtrate of the first crop of crystals dissolved in ethanol yielded a crop of crystals, m.p. and mixed m.p. 175° - 176° with methyl β-D-galactopyranoside, $[\alpha]_D^{20}$ 0° (c, 1.0 in H_2O). The total weight of methyl galactosides (4.11 g.) represented 22.6% (calc. as $C_6H_{10}O_5$) of the reacted agar.

Fraction 4 / ...

Fraction 4 was a solid residue [11.4 g.; 62% (calc. as $C_{12}H_{18}O_9$) of the reacted agar], R_{Rh} 0.65 in solvent (c) which was crystallised and recrystallised from ethanol to yield beautiful colourless hexagonal plates, m.p. 163 - 166°, $[\alpha]_D^{18}$ -36° (c, 1.0 in MeOH) (Found: C, 45.5; H, 7.2; OMe, 16.9. Calc. for $C_{14}H_{26}O_{11}$: C, 45.5; H, 7.1; OMe, 16.8%). Araki and Hirase[21] report m.p. 162 - 164°, $[\alpha]_D^{20}$ -37.4° (c, 1.1 in MeOH) for agarobiose dimethyl acetal.

Some of this compound (0.8 g.) in 0.01N-oxalic acid (8.0 c.c.) was heated on a boiling water bath for 2.5 hr., $[\alpha]_D^{18}$ -25° $\longrightarrow$ -17° (constant value). The solution was neutralised (IR 4B resin) and concentrated to a white solid foam (0.55 g.) which reduced Fehling's solution in the cold and gave positive Schiff and Seliwanoff reactions. Developed on papers (solvent c) this sugar gave a long streaky spot of approximately R_{Gal} 0.27.

The phenylosazone prepared in the usual way was recrystallised from 96% ethanol, m.p. 222 - 223.5°, $[\alpha]_D^{20}$ -115° [c, 0.8 in pyridine-ethanol (2:3)] (Found: C, 54.6; H, 5.9; N, 10.9. Calc. for $C_{24}H_{30}O_4N_8$: C, 57.35; H, 6.0; N, 11.15%). Araki and Hirase[21] report m.p. 220 - 221°, $[\alpha]_D^2$ -136.8° $\longrightarrow$ -108.8° (after 24 hr.) [c, 0.432 in pyridine-ethanol (2:3)].

Agarobiose dimethyl acetal (0.55 g.) in dry pyridine (5 c.c.) was cooled to 5° and acetic anhydride (5 c.c.) in pyridine (5 c.c.) was cooled to 5° and the solutions were combined. The mixture was left at 5° for 3 days and at room temp. for 5 hr. and then poured into ice-water (200 c.c.); after 2 days the hexa-acetate of agarobiose dimethyl acetal crystallised out. Recrystallisation from methanol gave beautiful colourless crystals, m.p. 137.5 - 138.5°, $[\alpha]_D^{18}$ -13.5° (c, 1.2 in benzene) (Found: C, 50.2; H, 6.2; OMe, 9.8; Ac, 39.0. Calc. for $C_{26}H_{38}O_{17}$: C, 50.15; H, 6.15; OMe 10.0; Ac, 41.5%). Araki and Hirase[21] report m.p. 87 - 88°, $[\alpha]_D^{13}$ -12.5° (in benzene).

[The following experiment was carried out by Dr. J.R. Nunn:- A little of the free sugar in water was reduced with sodium borohydride

in / ...

in the usual way. The solution was neutralised (IR 120 resin) and concentrated to dryness. The residue was fractionated on a cellulose column with n-butanol-formic acid-water (45:4:1) as irrigating solvent. The carbohydrate material off the column crystallised from methanol m.p. and mixed m.p. 173 - 174° with the disaccharide glycitol previously obtained from agar.]

0.4M-Sodium metaperiodate (5.0 c.c.) was added to agarobiose dimethyl acetal (0.0977 g.) in water (ca. 25 c.c.) and the mixture made up to 50.0 c.c. Simultaneously a blank was prepared. Both these solutions were stored in the dark. In order to estimate the formic acid produced at time intervals, aliquots (5.0 c.c.), after the addition of excess ethylene glycol, were titrated against 0.01N-sodium hydroxide using bromocresol purple indicator[87]. The reaction was complete in 24 hr.

Time of Reaction	Formic Acid Produced
hr.	mol.
1	0.94
6	0.97
24	0.99

The usual method was employed to estimate periodate consumed. Aliquots (5.0 c.c.) of both sample and blank were treated with excess 0.1N-sodium arsenite (10.0 c.c.) and sodium bicarbonate (2 g.) and 20% potassium iodide. The mixtures were left for 15 minutes when the excess sodium arsenite was titrated with 0.02N-iodine. Formaldehyde could not be detected in the reaction products[85] (Found: 0.99 mol. formic acid produced; 2.02 mol. periodate consumed).

Very Short Methanolysis of Agar

Dry agar (10 g.) in 1% methanolic hydrogen chloride (200 c.c.) was refluxed for 10 min. The undissolved residue (2.3 g.) was filtered off and the filtrate neutralised (Ag_2CO_3). The solution was concentrated to small volume and treated with a slow stream of hydrogen sulphide, and

then / ...

then a stream of nitrogen was passed through the solution to remove excess hydrogen sulphide. The silver sulphide precipitate was filtered off and the filtrate concentrated to leave a syrup (9 g.). The syrup was fractionated on cellulose (5 x 34.5 cm.) using n-butanol-water (9:1) as irrigating solvent to yield the following materials: (i) 3:6-Anhydro-L-galactose dimethyl acetal [0.37 g.; 5.0% (calc. as $C_6H_8O_4$) of the reacted agar], R_{Rh} 1.5 in solvent (c). (ii) A mixture of methyl galactosides [1.16 g.; 19% (calc. as $C_6H_{10}O_5$) of reacted agar], mostly methyl galactofuranoside R_{Rh} 1.1 in solvent (c). (iii) Agarobiose dimethyl acetal [4.76 g.; 76% (calc. as $C_{12}H_{18}O_9$) of reacted agar] m.p. and mixed m.p. 164.5 - 166.5°.

