AN INVESTIGATION CONCERNING A WATER SOLUBLE COMPONENT EXTRACTED FROM THE MATURE SEED OF LOTUS PEDUNCULATUS (FAMILY : LEGUMINOSAE)

A thesis presented in partial fulfillment of the requirements for the degree of Master of Agriculture Science in Plant Biochemistry

by

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S U M M A R Y

(i) A new polysaccharide has been extracted from the mature seeds of Lotus Pedunculatus.

(ii) The homogeneity of the polysaccharide was determined by ultracentrifuge and fractional precipitation studies.

(iii) Ultracentrifuge studies indicated an average molecular weight of 1×10^5.

(iv) Acid hydrolysis experiment indicated a galactomannan with a molar ratio of D-galactopyranose to D-mannopyranose of 49 to 51.

(v) Methylation by methods of Haworth, Purdie, and Kuhn followed by hydrolysis yielded the following methyl ethers

2, 3, 4, 6 - tetra - O - methyl - D-galactopyranose.

2, 3, 6 - tri - O - methyl - D-mannopyranose.

2, 3 - di - O - methyl - D-mannopyranose. A fourth unidentified component (possibly a tri - methyl - D-galacto-pyranose) was shown to present in trace amounts. The molar ratio of "di-methyl sugar": "tetra-methyl sugar (plus tri-methyl sugar)" was 49 : 51. The hydrolysate was also examined by gas-liquid chromatography.

(vi) Further evidence as to the fine structure of the galactomannan was adduced from periodate studies. On periodate oxidation the galactomannan consumed 1.20 moles of periodate and released 0.49 moles of formic acid.

The hydrolysis of the reduced periodate oxidised polysaccharide yielded D-mannose, D-erythritol and ~~X~~-glycerol. The acetylated hydrolysate of the latter was examined by gas-liquid chromatography.

(vii) Mild acid hydrolysis of the galactomannan removed D-galactose residues only.

(viii) The results indicate that the galactomannan possesses $1 \rightarrow 4$ linked D-mannopyranose backbone and to C_6 of most of these mannose units are attached as single non-reducing D-galactopyranose residues.

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1.1 GENERAL SURVEY OF THE FIELD OF PLANT GUMS AND MUCILAGES

Smith and Montgomery (1) state in the preface to their monograph that plant gums and mucilages have been known and in use since very early times. References can be found to them in the Bible and they seem to have had commercial value for several 1000 years, especially for India, Asia, Africa, Australia and China. These products found a market in Europe where their use in industry has never ceased to expand. Seaweed gums have been used by the natives of the coastal regions of France, Wales, Ireland, Scotland and Scandinavia in food and medicinal preparations. (1)

Plant gums are defined by Jones, J.K.N. and Smith, F (2) as substances of plant origin, which are obtained as exudations from fruit, trunks or branches of trees, spontaneously or after invasion by bacteria or fungi. When exposed to air and allowed to dry they form clean, glassy masses, usually coloured from dark brown to clear yellow. The quantity of gum excreted is a function of (a) the species of plant, (b) the environmental conditions and state of health in which the species is found (1). Their most probable function appears to be directly concerned with the sealing off of injured parts and thus preventing the loss of moisture. This property would also serve to prevent the invasion of the tissue by bacteria and fungi (1, 2). Of the several theories (1) that exist to explain the biochemical origin of gums, the most probable is that they are a product of normal plant metabolism. Other

workers have suggested that they are a product of pathological conditions induced by the infection of the plant by micro-organisms (1). Whether or not gums are formed at the site of injury or synthesised elsewhere in the plant and then transported to the injured area has yet to be ascertained. Chemical structural work has shown that there is a striking similarity among plant gums isolated from the same plant species (2). This would seem to indicate that the structural pattern is under genetical control via protein enzymes.

Plant gums are amorphous substances containing carbon, hydrogen and oxygen and are members of the carbohydrate group. The small amount of nitrogen which is detectable has been attributed to protein debris arising from the synthesising enzymes, or to contact of the gums with other proteinaceous material of the plant. Gums are lyophilic substances and are characterised by dissolving in cold water or taking up water to form a mucilage. They are neutral salts of complex polysaccharide acids composed of hexose residues (D-galactopyranose and D-mannopyranose); uronic acid residues (D-galacturonic acid and D-guluronic acid which exist in the pyranose form); pentose residues (L-arabinose in the furanose form and D-xylose in the pyranose form); and methyl pentose residues (L-rhamnose and L-fucose which are assumed to be in the pyranose form). These residues are joined by glycosidic linkages in a diverse manner within the same molecule. The significance of this complexity is notknown unless it be that they are so constituted as to be able to resist the action of an organism

or series of invading organisms with their accompanying enzyme systems (2) and/or to prevent the loss of moisture. With the exception of gum tragacanth which has D-galacturonic acid units as its acid component all the other gums so far studied are based on D-glucuronic acid.

The so called plant mucilages are polysaccharides which form colloidal solutions in water from which they can be precipitated with ammonium sulphate, sodium chloride and the usual protein precipitants. They differ from pectin in that they do not form jellies although seaweeds are an exception to this, e.g. agar. They usually contain D-galactose as a structural building unit and exist either as a secondary membrane thickening material or as an intracellular material (or membrane mucilages and cell content mucilages respectively) (2). Membrane mucilages occur in the root (Althaea), the cortex (Cinnamonum), the stalk (Tragacanth), the leaves (Buccu), the flowers (Malvaceae, Tilia), the endosperm (Trigonella) and in the seed shell (cocoa). In seaweeds the mucilage is inter-cellular (Saminaria, Carragheen), while in the succulents (Aloe, Euphorbiaceae) the mucilage occurs in the cell content. In several bulbs (Scilla), and in orchis (salep), mucilage cells occur. The mucilages are sometimes a food reserve (Linum, Cruciferae) while in plants living in dry climate they may act as a water reserve (3).

The plant gums and mucilages exhibit such a wide spectrum of physical and chemical properties that it is difficult to devise a means of classification without considering a large number of

exceptions. From present knowledge (physical and chemical) there is no clear line of demarcation between the gums and mucilages. The physical properties of gums and mucilages, and related polysaccharides, depend not so much on the actual building units (or their source) as on the three-dimensional, molecular architecture of the complex molecules. Physical properties such as viscosity and solubility depend largely on whether the polysaccharide has a linear or a branched structure. Therefore at one end of the spectrum there are the linear molecules such as amylose and cellulose and at the other end such highly branched gums as gum arabic and mesquite gum. The latter is complex from the point of view of types of glycosidic linkage and the component building units, and will dissolve in water to give clear solutions. The introduction of partial irregularity into linear compounds by changing some of the sugar building units on the linkages, or replacing - CH_2OH by - COOH, or adding side chains, has a marked effect on their solubility in water (1). In general it appears that if the side chains or modifications occupy regularly spaced positions, mucilage-like properties result, whereas irregular substitutions or modifications lead to the manifestation of gum-like properties. That is, the more regular the branching, the better the chance of a three dimensional network which will give rise to thick mucilages or gels. The more irregular the branching, the less likely will be the association of parts of the molecule chains

and hence a three dimensional network cannot be formed. Thus a true solution with water can be obtained. The concept of the structure of gums and mucilages attempts to correlate gum- or mucilage-like properties with the whole molecular structure rather than with the detailed composition of the types of building units and linkages present in the polysaccharide.

Smith and Montgomery (1) have put forward a classification as follows:-

GROUP I

ACIDIC GUMS AND MUCILAGES CONTAINING

ACIDIC COMPONENT	NEUTRAL COMPONENT
L-guluronic acid	
D-glucuronic acid	Hexoses
D-galacturonic acid	Pentoses
Sulphate groups	6-Deoxyhexoses
Phosphate groups	Sugar alcohols
Ethers of all the above	Ethers of all the above

GROUP II

NEUTRAL GUMS AND MUCILAGES CONTAINING

Hexoses

6-Deoxyhexoses

Pentoses

Sugar alcohols

Ethers of all the above

As yet no basic gums and mucilages have been found in nature, but as it is envisaged that these will eventually be synthesised so provision is made for them.

GROUP III

BASIC GUMS AND MUCILAGES CONTAINING

Amino sugars

Amino alkyl ethers

Amino acids polypeptides or proteins

O - and N - Alkyl derivs. of the above

Hexoses

Pentoses

6-Deoxyhexoses

Sugar alcohols

Ethers of all the above

Jones, J.K.N. (2) roughly divides mucilages into three groups according to their chemical properties.

(a) The neutral polysaccharides, consisting of one or more sugar residues joined together through their reducing groups with the formation of substances of high molecular weight, e.g. mannans, galactomannans. A large proportion of these mucilages contain D-galactose, L-arabinose as the main sugars.

(b) The polysaccharides containing uronic acid residues as well as other sugar residues. In this class of materials are grouped the majority of the seed mucilages, the acidity of which is due to a uronic acid (usually D-galacturonic acid) or to a methyl ether derivative of a uronic acid. The acidity of the majority of gums is due to the presence of D-glucuronic acid or one of its methyl ethers and this property can be used to make an arbitrary distinction between gums and mucilages. These polysaccharides contain, on the whole, a wider variety of sugars than the neutral mucilages. Sugars present include D-galactose, L-arabinose, D-xylose, L-rhamnose, D-glucose and D-mannose.

(c) The mucilages found in seaweeds, and which consist in the main of the salts of sulphate esters of polysaccharides. They have high molecular weights and nearly all contain D-galactose.

1.2 GENERAL FEATURES, CHEMICAL AND PHYSICAL PROPERTIES OF GALACTOMANNANS ISOLATED FROM THE FAMILY LEGUMINOSAE

Water or alkali soluble galactomannans may be extracted from the mature seeds of many species belonging to the family Leguminosae (1, 3). As Lotus Pedunculatus is a species of this family it might be expected that the water soluble polysaccharide that can be extracted from its mature seeds could contain a galactomannan. The galactomannans are a characteristic of the plant kingdom where they act as food reserves in seeds. Their most common source is the endosperm of the family Leguminosae but they are also found to a lesser extent in other species, e.g. Phoenix dactylifera, Elaeis gumensis, Cocos nucifera and Coffea arabia, (3) and in bacteria (4, 5, 6, 7).

Much of the work carried out before 1950 on the elucidation of chemical structure and physical properties was stimulated by their increasing use in industry. New technological applications were being found, especially for the mucilages extracted from guar and carob tree. Hart, R. (8), in 1932, reported that number of patents had been taken out relating to the extraction of a mucilage substance from the endosperm of carob bean (Ceratonia siliqua). These patents related to different methods of obtaining the endosperm free from the tenacious husk and colouring impurities. As these polysaccharides have a mucilaginous property they find a use in the paper industry where, when added to the cellulose, they increase the strength of the resultant paper. They are used for

thickening the colourless paste in calico printing and as a thickener in foods (e.g. ice-cream). Owing to their high viscosity and the property of reverting to a continuous unbroken film on drying, the mucilages can be used in the sizing and finishing of yarns. Other uses include their incorporation as restraining agents in the tanning processes, and in pharmaceutical products (as a laxative and in the manufacture of pill capsules). Guar gum (Cyanopsis tetragonolobus) is used for human food and cattle feed in India while carob gum (Ceretonia siliqua) is grown in the Mediterranean regions, where it is used as a food called St. John's bread.

In 1949 Anderson, E. (9) conducted a survey on the presence or absence of water-soluble polysaccharides in legume species. Anderson showed that three-quarters of the 163 species he examined contained a mucilage, while examination of the hydrosylate indicated two monomers in large and varying quantities. These monomers were D-galactose and D-mannose. The other quarter of the species contained no mucilage. By cutting across the mature seeds and observing the cut surface he approximated the amount (if any) of endosperm present. The highest yields (38 per cent and 30 per cent by weight respectively) were obtained from carob seed (Ceretonia siliqua) and guar seed (Cyamopsis tetragonolobus). In general lowest yields (2 to 4 per cent) were found in species belonging to the genus Lespedeza and Lotus. From this survey Anderson concluded that in this family there is a high positive correlation between the presence of an endosperm and a water soluble

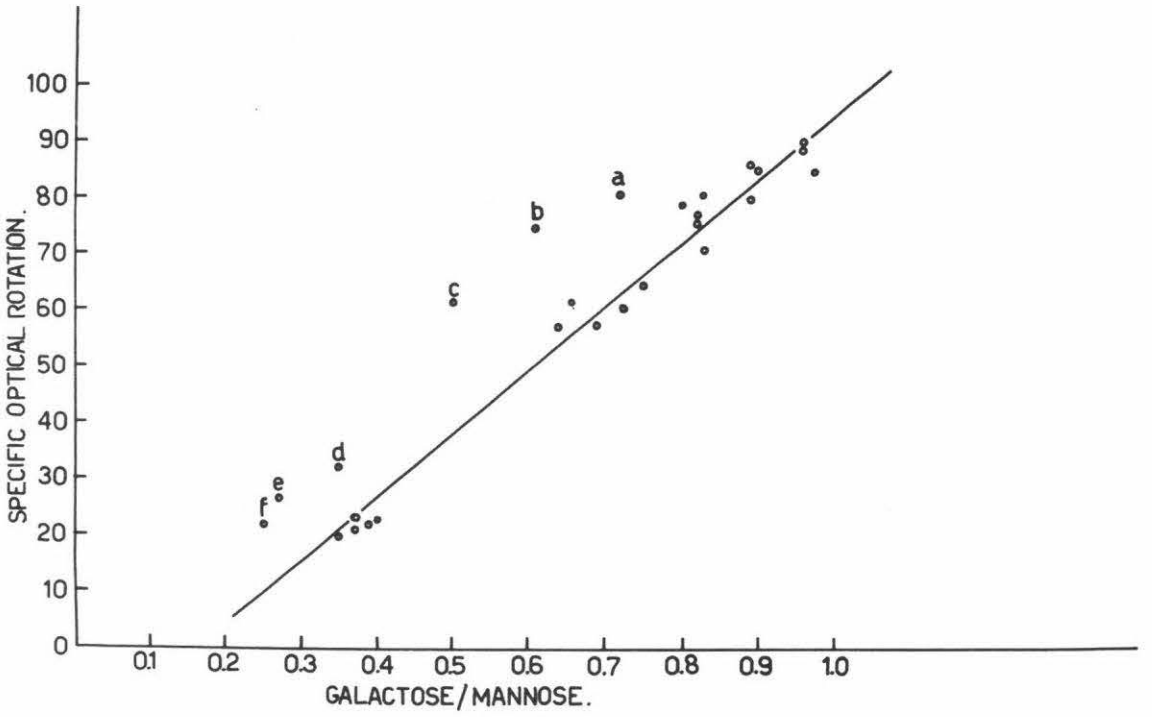
TABLE 1

COMPOSITION AND OPTICAL ROTATION OF GALACTOMANNANS (10)