The column was stripped with alcohol and water to give higher material (2.6 g.). This material together with the undissolved agar (2.3 g.) was subtracted from the weight of original agar to give the weight of reacted agar; the yields of methanolysate products, calculated as free anhydro-sugars, were then based on this figure.

Very Short Methanolysis of Higher Materials

Higher material (6.1 g.) from a methanolysis of agar experiment was methanolysed in 1% methanolic hydrogen chloride (61 c.c.) for 5 minutes (there was no residue), neutralised (Ag_2CO_3), and excess silver ion precipitated as the sulphide. The syrup (5.2 g.) obtained on concentration of the filtrate was fractionated on cellulose (3 x 31 cm.) using n-butanol-water (9:1) as irrigating solvent. The following products were obtained: 3:6-anhydro-L-galactose dimethyl acetal (0.16 g.), R_{Rh} 1.6 in solvent (c); methyl galactosides (0.42 g.); agarobiose dimethyl acetal (1.53 g.) R_{Rh} 0.65 in solvent (c); higher materials (3.0 g.) were eluted off the column with ethanol and water.

The / ...

The Hot-Water Soluble Polysaccharide of Hypnea specifera

Extraction of Hypnea specifera Polysaccharide

Hypnea specifera seaweeds were collected near Gordons Bay, Cape. The undried seaweeds were suspended in water, brought to pH 4 with acetic acid, and steam was passed in for one hour. The hot solution was filtered through a cotton cloth and the hot filtrate was centrifuged twice and then poured into a large volume of ethanol (5 vols) to precipitate the polysaccharide. The precipitate was filtered, washed with alcohol, acetone, and ether and dried in a vacuum desiccator. In another experiment oven-dried Hypnea specifera seaweed (50 g.) was extracted as above to give 8 g. polysaccharide.

For analytical purposes some polysaccharide (13.5 g.) was further purified by being dissolved in water (200 c.c.) and then precipitated from ethanol (1 l.). The precipitate was re-dissolved in water (3 l.), centrifuged hot, and then passed through a column (38.0 x 5.5 cm.) of Amberlite IR 120 resin, in the sodium form. The eluate was precipitated in acetone (12 l.), filtered, washed with acetone and ether and dried in a vacuum desiccator to give a product (8 g.), $[\alpha]_D$ +65° (c, 0.3 in H_2O) (Found: Moisture loss at 60°/1 mm. for 78 hr., 10.1; sulphated ash, 23.8; SO_4^{--}, 22.0; N, 3.85%).

Isolation and Estimation of D-Galactose

The polysaccharide (1 g.) in N-sulphuric acid was hydrolysed at 100° for 16 hr., and spotted on Whatman No.3 MM papers which were developed overnight in solvent (a). The portions of the papers containing galactose were cut out and Soxhlet extracted with methanol. The methanolic solution was concentrated to give a crystalline product which after one recrystallisation from methanol gave m.p. and mixed m.p. 166.5 - 167.5° with D-galactose, $[\alpha]_D^{18}$ +86° (c, 1.0 in H_2O).

The / ...

The polysaccharide was hydrolysed with N-sulphuric acid at 100° for 24 hr. A known amount of maltose[61] was added to the hydrolysate which was neutralised ($BaCO_3$) and chromatographed on paper in solvent (a). The strips containing galactose and maltose were cut out, macerated in water, filtered, and the sugars estimated by the micro-Somogyi method[55] [Found: galactose (calc. as $C_6H_{10}O_5$) 47% on a moisture-free and protein-free basis].

Potassium Chloride Fractionation of the Polysaccharide[36,37]

1M-Potassium chloride was added dropwise to a stirred solution of the sodium salt of the polysaccharide (1.55 g.) in water (540 c.c.) until the solution was 0.2M with respect to potassium chloride. The precipitate so obtained was removed by centrifugation and washed several times with 80% ethanol (till free of chloride ions), twice with absolute ethanol, with ether and dried at 60°/20 mm. to give a product (1.3 g.). The material remaining in the supernatant liquor was precipitated with alcohol (3 vols), centrifuged, washed as for the previous precipitate and dried at 60°/20 mm. to give a product (0.17 g.) (Found: ash, 40.9; N, 1.4%).

The potassium chloride precipitated polysaccharide (1.3 g.) was dissolved in hot water (200 c.c.) and passed through a column (2.4 x 0.15 cm.) of Amberlite IR 120 resin in the sodium form to regenerate the sodium salt of the polysaccharide. Alcohol (3 vols) was added to the eluate in order to precipitate the polysaccharide. The material was separated by centrifugation, washed several times with 80% ethanol, twice with absolute ethanol, twice with ether, and dried at 60°/20 mm. for two days to give a product (1.0 g.), (N, 0.6%), $[\alpha]_D^{17}$ +66.5° (c, 0.3 in H_2O) [Found (on a protein-free basis): galactose (calc. as $C_6H_{10}O_5$), 47.4; sulphate (calc. as $NaSO_3$), 21.2%].

The material (0.13 g.), that had not been precipitated by potassium chloride, was dissolved in water (30 c.c.) and passed through

a/ ...

a column (1.8 x 0.15 cm.) of Amberlite IR 120 resin in the sodium form. When alcohol (5 vols) was added to the eluate no precipitate was obtained.