Species	Sugars in Hydrosylate %		Specific Rotation in N NaOH
	Mannose	Galactose	
Cassia marilandica	79	21	+27
Cassia emarginata	73	27	+21
Crotalaria spectabilis	74	26	+20
Crotalaria retusa	74	26	+29
Crotalaria incana	73	27	+23
Crotalaria lanceolata	72	28	+22
Parkinsonia aculeata	73	27	-10
Cyamopsis tetragonolobus	63	37	+53
Astragalus sinicus	62	38	+74
Astragalus tenellus	58	42	+80
Astragalus nuttallianus	58	42	+60
Astragalus cicer	57	43	+64
Astragalus glycyphyllos	55	45	+72
Alysicarpus vaginalis	59	41	+57
Medicago orbicularis	61	39	+55
Medicago hispida	55	45	+76
Medicago lupulina	53	47	+85
Lotus scoparius	53	47	+79
Melilotus indica	51	49	+89
Trifolium hirtum	51	49	+88
Trifolium resupinatum	48	46	+84

FIGURE 1

The Relationship Between the Optical Rotation and D-galactopyranose And D-mannopyranose Ratio for Legume Seeds.



a. Astragulus tenellus.
b. Astragulus sinicus.
c. Guar gum (Cyamopsis tetragonolubus).
d. Crotalaria retuse.
e. Cassia marilandica.
f. Gymocladus dioica.

galactomannan mucilage. An exception to this is Circis canadensis (Red bud) which has an endosperm but is not mucilaginous.

More recently the U.S. Department of Agriculture has been engaged in an extensive search for potential industrial raw materials from plants not now under cultivation. A paper by Tookey and co-workers (10) reports the first phase of a survey of seeds for their content of water soluble polysaccharides. The seeds from 175 species (representing 26 families) were examined. Nearly all the seeds were from herbaceous annuals. The conclusions of this work were summarised as follows:-

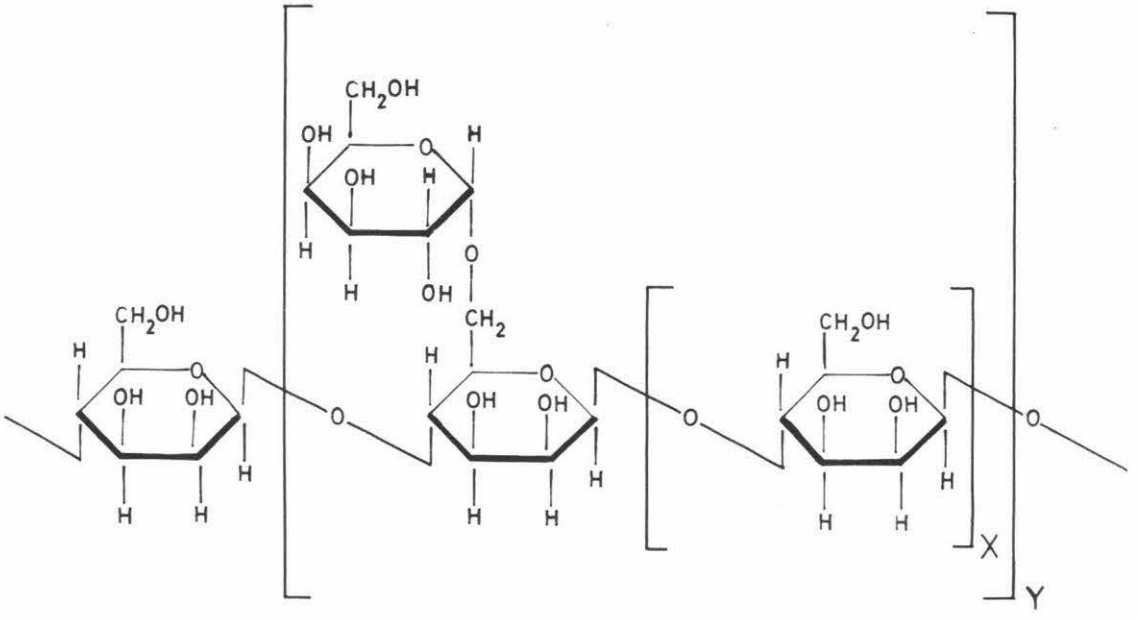
(a) Those seeds containing over 15 per cent mucilage were all from legumes.

(b) The molar ratio of D-galactose to D-mannose varied widely (1 : 4 to 1 : 1) among the species but the variation within a given species was not great. (See Table 1).

(c) The specific rotation of the mucilages increases regularly (with a few exceptions). (See Figure 1) as the ratio of D-galactose to D-mannose increases. This could indicate a similarity in the mode of linkage of various galactomannans and in particular reflects an increasing number of α glycosidic linkages as the D-galactose to D-mannose ratio increases. The discrepancies in Figure 1 might be the results of errors in the quantitative determination of D-galactose and D-mannose and optical rotation rather than because of structural differences.

FIGURE 2

General Structure of Galactomannans from Leguminosae as Indicated from Evidence.



The galactomannans extracted from the endosperms of species belonging to the family Leguminosae are defined as branched polysaccharides composed of the sugars D-galactose and D-mannose. The resistance of these polysaccharides to mineral acid hydrolysis suggests that the two sugars are present in the pyranose form rather than the furanose. More specifically then, these polysaccharides are chemically defined as having a D-mannopyranose backbone joined by β (1→4) glycosidic linkages to which the D-galactopyranose units are attached as single non-reducing side chains by α (1→6) glycosidic linkages. As the ratio of monomers is relatively constant for a species it is suggested that it is genetically controlled. The species differences in galactomannans is one of a difference in the number of D-galactopyranose residues per molecule (see Figure 2). Thus the D-mannopyranose residues have free hydroxyl groups at C_2 and C_3 and this accounts for the gelling properties of these mucilages with borax, and their ability to form insoluble copper complexes (1, 3). In nearly all cases the ratio of D-galactose to D-mannose is unity or less. Alfalfa seed (Medicago Sativa) galactomannan is an exception. When the milled seeds were extracted with hot potassium hydroxide the resulting polymer contained approximately 60 per cent D-galactose and 30 per cent D-mannose, and had $[\alpha]_D$ + 89° (Water). The results of methylation studies indicated a structure with (1→3) linked D-galactose units and (1→6) and (1→2) linked D-mannose units (11). However, when the milled seeds were extracted with hot

TABLE 2: COMPOSITION, HYDROLYSIS PRODUCTS FROM METHYLATION, PERIODATE CONSUMED AND FORMIC ACID AND OPTICAL ROTATION STUDIES OF

Family	Species	Ratio of D-galactose to D-mannose	Optical Rotation of Polymer	2,3,4,6-tetra-O-methyl-D-galactose	2,3,6-tri-O-methyl-D-mannose
Leguminosae	Ceratonia siliqua L. (Carob or Locust Bean)	1 : 5.25	+ 9° (N NaOH)	0.25	1.00
Leguminosae	Ceratonia siliqua L. (Carob or Locust Bean)	1 : 4.00		1.0	2.0 - 3.0
Leguminosae	Gymnocladus dioica (Kentucky Coffee Bean)	1 : 4.00	+ 29° (H_2O)	0.33	1.00
Leguminosae	Gleditsia amorphoides	1 : 2.50	+ 22.4° (H_2O)	0.48	1.00
Palmaceae	Borassus Flabellifer Limn. (Palmyra Palm Nut)	1 : 2.40	+ 8.5° (4% NaOH)	0.71	1.00
Leguminosae	Cyamopsis tetragonolobus (Guar Gum)	1 : 2.00	+ 60° (.6N NaOH)	1.00	1.00
Leguminosae	Glycine hispida Soybean Hulls	1 : 1.50	+ 65° (H_2O)	2.10	1.00
Leguminosae	Trifolium repens L. (White Clover)	1 : 1.30	+ 77.8° (H_2O)	3.38	1.00
Leguminosae	Trifolium pratence	1 : 1.28	+ 78 ± 11° (H_2O)	3.36	1.00
Leguminosae	Medicago sativa (Lucerne)	1 : 1.25	+ 118 ± 11° (H_2O)	3.80	1.00
Leguminosae	Trigonella Foecum-Graenum (Fenugreek)	1 : 1.20	+ 70 ± 10° (2N NaOH)	5.25	1.00

RELEASED PER HEXOSE UNIT (IN MOLES), PER CENT. END GROUPS (FROM COMPOSITION, METHYLATION AND PERIODATE RESULTS) GALACTOMANNANS OF THE TYPE COMMONLY EXTRACTED, FROM THE FAMILY LEGUMINOSAE.

2,3-di-O-methyl-D-mannose	Optical Rotation of Me. Polymer	Periodate cons.per hexose unit.	Theoretical periodate cons.per hexose unit	Formic acid released per hexose unit	Theoretical formic acid released per hexose unit.	Per Cent. End Groups			Refs
						By Periodate	By Methyl^n	From ratio of D-galactose to D-mannose	
0.25			1.16	0.168	0.160	16.8	16.7	16.0	13
1.00	-4° (Acetone)								14
0.35	± 0 (Chloroform)	1.20	1.20	0.200	0.200	20.0	19.6	20.0	15
0.52	$+28.3^\circ$ (Chloroform)	1.20	1.29	0.250	0.286	25.0	24.0	28.6	16
0.68	$+28.7^\circ$ (Chloroform)	1.30	1.30	0.270	0.294	27.0	29.8	29.4	17
1.00	$+42^\circ$ (Chloroform)	1.36	1.33	0.320	0.333	32.0	33.3	33.3	18
2.00	$+58^\circ$ (Chloroform)	1.15	1.40	0.368	0.400	36.8	41.1	40.0	19
3.00	$+74.4^\circ$ (Chloroform)	1.14	1.44	0.412	0.435	41.2	45.8	43.5	20
3.43	$+76 \pm 2^\circ$ (Chloroform)		1.44	0.432	0.438	43.2	43.2	43.8	12
3.82	$+66 \pm 3^\circ$ (Chloroform)		1.45	0.437	0.445	43.7	44.2	44.5	12
5.15	$+50 \pm 3^\circ$ (Chloroform)	1.49	1.46	0.435	0.455	43.5	46.0	45.5	21

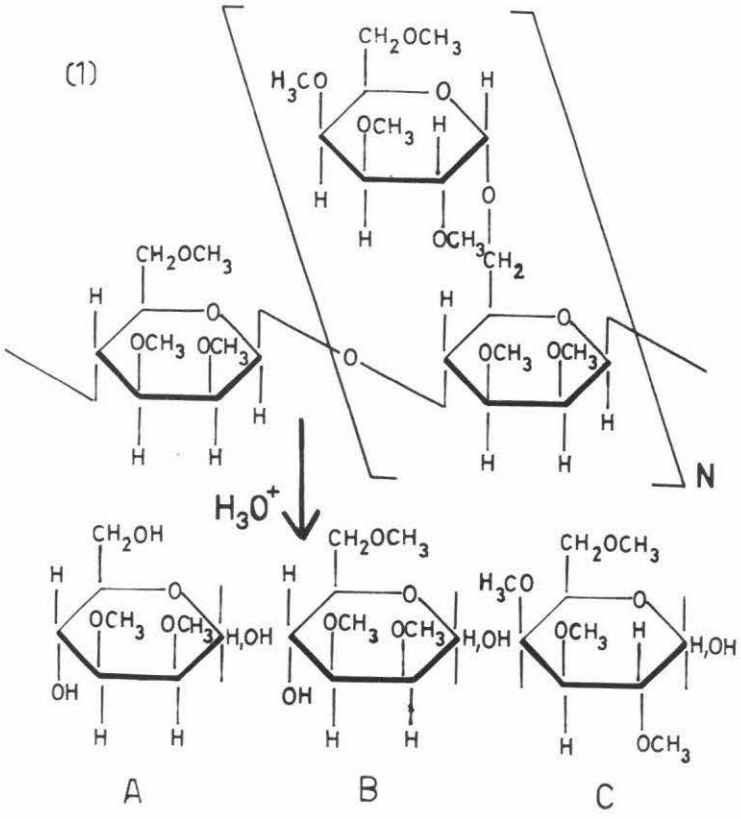
water the polymer which separated had a $[\alpha]_D^{19}$ + 118 ± 11° (water), a D-galactose to D-mannose ratio of 4 : 5, and a chemical structure, as defined above (12). Parkinsonia aculeata which has approximately 27 per cent D-galactose and 78 per cent D-mannose had $[\alpha]_D$ - 10° (sodium hydroxide). This negative optical rotation probably indicated structural differences (10).

The principal evidence which confirmed the general chemical mature of water or alkali-soluble polysaccharides has been obtained from methylation, periodate, enzymic and partial hydrolysis studies.

Methylation of galactomannans followed by mineral acid hydrolysis, the separation and identification of the resulting methyl ethyl monomers indicated the following general characteristics. The molar ratio of 2, 3 - di - O - methyl - D - mannopyranose to 2, 3, 4, 6 - tetra - O - methyl - D - galactopyranose was approximately equal to one (see Table 2). The molar proportion of the third major component 2, 3, 6 - tri - O - methyl - D - mannopyranose in the methylated hydrosylate varies according to the molar ratio of D-galactose to D-mannose in the non-methylated starting material, i.e. the lower the ratio of D-galactose to D-mannose in the starting material the higher will be the molar ratio of 2, 3, 6-tri - O - methyl - D - mannopyranose in the methylated hydrosylate. Several workers (15, 16, 17, 18, 21) have demonstrated the presence of other minor components. Larsen et al., (15) described the separation of 2, 3, 4, 6 - tetra - O - methyl - D - mannopyranose

FIGURE 4

Methylated Galactomannan and Hydrolysis Products.



A. 2, 3 - di - O - methyl - D-mannopyranose.

B. 2, 3, 6 - tri - O - methyl - D-mannopyranose.

C. 2, 3, 4, 6 - tetra - O - methyl - D-galactopyranose.

(3.3 per cent) from the galactomannan extracted from Gymnocladus dioica. Andrew et al., (21) found trace amounts of a substance they suggest to be a monomethyl hexose whereas Rafique et al., (18) chromatographically identified trace amounts of two other methyl hexoses which appeared to be partially methylated derivatives of D-mannose. In a recent paper (17) Mukherjee et al., tentatively identified a minor component as 2, 3, 6 - tri - O - methyl - D - galactopyranose. Cerezo (16) found evidence of a trace of a tri - methyl - galactose which he suggested to be 2, 3, 4 - tri - O - methyl - D - galactopyranose. This product could form part of a small number of side chains of D-galactopyranose residues by (1→6) linkages. Whether these minor components are of structural in vivo significance or merely a degradation product has not yet been ascertained. If the methylation of the mucilage has been complete and if the additional unidentified fragments have not resulted from the scission of methoxyl groups or from the condensation of hexose units to give oligosaccharides during methanolysis or hydrolysis, then these additional products will have to be taken into account when the finer points of the molecular structure of the mucilages are considered.

Periodate oxidation and subsequent work on the polyaldehyde has given valuable information for determining fine structure of these polymers. The measurement of the consumption of periodate and the formic acid released per hexose unit and the comparison of these results with a theoretical structure is of significance

(See Table 2). Theoretically one mole of periodate is consumed per D-mannopyranose unit and two moles of periodate are consumed per D-galactopyranose unit with the release of one mole of formic acid. The non-reducing D-galactose side units are the only source of formic acid. However, the resistance of some of the D-mannopyranose residues to periodate oxidation has been observed (12, 16, 21, 22). This effect has been attributed (12) to a steric effect resulting from the highly ramified structure of the galactomannan, but present knowledge (16) indicates that this phenomenon is most likely due to cyclic acetal formation and not to the presence of β (1→3) glycosidic linkages. Another explanation (12, 16, 21) attributes the effect to the use of low concentrations of periodic acid. By using excess of the periodate in the reaction mixture all D-mannopyranose units are oxidised. The polyaldehyde resulting from periodate oxidation may be used for elucidating fine structure by reducing it to the polyalcohol. Theoretically the components released on the acid hydrolysis of the latter are D-erythritol, D-glycerol and glycolaldehyde (see Figure 3). However, the glycolaldehyde is lost due to its lability and periodate-resistant D-mannopyranose residues may be present. The D-glycerol is derived from the non-reducing D-galactopyranose units while the D-erythritol is derived from the β (1→4) linked D-mannopyranose units.

The very important techniques of graded acid hydrolysis and enzymic hydrolysis for determining the chemical structure of the galactomannans have been used by several workers (17, 23, 24, 25, 36). Often the products are di-, tri-, and tetra-

saccharides which can be identified by the usual methods. Early work concerning the isolation and identification of oligosaccharides from these polymers was carried out by Whistler and his co-workers. A paper (23) describes a method for the isolation of a crude enzyme extract from the germinated seeds of guar gum (Cyamopsis tetragonolobus) and its subsequent use in the hydrolysis of a solution of guar gum. A crystalline disaccharide mannobiose [4 (β - D-mannopyranosyl) β - D-mannopyranose] was isolated and identified. The presence of a β glycosidic linkage was suggested by the fact that the disaccharide was not hydrolysed by emulsin which is a known source of α mannosidase. Since the mannobiose was not produced when the enzyme preparation acted on a solution of D-mannose the isolated disaccharide must have been a true fragment of the guar gum. Elsewhere (24) Whistler et al., described the isolation and identification of two disaccharides by partial acid hydrolysis of guar gum. One proved to be mannobiose and the other epimelibiose [6 (α - D-galacto-pyranosyl) β - D-mannopyranose]. Whistler et al., (25) have also identified a mannotriose [(β - D-mannopyranosyl (1→4) β - D-mannopyranosyl (1→4) β - D-mannopyranose)] in 7.5 per cent yield. By the use of partial acid hydrolysis Henderson et al., (26) extracted mannobiose, mannotriose, and epimelibiose from lucerne and fenugreek galactomannans. A more recent paper (17) described the isolation of the oligosaccharides mannobiose, epimelibiose and mannotriose from the galactomannan from the kernal of green Palmyra Palm nut (Borassus Flabellifer Limn).

From these results it can be deduced that the presence of mannobiose and mannotriose in relatively large amounts clearly indicates a β (1→4) linked D-mannopyranose chain in the molecule. The existence of α linked D-galactopyranose residues as non-reducing terminal units is confirmed by the isolation and characterisation of epimelibiose.

Galactomannans differing in gross structure from those described above have also been found in nature. The only galactomannan extracted from the family Leguminosae that differs from the structure expected is from Medicago sativa (11) (see page 11). Bishop et al., (4, 5, 6, 7) described the extraction of a group of water-soluble polysaccharides from dermaphytes, Trichophyton granulosum, T. interdigitale, T. rubrum, T. schonleinii, and Microsporum quinckeanum. Each contained a similar pattern of water soluble polysaccharides, specifically, two galactomannans and a glucan. Bishop et al., (7) also described the isolation of one of the groups of galactomannans (called galactomannan I) via their insoluble copper complexes from aqueous solutions of crude polysaccharides obtained from each of the organisms above. Results of methylation and periodate studies indicated the following basic structures: A basic chain of (1→6) linked α - D-mannopyranose units for approximately every 22 of which there is a (1→3) linked α - D-mannopyranose residue. Branch points occur along the (1→6) linked chain at C_2 positions of the D-mannopyranose units and once in every 45 units at the C_2 position of a (1→6) linked

D-mannofuranose residue. The D-galactose in the polysaccharide is present exclusively as non-reducing terminal furanose units; non-reducing terminal units of D-mannopyranose are also found. Rao et al., (27) described the isolation from the kernel of coconut (Cocos nucifera) of a water soluble galactomannan. Methylation studies indicated a highly branched structure with an average repeating unit of about nine hexose units. Equal amounts of D-galactopyranose and D-mannopyranose were present as non-reducing terminal units. The basic chain consisted of residues of D-galactopyranose and D-mannopyranose joined by (1→4) glycosidic linkages while an unidentified di - O - methyl - derivative of D-galactose (probably 3, 6, - di - O - methyl - D-galactose) showed that branching occurred at certain of the D-galactopyranose units of the chain. Unrau (28) employed methylation and periodate techniques to study the constitution of a galactomannan isolated from the seed of Leucaema glauca. Results indicated that all the D-galactose residues occupied terminal non-reducing positions (as did some of the D-mannose residues). The remaining D-mannose residues were predominantly joined by (1→4) linkages, with a small proportion of (1→3) linkages, while branch points occurred at the 6 position of the D-mannose residues.

1.3 ISOLATION FROM THE GROUND MATERIAL OF AN UNDEGRADED UNCONTAMINATED HOMOGENEOUS POLYSACCHARIDE SPECIES (29)

The first problem in the investigation of the fine structure of any natural polysaccharide is to separate from the ground material a homogeneous, undegraded polysaccharide species. A homogeneous polymer is defined as one consisting of molecules having identical chemical structures but not necessarily the same molecular weight (29). This variation in molecular weight is accounted for by (a) Enzymic synthesis may not be uniform; (b) Subsequent enzymic and chemical change..

The necessity for assessing homogeneity is that methods for determining the fine structure of polysaccharides are becoming increasingly accurate. Whether minor components are artifacts or not may depend on the purity of the starting material. A polysaccharide species is usually inlaid and overlaid with other polysaccharide species (similar or dissimilar) and other biological substances, e.g. fats, minerals, protein, etc. After the removal of non-polysaccharide material the problem is to separate a polysaccharide mixture (if more than one polysaccharide species is present) into component homogenous fractions which are large enough for structural work.

There is no single procedure for obtaining a polysaccharide species (one or more) from the ground material. Each botanical source presents its own problems. It must be realised that extraction techniques may have the effect of modifying -

(a) The structure of the molecule; (b) The molecular weight distribution.

Procedures for obtaining a homogeneous polysaccharide species include graded extraction, fractional precipitation, increasing salt concentration, precipitation by a specific complexing agent, chromatography, gel filtration, ultracentrifugation, electrophoresis and the use of enzymes. The success of fractionation (and hence homogeneity) may be ascertained by measurements of specific rotation, molecular weight, viscosity, sedimentation, light scattering, osmotic pressure, moving boundary electrophoresis and by the determination of the nature and percentage of the component sugars in each fraction.

The extraction of uncontaminated galactomannans from the endosperms of the ripe seeds of species belonging to the family Leguminosae does not present any problem. As these polysaccharides are water and alkali-soluble, they are easily dissolved out of the ground material. When using dilute alkali in the extraction procedure it is inadvisable to heat the mixture and also preferable to carry out the extraction in an inert atmosphere (free from oxygen). Although protein is removed in alkali extraction undesirable side reactions may occur (30). With larger seeds the seed covering may be manually or mechanically removed prior to the extraction of the endosperm. Using smaller seeds, however, this is impossible and the whole seeds have to be milled before extraction with water or alkali. Precautions must be taken to remove dust, colouring matter, etc.

1.4 DETERMINATION OF THE HOMOGENEITY OF A GALACTOMANNAN SPECIES (1, 4, 39)

The methods that can be employed to assess the homogeneity of a galactomannan species are:-

(a) Fractional precipitation.

(b) Precipitation using a specific complexing agent.

(c) Ultracentrifuge studies.

(d) Electrophoresis.

(a) Fractional precipitation: This involves the precipitation of the polysaccharide from aqueous solution by addition of a non-solvent. It is a method used for purifying natural plant gums and mucilages as well as their acetate, nitrate and methylated derivatives. By using acetate, nitrate and methylated derivatives the hydroxyl groups are masked and the problem of co-precipitation (by hydrogen bonding) and occlusion with other polysaccharide species is to some extent mitigated. This method is most successful when the solubilities of polysaccharides in a mixture are quite different (1, 4, 29).

(b) Precipitation using a specific complexing agent (1, 4, 29): Metallic salts have been widely used as specific precipitating agents for water soluble polysaccharides and hemicelluloses. Fehlings solution, barium hydroxide and boric acid can all be used. All galactomannans from leguminous seeds produce gels or mucilaginous precipitates when borax is added to these

aqueous solutions. This property is attributed to the presence of adjacent "cis" hydroxyl groups on the hexose units of the polymer which lead to extensive cross linking. Gelation does not occur in acidic solutions and may be reversed by the addition of low molecular weight substances such as mannitol and glycerol which form complexes with boric acid (4). These results indicate that the hydroxyl groups on carbons two and three of the D-mannopyranose residues are free. Fehlings solution has been widely used for purifying galactomannans. Most, if not all, the galactomannans extracted from seeds of the Leguminosae give copper complexes with Fehlings solution and this phenonomen is attributed to the D-mannopyranose residues having "cis" adjacent hydroxyl groups at carbons two and three. Work by Andrews et al., (21, 32) suggests that Fehlings solution may be used to distinguish galactomannans and mannans from galactans. These workers used Fehlings solution to separate a galactan from a mannan from a polysaccharide material extracted from Strychnos nux vomica seeds. The mannan (as do galactomannans from Leguminosae seeds) formed an insoluble copper complex while the galactan remained in solution. Mukherjee et al., (17) studying the constitution of a galactomannan from the kernel of green Palmyra Palm nut, observed that the proportion of D-galactose gradually decreased (D-galactose to D-mannose ratio increased from 1 : 24 to 1 : 34) during five treatments with Fehlings solution. The mild hydrolysis of the polymer using oxalic acid (.02N) resulted

only in the removal of D-galactose residues. These workers suggested that the increase in the D-mannose content during purification via the copper complex may be due to the fact that some of the α linked galactose residues (which are more labile than β linked residues) were removed during the acid treatment employed to break down the Fehlings solution.