Methanolysis of Polysaccharide

The polysaccharide (21 g.) was refluxed with 2% methanolic hydrogen chloride (210 c.c.) for 20 min. and filtered. The insoluble residue (17 g.) was again refluxed in 2% methanolic hydrogen chloride (170 c.c.) for 20 min. and filtered. This process was repeated another four times. The filtrates were neutralised (Ag_2CO_3), combined, and concentrated to a syrup (10.2 g.). Paper chromatograms run in solvent (c) disclosed spots of R_{Rh} (relative to rhamnose) 0.8, 1.3 and 1.7. The syrup (10 g.) was fractionated on a cellulose column (33 x 5 cm.) with n-butanol-water (9:1). Fractions were spotted on paper chromatograms and like fractions were combined and concentrated. The first fraction, a syrup (0.96 g.), was chromatographically identical with 3:6-anhydro-D-galactose dimethyl acetal, R_{Rh} 1.7 in solvent (a), $[\alpha]_D^{17}$ +29° (c, 1.0 in H_2O), $[\alpha]_D^{17}$ +33° (c, 1.1 in MeOH); Haworth, Jackson and Smith[64] report $[\alpha]_D^{18}$ +36.5° (c, 0.9 in H_2O) for 3:6-anhydro-D-galactose dimethyl acetal. The material gave a positive Seliwanoff reaction.

The syrup (0.8 g.) was hydrolysed in 0.1N-sulphuric acid at 20° for two days, neutralised ($BaCO_3$), and concentrated to dryness. The residue was shaken with acetone (50 c.c.) containing conc. sulphuric acid (2 c.c.) and anhydrous copper sulphate (5 g.) for 5 hr. The mixture was neutralised (anhydrous K_2CO_3), filtered, boiled with charcoal and filtered again. The filtrate was concentrated to dryness and the residue dissolved in water. Ether extraction of the aqueous solution was carried out, the ethereal solution evaporated and the residue crystallised from petroleum ether (b.p. 60 - 80°). After recrystallisation from the same solvent the derivative gave m.p. and mixed m.p. 90 - 91° with 1:2-O-isopropylidene-3:6-anhydro-D-galactose,

$[\alpha]_D^{18}$ / ...

$[\alpha]_D^{18}$ +20° (c, 1.0 in $CHCl_3$). Araki and Arai[83] report m.p. 92° (Found: C, 53.4; H, 7.15. Calc. for $C_9H_{14}O_5$: C, 53.5; H, 7.0%).

The next fraction (0.14 g.) off the column, R_{Rh} 1.3, probably methyl galactofuranoside, was not investigated.

This was followed by an amorphous substance (3.88 g.), $[\alpha]_D^{16}$ +20° (c, 1.1 in H_2O); it was non-reducing to Fehling's and gave a positive Seliwanoff test. It showed up on paper chromatograms sprayed with p-anisidine hydrochloride and heated in an oven (110°) for 15 min. as a yellow spot with the same R_{Rh} (0.8) as agarobiose dimethyl acetal. Some of this material (0.25 g.) was dissolved in pyridine (5 c.c.), cooled to 5°, and treated with a mixture of acetic anhydride (5 c.c.) and pyridine (3 c.c.) at 5°. The mixture was left at 5° for one day and at room temperature for two days. Poured into ice-water (250 c.c.) a syrup settled out which could not be induced to crystallise. The remainder of the material (2.8 g.) was hydrolysed in 0.01N-oxalic acid (100 c.c.) at 100° for 3 hr., $[\alpha]_D^{18}$ +20 ⟶ +27° (constant value). The solution was neutralised (Amberlite IR 4B resin) and concentrated to dryness. Spotted on a paper developed in solvent (c) it gave a long streaky spot of R_{Rh} 0.45. The residual material dissolved in methanol (100 c.c.) was reacted with sodium borohydride (0.35 g.) in methanol (3.5 c.c.) and left overnight. The solution was neutralised (Amberlite IR 120) and concentrated to dryness. On papers developed in solvent (c) it gave a spot of R_{Rh} 0.51 together with a smaller spot of R_{Rh} 0.60, probably dulcitol.

The material was packed on a cellulose column (27.5 x 3.0 cm.) and eluted with n-butanol-formic acid-water (45:1:4). The main fraction (2.1 g.), R_{Rh} 0.5, was collected and concentrated. A small amount of dulcitol was also obtained, m.p. and mixed m.p. 188°.

Disaccharide glycitol

This substance crystallised from methanol m.p. 173 - 174°, mixed m.p. with agar disaccharide glycitol (of m.p. 173 - 174°)

161 - 163°, $[\alpha]_D^{21}$ +15° (c, 1.0 in H_2O) (Found: C, 44.2; H, 7.4; $C_{12}H_{22}O_{10}$ requires C, 44.2; H, 6.8%).

Disaccharide glycitol (0.3261 g.) in 2N-sulphuric acid (25 c.c.) was heated at 100° for 24 hr., $[\alpha]_D^{20}$ +14 ⟶ +45.5° (constant value), the final value corresponding to the specific rotation of an equimolar mixture of D-galactose and 1:4-anhydro-L-galactitol (=3:6-anhydro-D-galactitol). The hydrolysate was neutralised ($BaCO_3$) and concentrated; on papers developed in solvent (c) it disclosed two spots R_{Gal} 1.0 and 2.2. The concentrate was spotted on two papers developed in solvent (c) and the paper strips containing the hydrolysate products were cut out, macerated in water, filtered and concentrated. D-Galactose (0.0799 g.; yield, 44.4%) was obtained crystalline from methanol, m.p. and mixed m.p. 166 - 167°, $[\alpha]_D^{22}$ +82° (c, 0.8 in H_2O). The aglycone was a syrup (0.0769 g.; yield 47.0%), $[\alpha]_D^{22}$ +12° (c, 1.0 in H_2O), identical with 1:4-anhydro-L-galactitol on paper chromatograms. Hocket, Conley, Yusem and Mason[67] report $[\alpha]_D$ +16.1° (H_2O) for 1:4-anhydro-L-galactitol.