(c) Ultracentrifuge: This is perhaps the most generally-applicable method of assessing homogeneity but it has a limited use as a separation method on a preparative scale. If the molecular weight of a homogeneous polysaccharide is sufficiently large, sedimentation occurs in the field. The presence of a single peak usually suggests a homogeneous polysaccharide, but many complications can occur (29). In the case of an apparently homogeneous peak, care has to be taken that the distortation of the base line due to solvent does not disguise the presence of degraded material of low molecular weight. Again one peak may, in reality, represent two components of closely overlapping molecular weight distributions. The spread of the "peak" is related to the molecular weight distribution, as the small molecules tend to trail behind those of average size and the larger ones tend to move ahead. The molecular weight may be calculated according to the Archibold method as described in a review article by Schachman (32).

(d) Electrophoresis: Free boundary electrophoresis is an essential diagnostic tool for acidic polysaccharides, particularly for following the success of a separation procedure. Neutral polysaccharides such as galactomannans, mannans, glucomannans, etc., will move electrophoretically in the presence of a borate buffer (33). A reaction between borate ions and carbohydrates in aqueous solution leads to the formation of negatively-changed complexes that migrate in an electric field.

1.5 IDENTIFICATION OF THE COMPONENT MONOMERS PRESENT (1, 4, 34)

After the criteria of homogeneity of the polysaccharide have been satisfied, the first determination in the elucidation of the fine structure is the identification of the monomer/s released on mineral acid hydrolysis. Since acid hydrolysis will give an equilibrium mixture of α and β anomers of the monosaccharides any decrease in optical rotation will indicate a predominance of α glycosidic linkages in the polysaccharide; likewise any increase will provide evidence of β glycosidic linkages. The significance of these results is decreased if changes occur (other than the scission of glycosidic linkages). The extent of hydrolysis may be followed optically or by iodometry (35). When these values remain constant hydrolysis may be assumed to be complete. For the hydrolysis of normal pyranose forms it is standard practise to use sulphuric acid (1 - 2N) at 100°C. If furanose forms are present less vigorous conditions may be employed. Reaction conditions

are controlled both to reduce to a minimum the degradation of sugar units and to minimise the repolymerisation of sugar units to produce inconsonant oligosaccharides. One of the linkages most resistant to acid hydrolysis is that in which a glycuronopyranoside unit is linked to a sugar unit.

Sheet paper chromatography, thin-layer chromatography and paper-ionophenetic analysis of the polysaccharide hydrosylate allows a tentative identification of the specific sugars present. When examining a hydrosylate by sheet paper partition chromatography it is necessary to use two or three solvent systems to identify the components present (1).

The quantitative separation of the sugars of a hydrolysate is usually achieved by a technique of column chromatography (36) using cellulose (37, 38) or a mixture of cellulose and hydrocellulose (39). The purpose is to provide relatively large amounts of the components of the hydrolysate in a pure state. Although it is preferable to crystallize the monosaccharide itself difficulty is encountered because of the existence in solution of α and β isomers. Monomers are usually identified as crystalline derivatives. For initial crystallization of a neutral monosaccharide it is sometimes easiest to crystallize from a syrup, preferably one which has been dried by repeated distillation from methanol. The favoured solvents used for recrystallization are methanol and ethanol but occasionally it is preferable to use acetone, (L-Rhamnose) or glacial acetic acid (D-galactose and D-mannose). D-galactose may be identified as the free sugar,

α - methyl phenylhydrazone, galactaric acid and galactitol hexa-acetate. D-mannose may be identified as its free sugar, phenylhydrazone, N-(p-nitrophenyl) glycosylamine, methyl - α - pyranoside, mannitol hexa-acetate and N-phenylglycosylamine(1)

1.6 QUANTITATIVE DETERMINATION OF THE MOLAR RATIO OF THE MONOMERS PRESENT

The composition of gums and micilages is best determined by descending paper chromatography in conjunction with a micro-volumetric or colorimetric method of sugar analysis. The most accurate methods involve the dissection of a paper chromatogram into sections containing each component, elution of the sugar from each section, followed by microanalysis of the eluate.

Elution of sugars followed by microcolorimetric or micro-volumetric techniques include methods involving use of phenolsul-phuric acid, Somogyi reagent, hypoiodate, alkaline ferricyanide and sodium metaperiodate. Methods involving the use of sprays and devel-opment prior to elution include the use of aniline hydrogen phthalate, benzidine, panisidine hydrochloric acid, p-aminodiphenyl amine, anthrone and chromotropic acid (1, 3, 40). Other procedures involving photometry are also employed (40).

1.7 METHYLATION PROCEDURES: WHICH INCLUDES METHODS OF METHYLATION, ACID HYDROLYSIS, QUALITATIVE AND QUANT-ITATIVE IDENTIFICATION OF THE MONOMERS RELEASED (34, 41a, 42a, 42b)

This procedure involves the preparation of an exhaustively methylated polysaccharide, acid hydrolysis to a mixture of monomers,

and the separation, identification, and quantitative estimation of the components of the mixture. From these results it is deduced that the original points of substitution in the natural polymer will correspond to the unsubstituted hydroxyl groups in these monomeric methyl ethers. Quantitative estimation of these methyl ethers will give some indication of the proportion of glycosidic linkages present. Usually the results are summarised in terms of a "repeating unit" which is defined as the simplest repeating structure which defines the polysaccharide molecule. Unless the polysaccharide structure is relatively simple methylation studies alone provide little evidence as to the exact sequence of the building units. Methylation studies are most useful when used in conjunction with partial hydrolysis and periodate studies.

The aim of methylation is to achieve an etherification of all free hydroxyl groups in the polysaccharide. To achieve this a number of methods have been devised.

The classical method of Haworth (43), and Denham and Woodhouse (44) is still widely used. The polymer is simultaneously treated with alkali and dimethyl sulphate. Slight modifications to the method may be made according to habit and experience. The use of more concentrated sodium hydroxide has been reported to give better results (45) and higher temperatures may facilitate the methylation of resistant polysaccharides (46). Replacing sodium hydroxide by potassium hydroxide has been reported

to give improved results but the reason is obscure. Freudenberg and Boppel (47) devised an important method where the polysaccharide is treated with metallic sodium to give the sodio salt, which is then methylated with methyl iodide. In general the above methods and their modifications do not give complete methylation and the product has to be methylated by some other method. The methods of Purdie and Irvine (48) and Kuhn and his co-workers (49, 50, 51, 53, 54) are frequently employed. The latter is really only an improvement of the Purdie method. In the latter method, the partially methylated material (usually) is refluxed in methyl iodide and treated with silver oxide. This method generally has to be repeated several times to achieve complete methylation andhas even been reported to fail completely (55). One of its advantages is the ease with which the methylated product may be recovered. In the Kuhn procedure the polysaccharide is dissolved in the polar solvents N, N-dimethylformamide and/or dimethyl sulphoxide. Silver oxide (49, 50, 51, 52) may be replaced by barium oxide and/or barium hydroxide (53, 54) while dimethyl sulphate is sometimes more effective than methyl iodide (54).

The success of methylation can be followed by methoxyl-content determination and comparison of this with a theoretical determination of the suspected repeating unit. A slight modification to this is to repeat methylation procedures until there is no change in methoxyl content on consecutive methylations. Another method involves observing absorption in the 3400 - 3600 cm^{-1}

region of the infrared spectrum (hydroxyl group stretching vibrations occur between 3400 and 3600 cm^{-1}). This peak should be virtually absent when methylation is complete but it is often difficult to obtain a sample perfectly free from water.

Often the fully methylated product may be purified (or less often fractionated into distinct methylated-polysaccharide species) by fractional precipitation methods. The purification serves to remove non-polysaccharide materials (salts, etc.) and incompletely methylated material. The fractional precipitation is carried out from acetone, ethanol or chloroform solutions either by addition of light petroleum, or cooling (56), or the material may be extracted under reflux with different proportions of these two solvents (57).

The purpose of hydrolysing a methylated polysaccharide is to depolymerise it to the monomeric state under conditions which will give the least degradation of the resultant monomers (42b). As the ethers are generally insoluble in hot water (and sometimes even in cold) it is usually necessary to use a non aqueous (or only partially aqueous) medium for initial hydrolysis. One method generally employed is that of methanolysis where the methylated product is refluxed with methanolic hydrochloric acid and then undergoes by mineral acid hydrolysis (44). Other methods include formolysis (58) and the 72-8 per cent sulphuric acid procedure (59).

A mixture of methyl sugars is generally fractionated by partition chromatography on cellulose columns (41a). Lemieux

and co-workers (60) have found that columns of diatomaceous earth (e.g. celite) are as good as, or better than, cellulose columns. Separation can also be achieved by absorption chromatography on carbon-celite columns preferably using gradient elution. The capacity of carbon columns is high and a wide range of substances from unsubstituted to fully methylated sugars, can be fractionated in the same run. Mixtures that are difficult to separate by partition chromatography can often be resolved on carbon columns. Since the reverse is often true, the methods supplement each other.

Many methylated sugars are known only in the amorphous state, but even those that are known in the crystalline state are difficult to obtain solid unless material is available for seeding. It is common practise to identify methylated sugars as their crystalline derivatives. The identity of the parent sugar of a methyl ether is established by demethylation with hydrobromic acid (61) or boron trichloride (62).

Quantitative determination of methyl derivatives involves either paper partition chromatography, elution and a micro-volumetric or micro-colorimetric procedure (41a), or gas-liquid partition chromatography (63, 64). The amounts of methyl sugars in the fractions eluted from the paper chromatogram can be determined by a volumetric procedure using sodium hypoiodate, or by colorimetric methods involving the use of aniline phthalate, benzidine, 3, 5 - dinitrosalicylic acid or phenol-sulphuric acid reagent (41a).

1.8 PERIODATE OXIDATION: INCLUDING PERTINENT THEORETICAL CONSIDERATIONS, THE DETERMINATION OF PERIODATE UPTAKE AND FORMIC ACID RELEASE, REDUCTION OF POLYALDEHYDE AND THE IDENTIFICATION OF PRODUCTS RELEASED ON MINERAL ACID HYDROLYSIS (1, 3, 35, 41b, 42c, 42d, 65)

Periodic acid and its salts (generally sodium periodate because of its greater solubility in water) bring about the cleavage of a carbon chain whenever two or more adjacent hydroxyl groups are present.

e.g. $R_1\ CHOH\ CHOHR_2 + IO_4^{\prime} \longrightarrow$

$$R_1\ CHO + R_2CHO + H_2O + IO_3^{\prime}$$

In this reaction there is a cleavage of the carbon chain, the consumption of one molecular proportion of periodate and the formation of two aldehyde groups. In the galactomannan under study this reaction involves the hydroxyl groups on the C_2 and C_3 of the D-mannopyranose units.

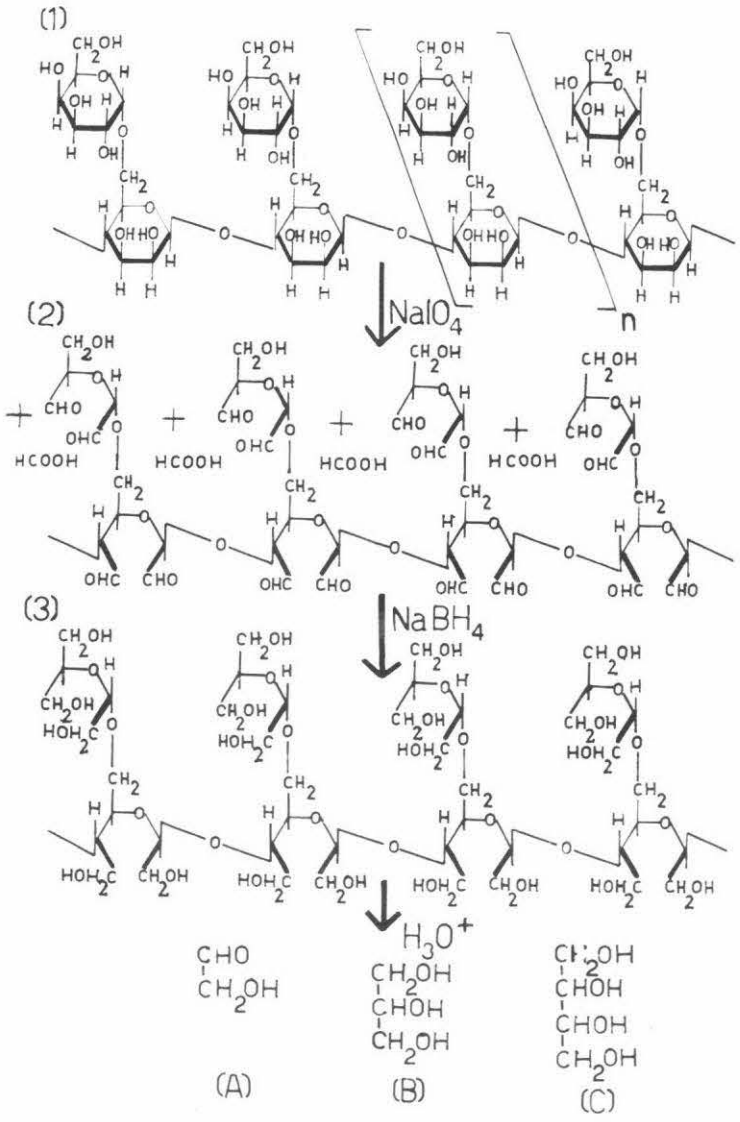
also: $R_1\ CHOH\ CHOH\ CHOHR_2 + 2IO_4^{\prime} \longrightarrow$

$$R_1CHO + R_2CHO + HCOOH + H_2O + 2IO_3^{\prime}$$

There is, in this reaction, a double cleavage with the formation of two aldehyde groups, the liberation of one molecular proportion of formic acid and the consumption of two molecular proportions of periodate. Theory indicates that this reaction would involve the carbons, C_2, C_3, and C_4 on the non reducing D-galactopyranose units of the galactomannan (See Figure 3).

FIGURE 3

Periodate Oxidation, Reduction of Polyaldehyde, Acid Hydrolysis of Polyalcohol.



(1) Galactomannan. D-galactose/D-mannose = 1.0
(2) Galactomannan Polyaldehyde.
(3) Galactomannan Polyalcohol.

(A) Glycolaldehyde (B) Glycerol

(C) D-erythritol

The rate of oxidation varies for different types of glycol groupings and the succeeding generalisations should only be taken as a rough guide. The most readily oxidised are the open chain glycols, followed by cyclic "cis" glycols. Cyclic "trans" glycols are oxidised more slowly and are not oxidised at all if fixed in unfavourable conformations as in some bicyclic anhydroxhexoses. Neighbouring groups may also affect the reactivity (41b). The reaction conditions can be chosen to favour either partial or complete oxidation, with or without the hydrolysis of intermediate formate ester groups and even in some cases, "overoxidation". Overoxidation mainly occurs when oxidation of the 1, 2 - glycol groupings in the reducing end group produces a malondialdehyde derivative possessing an activated hydrogen atom and thus the oxidative specificity of the reagent is lost (66). This flexibility, combined with the easy determination of the oxidant consumption and reaction products (formaldehyde, formic acid and carbon dioxide), makes these oxidising agents valuable and versatile tools in carbohydrate chemistry.

It was originally thought (41b) that the oxidation would only cleave α glycol groups but it is now known that periodate cleaves carbon bonds in for example α hydroxyaldehydes, α hydroxyketones and α aminoalcohols.

The selectivity of the above reactions is the outstanding characteristic of periodic acid oxidations when applied under the proper conditions. These conditions are selected so as to

minimise side reactions. Thus the reaction is usually carried out at room temperature (20 - 25°C), for at higher temperatures the oxidative specificity of the reagent is lost. At lower temperatures (5°C) the rate of oxidation is lower but side reactions are eliminated. The reaction is carried out in the dark as sodium metaperiodate in the light decomposes at a measurable rate with the production of ozone and sodium iodate. Also formic acid and formaldehyde are oxidised in the presence of periodic acid and light but are unaffected by the oxidant in the dark. Of all the various conditions of oxidation the effect of the pH of the medium upon the rate of oxidation is most marked. In general α glycols, hydroxyaldehydes and hydroxyketones are oxidised most rapidly in a slightly acid medium (pH 3 - 5) whereas α amino ketones and diamines require a neutral or slightly alkaline medium (pH 7 - 8) for optimum rate of oxidation. The concentration of periodate usually employed ranges between 0.01M and 0.1M. At concentrations greater than 0.1M non-specific oxidation may occur and at less than 0.01M the rate of primary oxidation is so slow that data obtained may be difficult to interpret.

As galactomannans are polyhydroxyl in nature periodate oxidation can be used effectively as a tool in the elucidation of fine structure. In a polysaccharide molecule the number of moles of periodate consumed and the number of moles of formic acid produced will depend on the types of glycosidic linkage, the number of carbon atoms in the sugar unit and the ring form.

Hexopyranose polymers (e.g. galactomannan), with non reducing terminal units (the D-galactopyranose units of galactomannans from Leguminosae seeds) or (1 → 6) linked non-terminal units having three adjacent hydroxyl groups, will be cleaved by two molecular proportions of periodate and release one molecular group of formic acid. Non-terminal units joined by (1 → 2) or (1 → 4) linkages undergo cleavage by one molecular proportion of periodate but no formic acid is produced (as with the D-mannopyranose units in Figure 3). Non terminal units joined by (1 → 3) linkages or units involved in branching at C_2 and C_4 are not affected by periodate. Theoretically those units of polysaccharide gums and mucilages not cleaved by periodate oxidation will be recognised in the hydrolysis products of the polyaldehyde or polyalcohol by the presence of intact sugar residues. Thus the determination of the periodate consumed, formic acid generated and the proportion of surviving sugar units will give information concerning the nature and proportion of glycosidic linkages present in the polysaccharide.

The polyaldehyde (Figure 3) formed by periodate oxidation may be further used in giving information in structural studies. The polyaldehyde does not undergo smooth hydrolysis unless special techniques are used (82) and affords carbonyl fragments which are difficult to manipulate. It is more convenient to reduce the polyaldehyde to polyalcohol using sodium or potassium borohydride (95, 96) or Raney nickel catalyst and a pressure of hydrogen (97)

prior to hydrolysis by mineral acids. This hydrolysis will afford a mixture of one or more simple polyhydric alcohols, glycolaldehyde and free sugars. The identification and quantitative analysis of these components provides information about the nature of the linkages in the parent polysaccharide. The polyalcohol may be methylated prior to hydrolysis and the partly methylated products may be identified (1).

The formic acid released is titrated with sodium hydroxide or barium hydroxide using as indicators methyl red, thymolphthalein, phenol red and methyl red-methylene blue. This acid may also be determined by potentiometric titration, iodometrically and by the Warburg respirometric method. There are three methods for determining periodate consumption. The arsenite method, the iodometric method and a spectrometric method (41b).

CHAPTER II

METHODS AND RESULTS

Melting point determinations were carried out using a Gallenkamp Electrical Melting-point Apparatus. Optical Rotations were determined using a Hilgar Standard Polarimeter. Optical density measurements were made using a Hilger 810 Biochem. Absorptiometer. Microanalyses were carried out at the University of Otago by Dr. A.D. Campbell.

2.1 EXTRACTION OF THE GALACTOMANNAN FROM LOTUS PEDUNCULATUS:

The milled seeds (300 g) were extracted with water (1.5 l) at room temperature for four hours with occasional stirring. The mixture was agitated (5 mins.) in a Waring blender and then centrifuged at 2500 r.p.m. and the supernatant was poured off and retained. This procedure was repeated on the remaining crushed seeds, dust, etc., and the supernatant collected was compounded with that from above. The solution was clarified, firstly by storing (24-36 hours) in centrifuge bottles (220 ml) (which allowed most of the fine particles to settle out), and then by centrifuging (2500 r.p.m.) This supernatant was further cleared (fine dust, etc.) by centrifugation at 30000 r.p.m. (1 hour)

using a Beckman Model L Preparative Ultracentrifuge.

The clear brown liquid (2 l) was added to an equal volume of acidified 95% ethanol (10 ml conc. HCl per l) when a white, fibrous precipitate appeared. This precipitate was collected by centrifugation (2500 r.p.m.), triturated with acidified ethanol (1x), absolute ethanol (2x) (to free from acid), diethyl ether and finally taken up in water and freeze dried to give a white, horny, matted polymer (6.7 g). The per cent yield of soluble mucilage from the whole seed was 2.2 (i.e. $\frac{6.7}{300} \times \frac{100}{1}$).

The polysaccharide was then purified by complexing with Fehlings solution (12). The polymer (6.7 g) was dissolved in water (500 ml) to give a viscous solution, which was centrifuged (2500 r.p.m.) to remove insoluble material. The resultant solution was treated in turn with freshly prepared Fehlings solution A (500 ml) and then Fehlings solution B (500 ml). After agitating the mixture in a Waring blender (5 mins) the blue gel-like precipitate was recovered by centrifugation (2500 r.p.m.). The precipitate was triturated with dilute Fehlings solution (2x) (10 ml of Fehlings solution per 200 ml water) and water (1x). The light blue precipitate was dispersed in water (200 ml) in a Waring blender and the complex decomposed by the addition of 2.0 N HCl (100 ml). This clear solution (1 l) of regenerated polysaccharide was poured into 95% ethanol (1 l) when a white, fibrous precipitate appeared. The precipitate was collected by centrifugation (2500 r.p.m.) washed successively with acidified

95% ethanol (2x) to remove copper ions, absolute ethanol (1x) and finally with diethyl ether (1x). These last two washings removed the acid (HCl) from the precipitate. The resulting solid was redissolved in water (500 ml), precipitated by adding acidified 95% ethanol (10 ml conc. HCl per l), washed as above to remove copper ions and HCl, and then the product was taken up in water and freeze dried (5.6 g); $[\alpha]_D^{20}$ + 82.5° (water).

2.2 ASSESSMENT OF HOMOGENEITY OF THE GALACTOMANNAN (68)

(a) Fractional Precipitation Method (68)

A portion (3.4 g) of purified polysaccharide was dissolved in water (350 ml). On centrifuging (2500 r.p.m.) the small amount of insoluble material that separated out was discarded. Aliquots (10 ml) of absolute ethanol were slowly added to the solution (while stirring in a Waring blender) until the clear solution became milky white. After the addition of each 10 ml portion of ethanol the solution was centrifuged (2500 r.p.m.) and the small insoluble scums were discarded. After 160 ml of absolute ethanol (or 31.4 per cent ethanol by volume) had been added to the solution additions were made as shown in Table 3. From this table it can be calculated that the aggregate weight of the fractions (1, 1A, 2, 3 and 4) represent 93.0 per cent recovery of the starting material. Furthermore 96.8 per cent of the total polysaccharide precipitated in the narrow range of 31.6 to 33.0 per cent ethanol by volume. These results together with the optical rotation,

TABLE 3

FRACTIONAL PRECIPITATION OF GALACTOMANNAN

Ml. Absolute Ethanol	Per Cent Ethanol by Volume	Fraction Number	Fraction Weight (g)	Per Cent Total P.S. Ppte.	Optical Rotation (Water)	D-galactose to D-mannose Ratio	Intrinsic Viscosity (Water)
162.0	31.6	1	1.19	38.0	82.0	0.98	4.1
162.0	31.6	1A	1.01	32.0	84.0	0.97	4.0
164.0	32.0	2	0.19	6.0	83.5	0.96	
172.0	33.0	3	0.65	20.7	82.0	0.98	4.0
200.0	36.4	4	0.10	3.2	83.5	0.98	3.2

FIGURE 5

Per Cent. Polysaccharide Precipitated at Various Alcohol Concentrations (68)

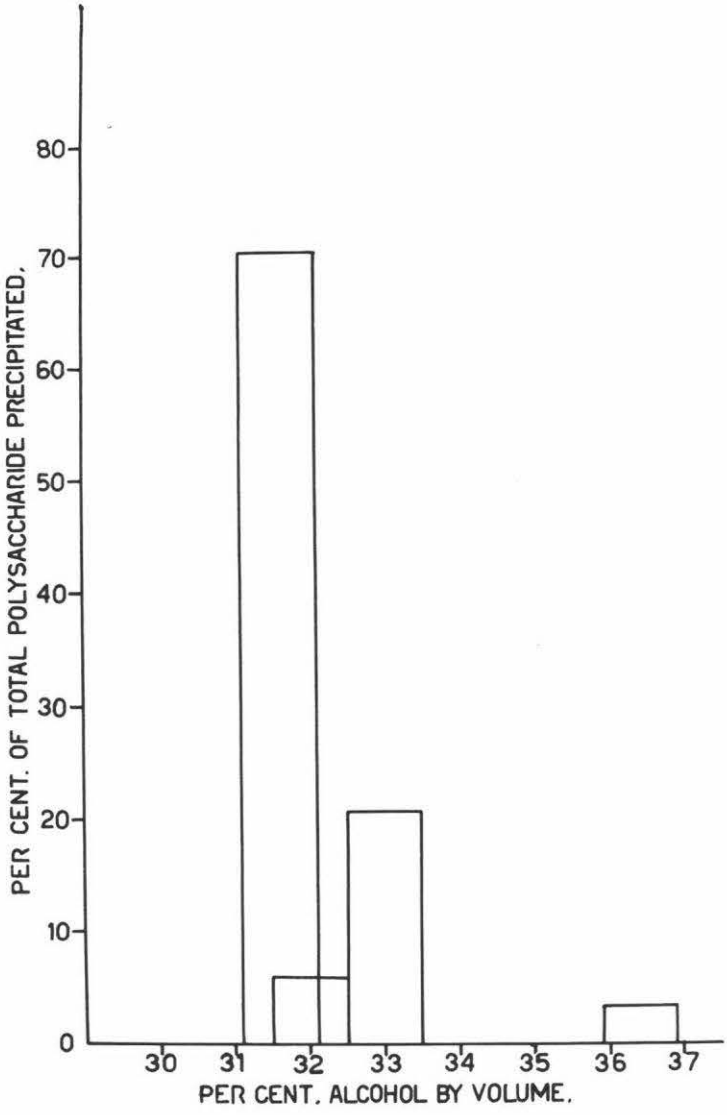
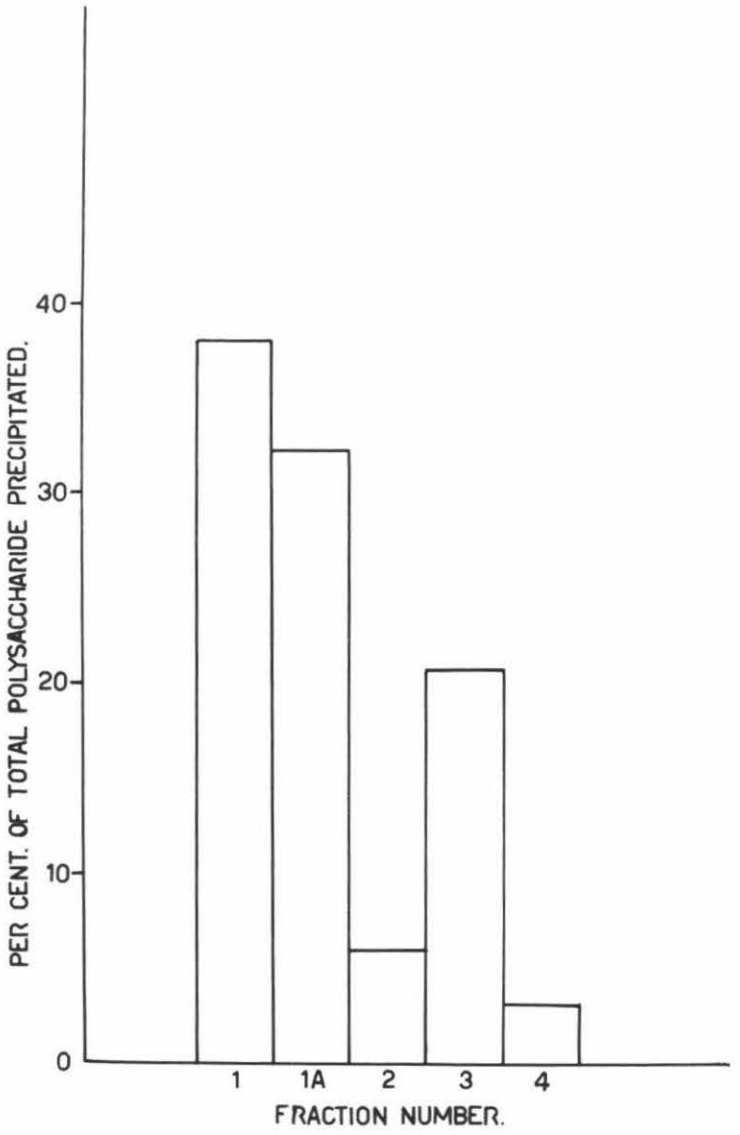


FIGURE 6

Per Cent. of Galactomannan in Each Fraction (68).