The disaccharide glycitol (0.1524 g.) was hydrolysed as above and the galactose estimated by the micro-Somogyi method[55], maltose being used as a standard[61] (Found: 1.11, 1.04 mol. galactose from 1 mol. disaccharide glycitol).

Disaccharide glycitol (0.3 g.) in pyridine (5 c.c.) was treated with acetic anhydride (5 c.c.) in pyridine (3 c.c.) at 5°; the mixture was left at 5° for two days and at room temp. for two days. It was then poured into ice-water (200 c.c.) with stirring. After standing a short while crystals appeared which were filtered and recrystallised from 96% ethanol, m.p. 144 - 145°, $[\alpha]_D^{23}$ -7.6° (c, 1.05 in $CHCl_3$) (Found: C, 50.8; H, 6.4; Ac, 49.9. Calc. for $C_{26}H_{36}O_{17}$: C, 50.3; H, 5.9; Ac, 48.5%).

Periodate / ...

Periodate Oxidation of Disaccharide Glycitol

The disaccharide glycitol (0.1234 g.) was dissolved in water (25 c.c.), 0.4M-sodium metaperiodate (5.0 c.c.) added, and the solution made up to 50.0 c.c. A blank solution was also prepared. The reaction mixture and the blank were kept in the dark for 24 hr. In order to estimate the formic acid produced, aliquots (5.0 c.c.) of the oxidation mixture were withdrawn at time intervals, treated with ethylene glycol (0.25 c.c.), stood for 5 min. and titrated with 0.01N-sodium hydroxide, using bromocresol purple indicator[87]. After 24 hr. when the formic acid produced had reached a constant value the periodate consumed was determined. Aliquots (5.0 c.c.) were treated with excess 0.1N-sodium arsenite (5.0 c.c.) in the presence of potassium iodide and sodium bicarbonate buffer and back-titrated with 0.01N-iodine. The blank solution was similarly treated. Formaldehyde produced was determined by the method of Reeves[85]; the disaccharide glycitol (0.0531 g.) in water (2 c.c.) yielded the dimedone derivative of formaldehyde, m.p. and mixed m.p. 188 - 189° (Found: 0.95 mol. formic acid, 0.75 mol. formaldehyde produced, 2.98 mol. periodate consumed).

Methylated Polysaccharide

The polysaccharide (15 g.) was dissolved in hot water (150 c.c.) and was treated with methyl sulphate (225 c.c.) and 30% sodium hydroxide (675 c.c.) added dropwise over 7 hr. (A little acetone was added to the mixture during methylation to help dissolve the polysaccharide.) The mixture was heated at 80° for 30 min. and then neutralised (50% sulphuric acid; external cooling). The solution was filtered free of precipitated sodium sulphate, concentrated, and freed of more sodium sulphate by the addition of 60% ethanol. The filtrate, though still not free of salts, was again methylated three times, being worked up every time as above.

The final methylation product was dissolved in water and dialysed against running tap water for ten days and then concentrated

to / ...

to a residue (2 g.). Those sodium sulphate precipitates which gave a positive Molisch test were also dissolved in water and dialysed against running tap water for ten days; the dialysate was concentrated to give a product (0.8 g.) which was combined with the main batch of methylated material (Found: OMe, 20.9; SO_4^{--}, 18.3%).

The methylated polysaccharide (2.3 g.) in N-sulphuric acid (23 c.c.) was hydrolysed at 100° for 20 hr., neutralised ($BaCO_3$), and concentrated to a syrup (1.34 g.). Chromatography on paper [solvent (c)] revealed the presence of 2:6-di-O-methylgalactose, R_{TMG} (relative to tetramethylglucose) 0.64, together with small amounts of mono-O-methylgalactose, R_{TMG} 0.41, tri-O-methylgalactose, R_{TMG} 0.77, and tetra-O-methylgalactose, R_{TMG} 0.90 and also a furfural derivative just behind the solvent front. The syrup (1.3 g.) was packed on a cellulose column[61] (35 x 3.5 cm.) irrigated with n-butanol-water (9:1) and yielded mainly a syrup (0.7 g.), R_{TMG} 0.64. The syrup crystallised from ethyl acetate and after recrystallisation from the same solvent gave m.p. 128.5 - 130°, $[\alpha]_D$ +45 ⟶ +88° (c, 0.8 in H_2O). Oldham and Bell[89] record m.p. 128 - 130°, $[\alpha]_D$ +46.8 ⟶ +87.5° (H_2O) for 2:6-di-O-methyl-β-D-galactose.