D-galactose to D-mannose ratio, and intrinsic viscosity for each fraction, indicate a highly homogeneous polymer.

Note: In Table 3, Fractions 1 and 1A were collected at 31.6 per cent ethanol by volume as follows. Fraction 1, was collected at this ethanol percentage and the supernatant was left to stand overnight after which time an additional precipitate was collected and designated Fraction 1A. (See also Figures 5 and 6).

(b) Ultracentrifuge Studies. Assessment of Molecular Weight of Polymer (32)

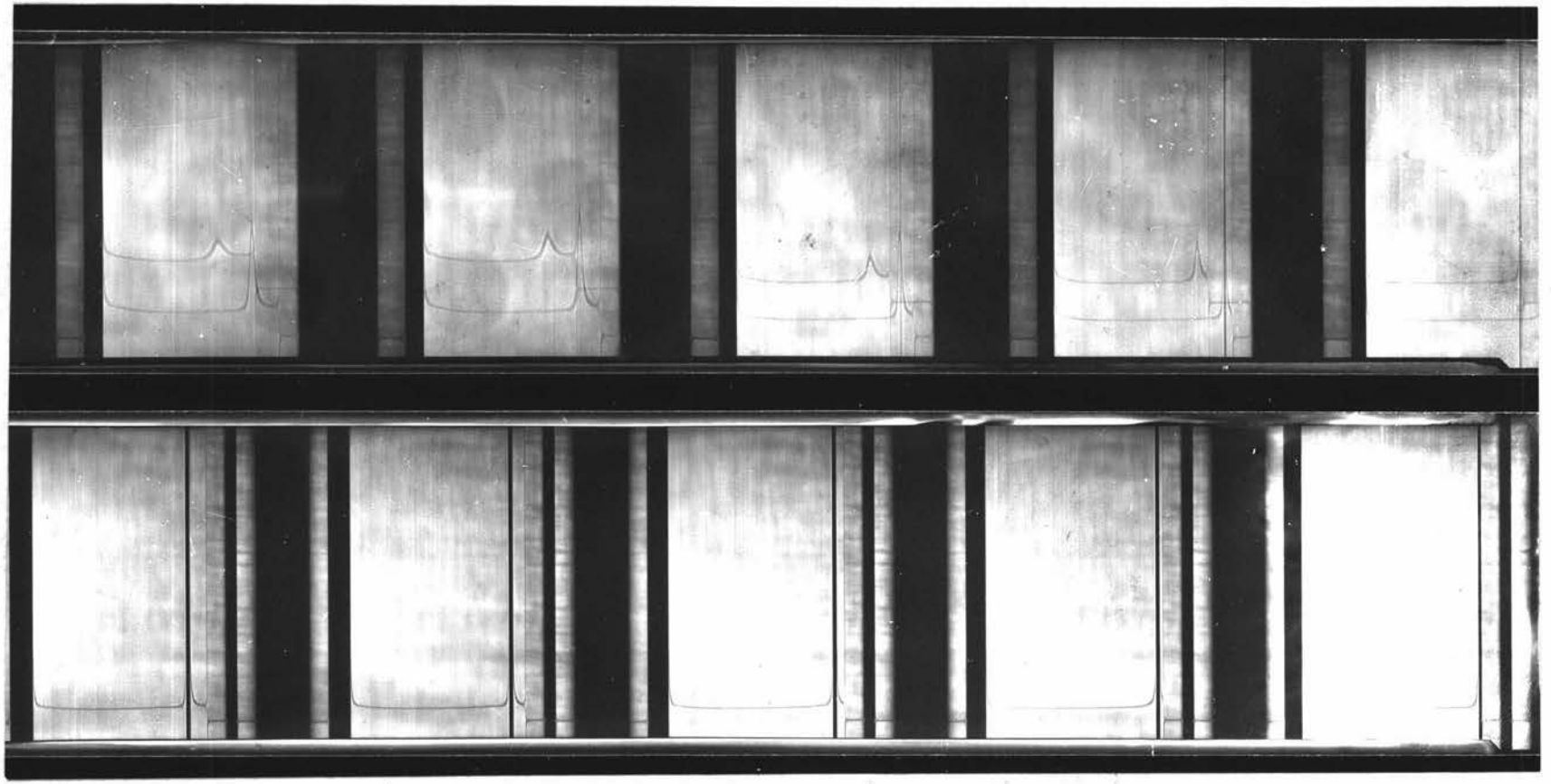
The sedimentation pattern (See Figure 7) was obtained in a Beckman Model E Ultracentrifuge at a rotor speed of 59,780 r.p.m. Photographs were taken at 16 min. intervals after attaining full speed. The sedimentation patterns a, b, and c correspond to polymer concentrations of 10.0, 5.0 and 2.5 mgs per ml Buffer (Buffer: pH 6.98, N/15 KH_2PO_4 (4.0 ml) and N/15 Na_2HPO_4 (6.0 ml)). These photographs indicate a homogeneous polymer.

An assessment of molecular weight was made according to the Archibold method (32). The partial specific volume of the solution ($\bar{V}$ soln) was calculated to be 0.625. ρ (density), buffer 1.0020 (pH 7.3, 0.01 M) ρ (density), solution 1.0055 (NaCl 0.1 M).

The sedimentation coefficient S_{20W} at zero concentration was calculated by extrapolation of the following S values.

FIGURE 7

Sedimentation Patterns



(a) Concentration 10 mg/ml
(b) Concentration 5.0 mg/ml
(c) Concentration 2.5 mg/ml

Photographs taken at 16 minute intervals.

S	Concentration
1.43	10.0 mg per ml.
2.26	5.0 mg per ml.
3.05	2.5 mg per ml.

and found to be 3.7 S.

Range of molecular weight distribution:

	Time after start	M. Wt. at Meniscus
Exposure (2)	60 mins	1.14×10^5
Exposure (3)	90 mins	1.03×10^5
Exposure (4)	120 mins	9.8×10^4
Exposure (5)	200 mins	9.75×10^4

Thus the average value for the molecular weight of the polymer was 1.0×10^5.

2.3.1 IDENTIFICATION OF MONOMER COMPONENTS RESULTING FROM COMPLETE ACID HYDROLYSIS OF THE GALACTOMANNAN:

A small quantity of the polysaccharide (0.6 g) was dissolved in N H_2SO_4 (25 ml) and heated in a series of sealed tubes at 100°C for eight hours. The hydrolysate was removed from the tubes, filtered, neutralised with $BaCO_3$ and deionized by passage through the ion exchange resins IR-45 and IR-120 respectively. This hydrolysate solution was evaporated at 40°C *in vacuo* to a syrup (0.53 g).

The hydrolysate was resolved into its monomer components using a cellulose column (37). A column (3 x 33 cm) was "dry packed" with cellulose powder (Dry Standard Grade) and irrigated in turn with water, (200 ml) absolute ethanol (200 ml), n-butanol (200 ml), and finally with n-butanol half-saturated with water. The syrup was taken in a small amount of dry cellulose powder, added to the top of the column and lightly tamped down. Dry cellulose (one inch) was added to this and tamped down. The column was developed using n-butanol half-saturated with water as irrigant and aliquots (10 ml) were collected using a fraction collector.

The fractions were examined by paper chromatography using Whatman No. 1 paper (Solvent system:- n-butanol : ethanol : water, 3 : 1 : 1), and D-galactopyranose and D-mannopyranose as markers. Only two components were apparent and these corresponded to the D-galactopyranose and D-mannopyranose markers respectively (spray:- aniline - phosphoric acid (83)). The appropriate fractions were combined into two fractions and evaporated <u>in vacuo</u> at 48°C.

<u>Fraction 1</u>. <u>D-mannopyranose</u>

The brownish syrup (246.0 mgs) was dissolved in water (5 ml), treated with charcoal, filtered and evaporated <u>in vacuo</u> at 35°C to give a clear syrup (232.2 mgs): $[\alpha]_D^{20}$ + 15° (water), lit., + 14.5° (12), + 15.2° (19), + 12° (69). D-mannopyranose was characterised as its phenylhydrazone (92). A portion of the syrup (50 mgs) was dissolved in absolute

ethanol, phenylhydrazone added (five drops) and the reaction mixture warmed (70-80°C) for 30 minutes. The crystals which formed when the solution was left overnight were removed by filtration, washed in turn with water, absolute ethanol and diethyl ether, and finally air-dried. The compound had a melting point of 191.5°C, undepressed in a mixed melting point determination with authentic D-mannose phenylhydrazone, lit., 186-188° (19), 197-199° (16), 194-196° (69), 199-200° (92). $[\alpha]_D^{20}$ + 26° (pyridine), lit., + 33° (28), + 32° (16), + 26.3° (92).

Fraction 2. D-galactopyranose

The syrup (216.1 mgs) was dissolved in water (1.0 ml) and methanol (9 ml) was added. On cooling the solution cyrstals separated out. These were removed by filtration, washed with methanol and dried at room temperature. The crystals had melting point 166°C undepressed in a mixed melting point determination with authentic D-galactopyranose: lit., 165° (12), 166-168° (19), 164-166° (69). $[\alpha]_D^{20}$ + 109° → + 79° (water), lit., + 79° (12), + 79.2° (19), + 80° (69). These optical rotation results suggest that the D-galactopyranose crystallised as the α anomer; $[\alpha]_D^{20}$ + 109° initially, changing in the course of several hours to an equilibrium value of + 79° (water).

2.3.2 QUANTITATIVE DETERMINATION OF MONOMER SUGARS PRESENT USING A SLIGHT MODIFICATION OF A METHOD DESCRIBED BY C.M. CURTIS (70)

On Whatman No. 1 paper (48 x 46 cm) a standard solution of D-galactopyranose and D-mannopyranose was spotted every 2.5 cm on a pencil line drawn 9 cm. from the edge such that the chromatogram was developed in a direction against the machine grain. The standard solution was applied with a microburette (20/ul) so that each sugar component was present in varying amounts over a range of 25 to 100/ugms (i.e. 25, 50, 75 and 100/ugms) and each concentration was replicated three times. On the same paper five spots of the unknown (hydrosylate from Section 2.3.1) were made so that the concentration of each component was approximately intermediate on the standard curve (i.e. 30 to 60/ugms). Because the standards were determined simultaneously with the unknowns more accurate results could be obtained and the running of a paper blank was rendered unnecessary. As there was some variation in the standard curves between papers it was necessary to run a standard curve with the unknowns for every paper. Each paper was developed for 36 hours (solvent:- n-butanol : ethanol : water, 3: 1 : 1), air dried, evenly sprayed with aniline-phosphoric acid spray (83) (1 ml aniline in 100 ml n-butanol (wet) and 1 ml phosphoric acid in 100 ml n-butanol (wet) mixed and stood overnight), dried and then heated at 110°C for 10 minutes. The areas of paper containing the components were immediately cut out in such a fashion that

all areas were as equal and as small as possible. Each area was further cut into small pieces and placed in test tubes (15 x 2 cm), in which the compounds were extracted from the paper by shaking the test tubes with acidified ethanol (5 ml) (0.7N HCl in 80 per cent ethanol ($^{v}/v$) was prepared by adding 36 per cent (29 ml) to 95 per cent ethanol (420 ml) and making up to 500 ml with water). After allowing each tube to stand for one hour (with occasional shaking) the optical density was read at 430 $m\mu$ (using a Hilger 810 Biochem. Absorptiometer). The concentration of each sugar in the unknown was determined by reference to the standard curve for D-galactopyranose and D-mannopyranose. The results of eight replications indicated that the molar ratio of D-galactopyranose and D-mannopyranose was 0.98 (or 49 to 51).