Synthesis of 2:6-Di-O-Methyl-D-Galactose

The sugar was synthesised by the method of Bell[70]. D-Galactose (50 g.) in 1% methanolic hydrogen chloride (400 c.c.) was refluxed for 14 hr.[90]. The methanolysate was neutralised (Ag_2CO_3), filtered, decolorised with charcoal (Darco G-60), and concentrated to a syrup. The syrup was dissolved in hot isopropyl alcohol (200 c.c.) and left to crystallise. The first crop of crystals (4.1 g.) containing methyl β-D-galactopyranoside was recrystallised from absolute ethanol, giving m.p. and mixed m.p. 178 - 179°, $[\alpha]_D^{18}$ +0.1° (c, 1.0 in H_2O). Methyl β-D-galactopyranoside (6.5 g.) in dry acetone (1540 c.c.) was shaken with anhydrous copper sulphate (28 g.) for six days at 30°. The mixture was clarified with a little Celite 535, filtered, and

concentrated / ...

concentrated (8.4 g.). A little of the material recrystallised from benzene gave m.p. 134 - 135°. Micheel[91] reported m.p. 135° for methyl 3:4-O-isopropylidene-β-D-galactoside (Found: C, 51.7; H, 7.9; OMe, 12.8. Calc. for $C_{10}H_{18}O_6$: C, 51.3; H, 7.75; OMe, 13.3%). The last product (8 g.) in anhydrous acetone (10 c.c.) and with Drierite (3 g.) was methylated twice with Purdie's reagents, methyl iodide (42 c.c.) and silver oxide (42 g.). The product (5.4 g.) was recrystallised from pentane m.p. 53 - 55°, $[\alpha]_D^{18}$ -4° (c, 3.0 in $CHCl_3$) (Found: OMe, 33.6. Calc. for $C_{12}H_{22}O_6$: OMe, 35.5%). Oldham and Bell[89] report m.p. 56 - 57°, $[\alpha]_D$ -4.46° ($CHCl_3$) for methyl 2:6-di-O-methyl-3:4-O-isopropylidene-β-D-galactoside. This material was hydrolysed in N-sulphuric acid at 95° overnight, neutralised ($BaCO_3$), and concentrated to dryness. The residue was extracted with dry acetone and the resultant solution concentrated to give a syrup. The material was crystallised and recrystallised from anhydrous ethyl acetate, m.p. 128.5 - 130°, $[\alpha]_D^{18}$ +47.5 $\longrightarrow$ 87.0° (c, 0.5 in H_2O). Oldham and Bell[89] report m.p. 128.5 - 130°, $[\alpha]_D$ +46.8 $\longrightarrow$ 87.5° (H_2O) for 2:6-di-O-methyl-D-galactose (Found: C, 46.4; H, 7.9; OMe, 28.9. Calc. for $C_8H_{16}O_6$: C, 46.2; H, 7.75; OMe, 29.8%).

Attempted Desulphation of Polysaccharide

Dry powdered Hypnea polysaccharide (12 g.) in 1% methanolic hydrogen chloride (1200 c.c.) was shaken for two days at 30°. The mixture was centrifuged and the residue (4.2 g.) was again treated with 1% methanolic hydrogen chloride (600 c.c.) for two days at 30°. The mixture was centrifuged and the residue dissolved in warm water in the presence of barium chloride and centrifuged; concentration of the clear supernatant solution and precipitation from alcohol gave a product (0.6 g.; SO_4^{--}, 2.85%).

In another similar experiment dry powdered Hypnea polysaccharide (16 g.) in 1% methanolic hydrogen chloride (900 c.c.) was shaken at 20° for 24 hr. leaving a residue. The residue was then

given / ...

given two further treatments with methanolic hydrogen chloride. The final residue was dissolved in warm water in the presence of barium chloride and centrifuged; concentration and precipitation in ethanol gave a material (2.0 g.; SO_4^{--}, 7.17%).

Methylation of Degraded Polysaccharide

In an unsuccessful attempt to remove sulphate groups prior to methylation, Hypnea polysaccharide was treated with sodium borohydride; the sodium borohydride reduction of polysaccharides is employed to prevent possible alkaline breakdown during methylation, any reducing end groups being reduced to hexitol end groups before methylation[73].

Hypnea polysaccharide (36 g.) in hot water (450 c.c.) was passed through a column of Amberlite IR 120 resin and then treated with a solution of sodium borohydride (3.6 g.) in water (20 c.c.), the mixture being stirred for 4 hr. and then left overnight. The mixture was neutralised (acetic acid) and then dialysed for two days against running tap water and for one day against distilled water. The dialysate was concentrated to small volume, precipitated in alcohol, filtered, and washed with alcohol and ether and dried in a vacuum desiccator (11.6 g.) (Found: SO_4^{--}, 19.3; N, 0.4%). This indicates the stability of the sulphate group in alkali.

The polysaccharide (11 g.) in water (70 c.c.) was methylated with methyl sulphate (150 c.c.) and 30% sodium hydroxide solution (450 c.c.) added dropwise over 6 hr. The mixture was left overnight, heated at 80° for 30 min. and then neutralised (50% sulphuric acid; external cooling). Precipitated sodium sulphate was filtered off and washed with cold water. The filtrate and washings were concentrated to a small volume. The concentrated solution was again methylated as before and this procedure was repeated a further five times. Whenever sodium sulphate precipitates that had been removed at the filter gave a positive Molisch test for carbohydrate they were dialysed against tap water, concentrated, and combined with the methylation mixture.

After / ...

After four methylations a negligible amount of chloroform soluble material was obtained (OMe, 21.4%). The hydrolysate of this material on a paper chromatogram [solvent (c)] gave spots for di-O-methylgalactose, R_{TMG} 0.62, and tetra-O-methylgalactose, R_{TMG} 0.88.

After a further three methylations the material was still not chloroform soluble to any appreciable extent. The material was dialysed against running tap water for three days and against distilled water for 8 hr. The dialysate was concentrated to give a residue (6.6 g.) (Found: OMe, 21.4; SO_4^{--}, 15.8%). Paper chromatograms in solvent (c) of this material when hydrolysed showed the presence of di-O-methylgalactose, R_{TMG} 0.70, and some tri-O-methylgalactose, R_{TMG} 0.81.

The methylated polysaccharide (6.2 g.) in 1% methanolic hydrogen chloride (300 c.c.) was shaken for 16 hr. at 30° when nearly all the material had dissolved. The solution was centrifuged, the clear solution neutralised (Ag_2CO_3) and concentrated to a syrup (5.4 g.) (Found: OMe, 32.0; SO_4^{--}, 7.7%).

Some of the syrup (5.0 g.) in dry methanol (10 c.c.) together with Drierite (1 g.) was methylated with methyl iodide (30 c.c.) and silver oxide (30 g.) added in portions over 8 hr. The reaction mixture was left overnight, then filtered and the silver oxide residue washed with hot methanol. The combined filtrate and washings was concentrated to a syrup.

The syrup was then remethylated as before and worked up to give a syrup (3.4 g.), $[\alpha]_D^{28}$ +7° (c, 1.2 in H_2O) (Found: OMe, 35.8; SO_4^{--}, 7.4%). This syrup (2.9 g.) was hydrolysed in N-sulphuric acid at 95° overnight, and neutralised ($BaCO_3$) when it showed on paper chromatograms [solvent (c)] spots of R_{TMG} 0.69 (di-O-methylgalactose), 0.82 (tri-O-methylgalactose), 0.90 (tetra-O-methylgalactose) as well as a very small spot R_{TMG} 0.38 (mono-O-methylgalactose).

The hydrolysate (2.1 g.) was fractionated on a cellulose column (35 x 3.5 cm.) irrigated with n-butanol-petroleum ether[61] (b.p. 100 - 120°) (2:3). The first fraction, a syrup (0.4 g.) was chromatographically identical with tetra-O-methyl-D-galactose,

R_{TMG} / ...

R_{TMG} 0.90 in solvent (c). The syrup (0.4 g.) was refluxed with aniline (0.3 c.c.) in ethanol (5 c.c.) for 3 hr. After some time at room temp. crystallisation set in and the crystals were filtered off and recrystallised from ethanol, m.p. and mixed m.p. 189 - 190° with authentic 2:3:4:6-tetra-O-methyl-N-phenyl-D-galactosylamine. The second fraction off the column, a syrup (0.35 g.) was shown by paper chromatography to contain mainly tri-O-methylgalactose, R_{TMG} 0.73 in solvent (c), but contaminated with a small amount of tetra-O-methylgalactose. The material was spotted on several Whatman No.20 papers, developed in solvent (c) over 40 hr. and the strips of paper containing tri-O-methylgalactose were cut out and extracted by maceration in water. The material (0.2 g.) obtained in this way was refluxed with aniline (0.15 c.c.) in ethanol (5 c.c.) for 3 hr. After several days at 5° crystals formed, m.p. 163 - 164.5°, raised to m.p. 170.5 - 171.5° after recrystallisation from ethanol, $[\alpha]_D^{18}$ -90 ⟶ +35° (c, 0.5 in acetone); the product, on admixture with 2:3:4:tri-O-methyl-N-phenyl-D-galactosylamine (m.p. 163 - 164°) (kindly supplied by Dr. A.M. Stephen) gave m.p. 151 - 153.5°. Hirst and Jones[92] report m.p. 179° (sometimes 169°), $[\alpha]_D$ -92 ⟶ +38° (acetone) for 2:4:6-tri-O-methyl-N-phenyl-D-galactosylamine.

At this stage the irrigating solvent for the column was changed to butanol-ethanol-water (18:5:2). The first fraction obtained after this change of solvent was shown by paper chromatography to contain di-O-methyl- and tri-O-methylgalactose, R_{TMG} 0.58 and 0.75. The mixture (0.2 g.) was chromatographed on Whatman No.3MM papers [solvent (c)] and the material of R_{TMG} 0.58 was extracted in the usual way to give a syrup (0.09 g.). The syrup was well dried and then left covered with anhydrous ethyl acetate. Crystals were obtained, which after recrystallisation from the same solvent gave m.p. and mixed m.p. 128 - 130° with authentic 2:6-di-O-methyl-D-galactose. The following fraction off the column (0.1 g.) contained mono-O-methyl-galactose, R_{TMG} 0.36. This fraction appeared to be a mixture and a crystalline anilide could not be obtained.

REFERENCES / ...

REFERENCES

1 Dr. F.J. Joubert, private communication.

2 Mori, Adv. Carbohydrate Chem., 1953, 8, 315.

3 Tseng, Science Monthly, 1944, 58, 24.

4 Bauer, J. prakt. Chem., 1884, (2), 30, 367.

5 Neuberg and Ohle, Biochem. Z., 1921, 125, 311.

6 Hoffman and Gortner, J. Biol. Chem., 1925, 65, 371.

7 Percival, Nature, 1944, 154, 673.

8 Barry and Dillon, Chem. and Ind., 1944, 167.

9 Percival and Somerville, J. Chem. Soc., 1937, 1615.

10 Hands and Peat, Nature, 1938, 142, 797.

11 Percival, Somerville and Forbes, Nature, 1938, 142, 797.

12 Pirie, Biochem. J., 1936, 30, 369.

13 Cottrell and Percival, J. Chem. Soc., 1942, 749.

14 Forbes and Percival, J. Chem. Soc., 1939, 1844.

15 Percival and Thomson, J. Chem. Soc., 1942, 750.

16 Araki, J. Chem. Soc. Japan, 1940, 61, 775; Chem. Abs., 1943, 37, 90.

17 Jones and Peat, J. Chem. Soc., 1942, 225.

18 Duff and Percival, J. Chem. Soc., 1941, 830.

19 Araki, Mem. Coll. Sci. Tech., Kyoto, 1953, 2, B, 17.

20 Araki, J. Chem. Soc. Japan, 1944, 65, 533.

21 Araki and Hirase, Bull. Chem. Soc. Japan, 1954, 27, 109.

22 Araki and Hirase, ibid, 1953, 26, 463.

23 Hirase and Araki, ibid, 1954, 27, 105.

24 Araki and Hirase, ibid, 1956, 29, 339.

24a Araki and Arai, ibid, 1957, 30, 287.