T A B L E 4

OPTICAL DENSITY VALUES FOR DIFFERENT CONCENTRATIONS OF D-GALACTOPYRANOSE AND D-MANNOPYRANOSE AFTER REACTION WITH ANILINE-PHOSPHATE SPRAY (FOR CALIBRATING STANDARD CURVES)

Conc. of Standard Solution of D-galactopyranose and D-mannopyranose	Optical Density of D-galactose-aniline Phosphate Derivative	Optical Density of D-mannose-aniline Phosphate Derivative
25 /ugms	0.061	0.059
50 /ugms	0.140	0.136
75 /ugms	0.208	0.201
100 /ugms	0.273	0.263

These results illustrate that changes in optical density with concentration (over the range given) for the aniline-phosphate derivatives of D-galactopyranose and D-mannopyranose obey Beer's law. (See Figure A).

2.4.1 METHYLATION OF GALACTOMANNAN BY METHODS AFTER HAWORTH, KUHN AND PURDIE

A solution of the polysaccharide (4.0 g) in water (50 ml) was added to a 500 ml, three-necked flask equipped with a glass stirrer, separating funnels and a condenser.

Haworth Procedure (41a, 43): To the constantly stirred solution (at room temperature) 30 per cent sodium hydroxide (160 ml) and dimethyl sulphate (64 ml) were added over a period of six hours. Care was taken to ensure that the reaction mixture was always alkaline. Stirring was continued for a further eighteen hours after which further aliquots of 30 per cent sodium hydroxide (160 ml) and dimethyl sulphate (64 ml) were added to the reaction mixture as above, but the temperature was raised to 50°C. The solution was then transferred to a litre beaker which was heated on a sand bath. On stirring, a gum-like precipitate (partly methylated polysaccharide) formed on the surface and was removed with a spatula. The gum-like syrup was dissolved in boiling water, dialysed against tap water for twenty-four hours and then evaporated at 40°C *in vacuo* to a syrup (3.6 g).

Kuhn Procedure (49, 50, 51, 52, 53, 54): The partially methylated polysaccharide (3.6 g) was dissolved in dimethyl formamide (100 ml) and added to the same apparatus as used in the Haworth method (at 30°C), after which methyl iodide (45 ml) and barium hydroxide (45 g) were added over a period of three hours. Stirring was continued for twelve hours by which time the partially-methylated polysaccharide had precipitated. Chloroform (100 ml) was added and stirring was continued for another twelve hours. The supernatant, was decanted from the barium hydroxide and the latter was extracted twice with chloroform (100 ml). These

chloroform extracts were added to the above supernatant and this was washed with equal volumes of water (6x). The chloroform solution was centrifuged (2500 r.p.m.) and the water layer removed with a pipette and then the extract was further dried by addition of anhydrous sodium sulphate. The filtered solution was evaporated in vacuo at 30°C to a syrup (3.5 g).

Another Kuhn procedure (52, 53, 54) was carried out on this syrup. The syrup was dissolved in dimethyl formamide (100 ml) and to the constantly-stirred solution (at 45°C), over a period of twenty four hours was added methyl iodide (40 ml) and silver oxide (30 g). Refluxing and stirring was continued for a further twelve hours after which the reaction mixture was centrifuged (2500 r.p.m.) and the supernatant (130 ml) was retained. The precipitate was washed with dimethyl formamide (50 ml) and chloroform (50 ml) and these two fractions were added to the above supernatant. Chloroform (350 ml) was added and the filtered solution was shaken with potassium cyanide (10 g) to remove silver ions. The filtered solution (520 ml) was washed with equal volumes of distilled water (8x) dried as described above and evaporated in vacuo to a syrup (3.4 g) (Found : OCH_3, 42.6%; Calc. for $C_{18}H_{32}O_{10}$ (6 : OCH_3 groups) OCH_3, 45.5%).

Purdies Procedure (48): The partially-methylated galactomannan from above was dissolved in methyl iodide (50 ml) and the solution was refluxed with silver oxide (5 g) which was added in six portions

over a period of twelve hours. The mixture was refluxed for a further twelve hours, the excess methyl iodide was distilled off in vacuo, and the residue was extracted three times with acetone (80 ml). The filtered solution, which yielded a light-brown glassy material on concentration, was subjected to a further Purdie's procedure. The final syrup was dissolved in chloroform (300 ml), shaken with potassium cyanide (10 g), filtered, washed with equal volumes of distilled water (6x) and reduced to a syrup in vacuo at 30°C (3.0 g). $[\alpha]_D^{20}$ + 71° (Chloroform). (Found: OCH_3, 44.4% Calc. for $C_{18}H_{32}O_{10}$ (6;OCH_3 groups). OCH_3, 45.5%).

2.4.2 HYDROLYSIS OF METHYLATED GALACTOMANNAN (71)

A portion of the methylated galactomannan (1.0 g) was dissolved in 3 per cent methanolic hydrochloric acid and refluxed (6 hours). After neutralisation with silver carbonate the solution was reduced to a syrup in vacuo (30°C). This was dissolved in N H_2SO_4 (40 ml) and heated in a series of sealed tubes at 100°C for eight hours. The hydrolysate was removed from the tubes, neutralised with silver carbonate, filtered, passed through the ion exchanges resins IR-45 and IR-120 respectively and finally the solution was evaporated in vacuo (30°C) to a syrup (0.875 g). A portion of this syrup (0.5 g) was separated on a cellulose column (33 x 3 cm) (see section 2.3.1 p. 39) (Irrigant:- methyl ethyl ketone : water, 89 : 11, weight/weight). The

TABLE 5

HYDROLYSIS PRODUCTS FROM METHYLATED GALACTOMANNAN

Fraction Number	Tube Nos	Weight (mgs)	Moles	MOLAR RATIO		SOLVENT (a)		SOLVENT (b)		Component
				Alkaline Hypoiodate	Gas-Liquid	R_F	R_G	R_F	R_G	
1	(18-25)	234.2	0.99	49	16.1	0.76	0.88	0.59	0.75	2, 3, 4, 6-tetra - O - methyl - D-galactopyranose
2	(28-32)	37.6	0.017		1.0	0.67	0.78	0.39	0.49	2, 3, 6-tri - O - methyl - D-mannopyranose
3	(36-44)	17.6								2, 3, 6-tri - O - methyl - D-mannopyranose plus tri - methyl - galactose (?)
4	(48-72)	217.6	1.05	51(plus Fraction 2)	7.7	0.42	0.57	0.14	0.18	2, 3-di - O - methyl - D-mannopyranose

Solvents

(a) n-butanol : ethanol : water, 4 : 1 : 5 (upper phase)

(b) methyl ethyl ketone : water, 89 : 11 ($^{w}/_{w}$)

R_G is relative to 2, 3, 4, 6 tetra - O - methyl - D-glucopyranose.

fractions were examined by paper chromatography on Whatman No. 1 paper (Solvents:- n-butanol : ethanol : water, 4 : 1 : 5 (upper phase) (Solvent A) and methyl ethyl ketone : water, 89 : 11, weight/weight (Solvent B)) using saturated aniline - oxalic acid as spray (72). Four spots were identified, two major, one minor and one as a trace (see Figure 8). On the basis of this chromatographic survey the appropriate fractions were combined into fractions and evaporated to syrups in vacuo at 35°C.

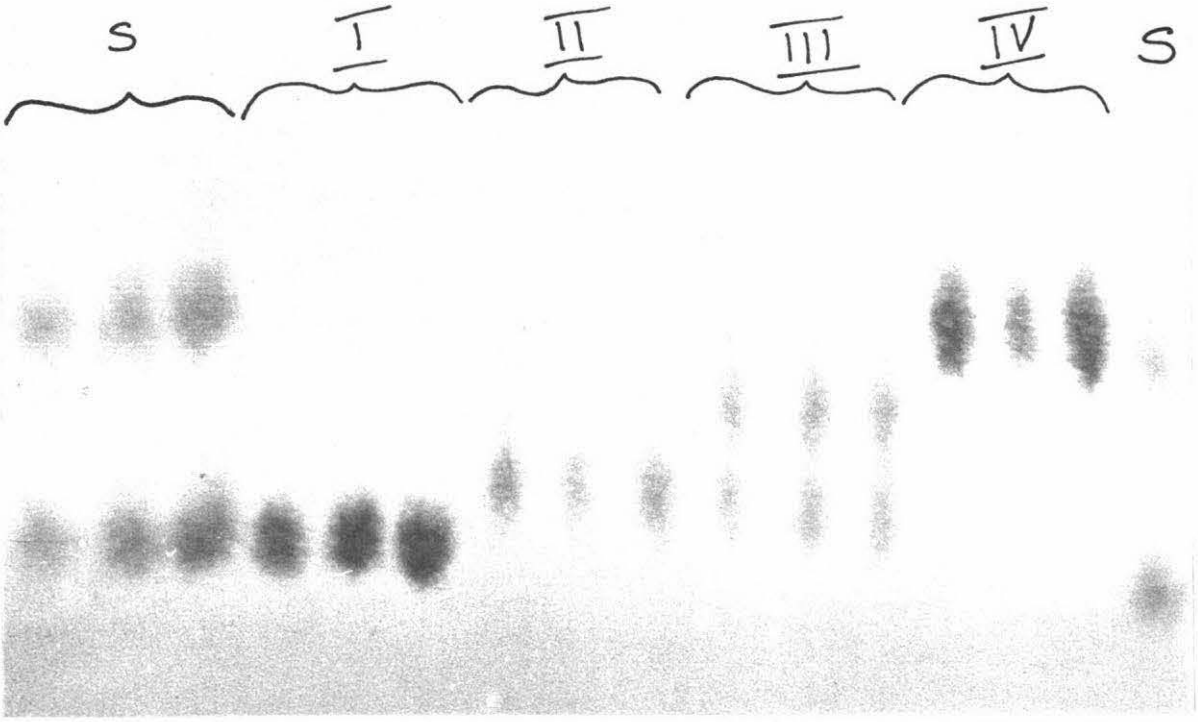
Fraction 1. Identification of 2, 3, 4, 6 - tetra - O - methyl - D-galactopyranose:

This component (0.234 mgs) had $[\alpha]_D^{20}$ + 107 (water), lit., for 2, 3, 4, 6 - tetra - O - methyl - D-galactopyranose, $[\alpha]_D$ + 110° (27), + 109.5 (73), + 112 (12), and + 106° (16). Paper chromatography showed R_F values of 0.76 (Solvent A) and 0.59 (Solvent B); lit., 0.86 (16), 0.69 (94) (Solvent A) and 0.68 (98), 0.69 (17) (Solvent B) and R_S values of 0.88 (Solvent A) and 0.75 (Solvent B), lit., 0.88 (80) (Solvent A). (See Table 5).

A portion of the syrup (15 mgs) was demethylated according to the method of Hough et al., (61) showing the parent sugar to be D-galactose. The syrup was dissolved in hydrobromic acid (48 per cent weight for weight) (2 ml) in a test tube (5 ml). After the sealed tube was heated for 20 to 30 minutes at 100°C the solution was removed, diluted to 10 ml with water and neutralised with

FIGURE 8

Unidimensional Chromatograph of Hydrolysis Products of Methylated Polysaccharide.



S. Sample of hydrolysis products of methylated polysaccharide.

I. 2, 3, 4, 6 - tetra - O - methyl - D-galactopyranose.

II. 2, 3, 6 - tri - O - methyl - D-mannopyranose.

III. 2, 3, 6 - tri - O - methyl - D-mannopyranose and tri - methyl - galactopyranose (?)

IV. 2, 3 - di - O - methyl - D-mannopyranose.

(Solvent: n-butanol : ethanol : water, 4 : 1 : 5, (upper phase)).

silver carbonate. To the filtered solution was added a small quantity of decolourising charcoal. The solution was filtered and the last traces of silver ions were removed by treatment with hydrogen sulphide. Finally this filtered solution was evaporated in vacuo to a small volume. Paper chromatography (Solvent:- n-butanol : ethanol : water, 4 : 1 : 5 (upper phase)) using D-mannose and D-galactose as markers showed the presence of D-galactose among a number of minor, presumably partially methylated products (Spray: aniline - phosphoric acid (83)).

Treatment of the methylated sugar with aniline yielded crystals of 2, 3, 4, 6 - tetra - O - methyl - N - phenylglycosylamine (74). A sample (50 mgs) was dissolved in absolute ethanol (5 ml), aniline (10 drops) was added and the mixture was refluxed for three hours and then cooled at - 10°C (12 hours). The white, flaky crystals which separated were filtered, washed with ethanol and air-dried. The mother liquor was concentrated to a small volume, cooled at - 10°C (12 hours) and the crystals were collected as above. The crystals (32.0 mgs) were compounded and recrystallised from acetone/ethanol. Melting point: 192-193°C, lit., 187-188° (27), 190° (12), 188° (21), 192-193° (19), 192° (18), 194° (14), 195° (15). The optical rotation $[\alpha]_D^{20}$ - 83° to -85° (acetone), lit., - 79° to + 39° (21), - 80° (75), - 84° (76).

(Found: C, 61.6; H, 8.2; N, 4.4; OCH_3, 39.9%. Calc. for $C_{12}H_{25}O_5N$: C, 61.7; H, 8.1; N, 4.5; OCH_3, 39.9%).

Fraction 2. Identification of 2, 3, 6 - tri - O - methyl - D-mannopyranose:

This component (37.0 mgs) when examined by paper chromatography had R_F values 0.67 (Solvent A) and 0.39 (Solvent B), lit., 0.80 (16) (Solvent A) and 0.48 (16), 0.51 (17), 0.50 (98) (Solvent B) and R_G values of 0.76 (Solvent A) and 0.49 (Solvent B), lit., 0.81 (80) (Solvent A). (See Table 5, p47a).