25 Araki, ibid, p. 543; Araki, Mem. Coll. Sci. Tech., Kyoto, 1956, 5, 21.

26 O'Neill and Stewart, Can. J. Chem., 1956, 34, 1700.

27 Schmidt, Ann. Chem. Pharm., 1844, 51, 29.

28 Stanford, J. Soc. Arts, 1862, 10, 185.

29 / ...

29 Dewar and Percival, J. Chem. Soc., 1947, 1622.

30 Haas, Biochem. J., 1921, 15, 469; Haas and Russel-Wells, ibid, 1929, 23, 425.

31 Buchanan, Percival and Percival, J. Chem. Soc., 1943, 51.

32 Peat, Adv. Carbohydrate Chem., 1946, 2, 38; Percival, Quart. Reviews, 1949, 3, 369.

33 Johnston and Percival, J. Chem. Soc., 1950, 1994.

34 Dillon and O'Colla, Proc. Roy. Irish Acad., 1951, 54, B, 51.

35 Young and Price, J. Biol. Chem., 1946, 164, 35.

36 Smith and Cook, Arch. Biochem. Biophys., 1953, 45, 232.

37 Smith, Cook and Neal, ibid, 1954, 53, 192.

38 Smith, O'Neill and Perlin, Can. J. Chem., 1955, 33, 1352.

39 Goring and Young, ibid, 1955, 33, 480.

40 Percival, Chem. and Ind., 1954, 1487.

41 O'Neill, J. Amer. Chem. Soc., 1955, 77, 2837.

42 O'Neill, ibid, p. 6324.

43 Yaphe and Baxter, Applied Microbiology, 1955, 3, 380.

44 Hassid, J. Amer. Chem. Soc., 1933, 55, 4163.

45 Hassid, ibid, 1935, 57, 2046.

46 Jones and Smith, Adv. Carbohydrate Chem., 1949, 4, 275.

47 Mori, J. Agric. Chem. Soc. Japan, 1943, 19, 297.

48 Mori, ibid, 1949, 23, 81.

49 Araki and Hirase, Bull. Chem. Soc. Japan, 1956, 29, 770.

50 Barry and Dillon, Proc. Roy. Irish Acad., 1945, 50, B, 349.

51 Dillon and McKenna, ibid, 1950, 53, B, 45.

52 Barry and McCormick, J. Chem. Soc., 1957, 2777.

53 Dillon and McKenna, Nature, 1950, 165, 318.

54 Isaac, Finlayson and Simon, Nature, 1943, 151, 532; Isaac and Molteno, J. S. African Botany, 1953, 19, 85.

55 Somogyi, J. Biol. Chem., 1952, 195, 19.

56 Carson and Maclay, J. Amer. Chem. Soc., 1946, 68, 1015.

57 Lindberg and Theander, Acta. Chem. Scand., 1954, 8, 1870.

58 / ...

58 Whistler and Durso, J. Amer. Chem. Soc., 1950, 72, 677.

59 Alm, Acta. Chem. Scand., 1952, 6, 1186.

60 Abdel-Akher, Hamilton and Smith, J. Amer. Chem. Soc., 1951, 73, 4691.

61 Hough, Jones and Wadman, J. Chem. Soc., 1949, 2511.

62 Ness, Fletcher and Hudson, J. Amer. Chem. Soc., 1951, 73, 3742.

63 Peat, Adv. Carbohydrate Chem., 1946, 2, 38.

64 Haworth, Jackson and Smith, J. Chem. Soc., 1940, 620.

65 Isaac and Hewitt, J. S. African Botany, 1953, 19, 73.

66 Fox and Stephens, S. African J. Sci., 1943, 39, 147.

67 Hocket, Conley, Yusem and Mason, J. Amer. Chem. Soc., 1946, 68, 922.

68 Barker, Bourne, Stacey and Whiffen, J. Chem. Soc., 1954, 171 and 3468.

69 Foster and Huggard, Adv. Carbohydrate Chem., 1955, 10, 356.

70 Bell, J. Chem. Soc., 1945, 693.

71 Kantor and Schubert, J. Amer. Chem. Soc., 1957, 79, 152.

72 Hoffman et al., Arch. Biochem. Biophys., 1957, 69, 435.

73 Jones and Perry, J. Amer. Chem. Soc., 1957, 79, 2787.

74 Jones, J. Chem. Soc., 1953, 1672.

75 Hough, Jones and Wadman, J. Chem. Soc., 1950, 1702.

76 Metzenberg and Mitchell, J. Amer. Chem. Soc., 1954, 76, 4187.

77 Rullen and Pacsu, Ind. Eng. Chem., Anal. Ed., 1942, 34, 1209.

78 Zemplen, Gerecs and Hadacsy, Ber., 1936, 69, 1827.

79 Teunissen, Rec. trav. chim., 1931, 50, 1.

80 Freudenberg and Hixon, Ber., 1923, 56, 2123.

81 Bell and Williamson, J. Chem. Soc., 1938, 1196.

82 Ohle and Thiel, Ber., 1933, 66, 525.

83 Araki and Arai, J. Chem. Soc. Japan, 1942, 63, 1720; Chem. Abs., 1947, 41, 3765b.

84 Halsall, Hirst and Jones, J. Chem. Soc., 1947, 1430.

85 Reeves, J. Amer. Chem. Soc., 1941, 63, 1477.

86 Cifonelli and Smith, Analyt. Chem., 1954, 26, 1132.

87 Hartman, J. Appl. Chem., 1953, 3, 308.

88 Nunn and von Holdt, J. Chem. Soc., 1957, 1094.

89 / ...

89 Oldham and Bell, J. Amer. Chem. Soc., 1938, 60, 323.

90 cf. Dale and Hudson, J. Amer. Chem. Soc., 1930, 52, 2534.

91 Micheel, Ber., 1929, 62, 687.

92 Hirst and Jones, J. Chem. Soc., 1939, 1482.

93 Kuhn, Analyt. Chem., 1950, 22, 276.

94 Barker, Bourne, Stacey and Whiffen, J. Chem. Soc., 1954, 171 and 3468.

95 Thompson, Nicholson and Short, Discuss. Faraday Soc., 1950, 9, 222.

96 Whistler and House, Analyt. Chem., 1953, 25, 1463.

97 Barnes, Liddel and Williams, Analyt. Chem., 1943, 15, 659.

98 Levine, Stevenson and Kabler, Arch. Biochem. Biophys., 1953, 45, 65.

99 Barker, Bourne and Whiffen, Methods of Biochem. Analysis, 1954, 3, 224.

100 Orr, Biochim. et Biophys. Acta, 1954, 14, 173.

101 Darmon and Rudall, Discuss. Faraday Soc., 1950, 9, 251.

102 Burket and Melvin, Science, 1952, 115, 576.

103 Bayley, Biochem. et Biophys. Acta, 1955, 17, 194.

104 Thompson and Torkington, J. Chem. Soc., 1954, 640.

105 Reeves, J. Amer. Chem. Soc., 1950, 72, 1499;
Adv. Carbohydrate Chem., 1951, 6, 107.

106 Isbell, Stewart, Frush, Moyer and Smith, J. Res. Nat. Bur. Stand., 1956, 57, 179.

107 Burket and Badger, J. Amer. Chem. Soc., 1950, 72, 4397.

108 Barker, Bourne, Stephens and Whiffen, J. Chem. Soc., 1954, 4211.

109 Barker, Bourne, Stacey and Whiffen, Chem. and Ind., 1952, 1156.

110 Barker and Stephens, J. Chem. Soc., 1954, 4550.

111 Tschamler and Voetter, Monatsh., 1952, 83, 303.

112 Barker, Bourne, Weigel and Whiffen, Chem. and Ind., 1956, 318.

113 Foster, Overend and Vaughan, J. Chem. Soc., 1954, 3625.

114 Cifonelli, Cifonelli, Montgomery and Smith, J. Amer. Chem. Soc., 1955, 77, 121.

115 Lemieux et al., J. Amer. Chem. Soc., 1957, 79, 1005.

116 Schwartz, Ann. Reports Chem. Soc., 1954, 51, 264.

117 Kirby, Colonial Plant Animal Products, 1951, 2, 150.

SUMMARY

Agar is the polysaccharide constituent of the cell wall of certain red seaweeds (Rhodophyceae) of the class Florideae. It is a polygalactan that contains half-ester sulphate associated with inorganic cations. The agar from Gracilaria confervoides, the chief agar-bearing seaweed of South Africa, has been investigated. Partial acid hydrolysis of the agar followed by reduction of the hydrolysate with sodium borohydride yielded 3:6-anhydro-4-O-β-D-galactopyranosyl-L-galactitol, a new disaccharide glycitol. Partial methanolysis of the agar yielded mainly 3:6-anhydro-4-O-β-D-galactopyranosyl-L-galactose dimethyl acetal (i.e., agarobiose dimethyl acetal). It would appear that at least 76% (by weight) of the agar molecule from Gracilaria confervoides is composed of agarobiose units probably united by 1 ⟶ 3'-links in an unbranched chain. The sulphate content indicated that there is about one sulphate ester for every 40 disaccharide residues. The agar of Gracilaria confervoides contains the same components as agar from other sources.

The water-soluble polysaccharide of Hypnea specifera, a red seaweed of the class Florideae that abounds in South African coastal waters, was also investigated. The polysaccharide was shown to contain D-galactose, 3:6-anhydro-D-galactose and half-ester sulphate.

Partial methanolysis of the polysaccharide followed by mild acid hydrolysis and reduction with sodium borohydride yielded 3:6-anhydro-4-O-β-D-galactopyranosyl-D-galactitol, the diastereoisomer of the disaccharide glycitol obtained from Gracilaria confervoides agar. Methylation studies on both the degraded and undegraded polysaccharide have lead to the conclusion that the principal D-galactopyranose linkage is 1 ⟶ 3 with the sulphate group on $C_{(4)}$. Thus besides the 1 ⟶ 4-links indicated by the isolation of the disaccharide, 1 ⟶ 3-links are also present in the polysaccharide.

Addition / ...

Addition of potassium chloride to an aqueous solution of the polysaccharide yielded a κ-fraction in high yield similar to that given by carrageenin. The ratio for D-galactose, 3:6-anhydro-D-galactose, and $NaSO_3$ in this material was 1.5 : 1.1 : 1.0. It has thus been shown that the hot-water soluble polysaccharide of Hypnea specifera is very similar to the κ-carrageenin isolated from Chondrus crispus.

The infra-red absorption spectra for a number of carbohydrates containing a hydrofuranol ring have been examined.

ACKNOWLEDGEMENTS / ...

ACKNOWLEDGEMENTS

This work was carried out in the National Chemical Research Laboratory of the Council for Scientific and Industrial Research.

I wish to thank Dr. W.S. Rapson, Director of the National Chemical Research Laboratory, for permission to present this work for a thesis. Thanks are also due to Dr. J.R. Nunn, who directed this investigation, for advice and encouragement, and to Dr. A.M. Stephen for his interest and helpful discussions.