The methylated sugar was converted to 2, 3, 6 - tri - O - methyl - D-mannopyranose 1, 4 bis - p - nitrobenzoate (56,93). A sample of methylated sugar (37.0 mgs) was dissolved in dry pyridine (6.0 ml) and treated with p-nitrobenzoyl chloride (200 mgs). The mixture was heated (70°-80°C) for two hours and then allowed to stand for eight hours at room temperature. A saturated solution of sodium bicarbonate (to destroy the excess of p-nitrobenzoyl chloride) was added until no further effervescence occurred, and the resulting solution was diluted to 50 ml with water. The product was extracted with chloroform (3x, 25 ml portions), dried with anhydrous sodium sulphate and the filtered solution was evaporated in vacuo. (30°C) to a small volume. Addition of petroleum ether (20 ml) and cooling (- 10°C) induced the crystallization of the 2, 3, 6 - tri - O - methyl - D-mannopyranose 1, 4 - bis - p - nitrobenzoate. The crystals were collected by filtration, washed with methanol and recrystallized from acetone/methanol (10 mgs). Melting point 185°, lit., 187-188° (56), 189-190° (27), 191° (78).

Fraction 3. Using paper chromatography (see Figure 8) two components were observed, one corresponding to 2, 3, 6 - tri - O - methyl - D-mannopyranose and the other suggested to be a tri - O - methyl - D-galactopyranose derivative.

Fraction 4. Identification of 2, 3 - di - O - methyl - D-mannopyranose:

This component had $[\alpha]_D^{20}$ - 16° to - 17° (water), lit., - 16° (79), - 17° (16). Paper chromatography showed R_F values 0.42 (Solvent A) and 0.14 (Solvent B), lit., 0.25 (17), 0.22 (98), 0.20 (16), (Solvent B) and R_G values of 0.57 (Solvent A) and 0.18 (Solvent B), lit., 0.54 (80) (Solvent A). (See Table 5, p47a).

A fraction of the syrup (66.0 mgs) was converted to 2, 3 - di - O - methyl - D-mannopyranose 1, 4, 6 - tri - p - nitrobenzoate as described for Fraction 2 above. However, the crystallization was carried out using methanol and not petroleum ether. The crystals collected (66.0 mgs) had a melting point 192-193° lit., 194° (1) and had $[\alpha]_D^{20}$ + 66° (chloroform), lit., + 66° (1).

(Found: C, 52.8; H, 4.0; N, 6.3; OCH_3, 8.6%. Calc. for $C_{29}H_{25}O_{15}N_3$. C, 53.0; H, 3.8; N, 6.4; OCH_3, 8.9%).

2.4.3 QUANTITATIVE DETERMINATION OF THE METHYL SUGARS:

The methyl sugars were quantitatively determined according to the method of Hirst et al., (80). A solution of the methylated hydrosylate (from Section 2.4.2, p. 47) was made such that its concentration was 30.0 mgs per ml of methanol.

A pencil line was drawn 9 cm from the edge of Whatman No. 3MM filter paper (48 x 46) such that the paper could be run against the machine grain. Two further pencil lines were made 7 cm from the edge to indicate the marker strips. Two margins of 6 cms were allowed on the 34 cm centre strip leaving 22 cm on the starting line. Along the latter 1 ml of the above solution was applied as a streak. The paper was developed for 14 hours, (Solvent:- n-butanol : ethanol : water, 4 : 1 : 5 (upper phase)), air dried, the marker strips cut away, sprayed with aniline - oxalic spray and finally heated at 100°C (5 minutes). Two major spots were recognised with R_F and R_G values corresponding to 2, 3, 4, 6 - tetra - O - methyl - D-galactopyranose and 2, 3 - di - O - methyl - D-mannopyranose. The minor third component, 2, 3, 6 - tri - O - methyl - D-mannopyranose was not obvious on the marker strips (even at high concentrations) but from its R_F and R_G values (see Fraction 2, p. 50) it was assumed to run close to 2, 3, 4, 6 - tetra - O - methyl - D-galactopyranose.

Appropriate strips and a blank were cut out and eluted (water) until 15 ml were collected. Each fraction (three) was

filtered through glass wool and made up to 20 ml (water). To each sample (5 ml containing approximately 3.0 mg of methyl sugar) was added 0.1 N Iodine (1 ml) followed by buffer (2 ml) in 15 x 2 boiling tubes with close fitting tops which were quickly closed with a stopper moistened with 10 per cent potassium iodide.

(Buffer: A solution containing 0.2 M-sodium hydrogen carbonate and 0.2 M-sodium carbonate (pH 10.6)). The tubes were kept in a dark place for 2-2½ hours, when the stopper was washed with water and the volume was then made up to 25 ml (water). The solution was acidified with 2N H_2SO_4 (2 ml) to liberate iodine and the excess iodine was titrated with 0.01N sodium thiosulphate using starch solution as end-point indicator.

The results were expressed as a ratio of 0.01 N Iodine (ml) consumed per sample of methyl sugar (assuming one mole of iodine was consumed per one mole of methyl sugar). The molar ratio of 2, 3 - di - O - methyl - D-mannopyranose to 2, 3, 4, 6 - tetra - O - methyl - D-galactopyranose (plus 2, 3, 6 - tri - O - methyl - D-mannopyranose) was 0.98 : 1. This result was an average from four determinations of 0.92, 0.98, 0.99 and 0.99 : 1 respectively.

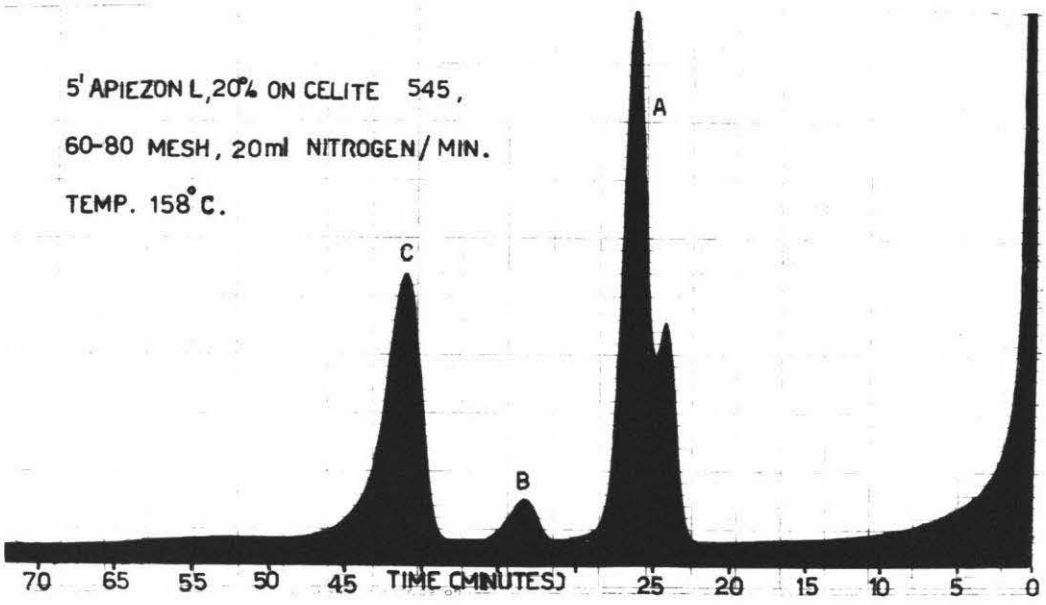
2.4.4 QUANTITATIVE AND QUALITATIVE DETERMINATION OF METHYL ETHER MONOMERS BY GAS-LIQUID CHROMATOGRAPHY:

The instrument used was a Model A 600 B. "Hy Fi" chromatograph with H. Flame Ionisation Detector. The column used was Apiezon. L. 20% on Celite 545 and packed into a 5' spiral column (81).

Samples of methyl 2, 3, 4, 6 - tetra - O - methyl - D-galactopyranose, methyl 2, 3 - di - O - methyl - D-mannopyranose (Fractions one and four respectively. (See Section 2.4.2, p. 47)) and methyl hydrolysate (see Section 2.4.2, p.47) were prepared. Each sample (50 mgs) was refluxed with 3 per cent methanolic hydrochloric acid (6 hours), cooled, neutralised (silver carbonate) and the filtered solution evaporated in vacuo (30°C) to a small volume. A typical chromatogram (see Figure 9) was interpreted as follows: Peaks A and C were identified as methyl 2, 3, 4, 6 - tetra - O - methyl - D-galactopyranose (α and β forms) and methyl 2, 3 - di - O - methyl - D-mannopyranose respectively by running the methyl ethyl hydrolysate with methylated samples corresponding to Fractions one and two (see Section 2.4.2, p.47). Figures 10 and 11 illustrate the results of this chromatography. The peak B was tentatively suggested to represent methyl 2, 3, 6 - tri - O - methyl - D-mannopyranose. By the measuring the area under the curves by the method of triangulation (height of the peak times width at half the height) the molar ratio of methyl 2, 3 - di - O - methyl - D-mannopyranose: methyl 2, 3, 6 - tri - O - methyl -

FIGURE 9

Gas-Liquid Chromatograph of Methyl Glycosides of Hydrolysis Products from the Methylated Galactomannan.



A. α and β methyl 2, 3, 4, 6 - tetra - O - methyl - D-galactopyranose.

B. Methyl 2, 3, 6 - tri - O - methyl - $\alpha\beta$ - D-mannopyranose (tentatively identified).

C. Methyl 2, 3 - di - O - methyl - $\alpha\beta$ - D-mannopyranose.

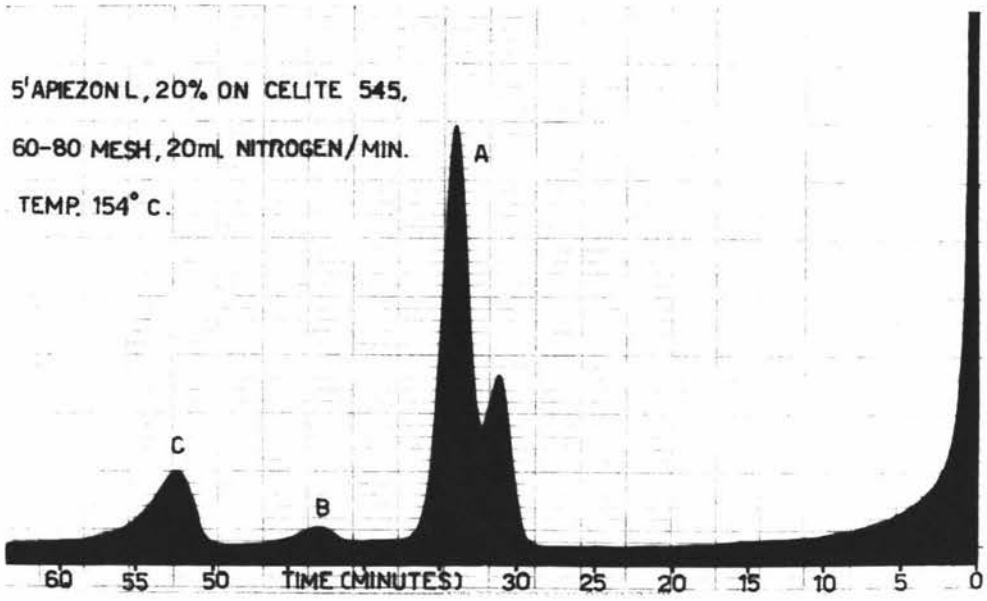
FIGURE 10

Gas-Liquid Chromatograph of Methyl Glycosides.

(1) Hydrolysis Products from Methylated Galactomannan plus methyl 2, 3, 4, 6 - tetra - O - methyl - $\alpha\beta$ - D-galactopyranose.

(2) Methyl 2, 3, 4, 6 - tetra - O - methyl - α/β - D-galactopyranose.

(1)



(2)

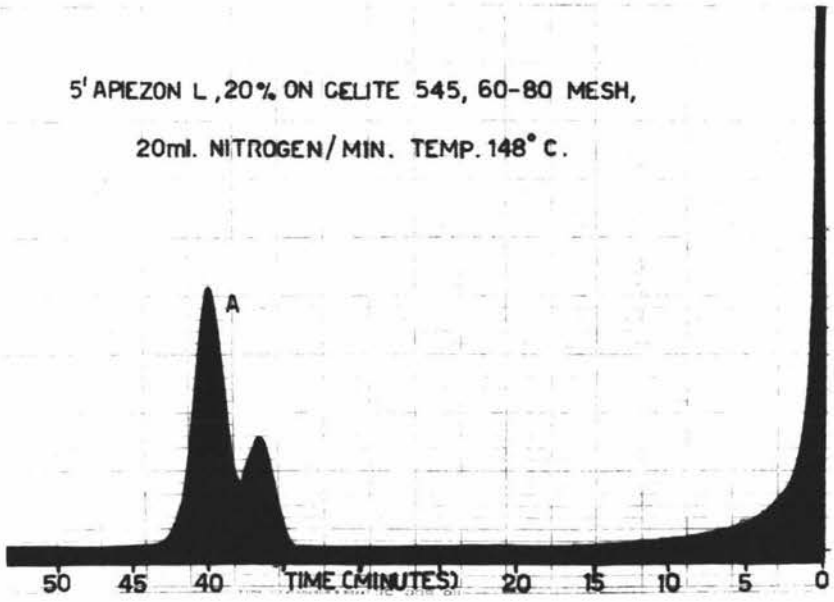


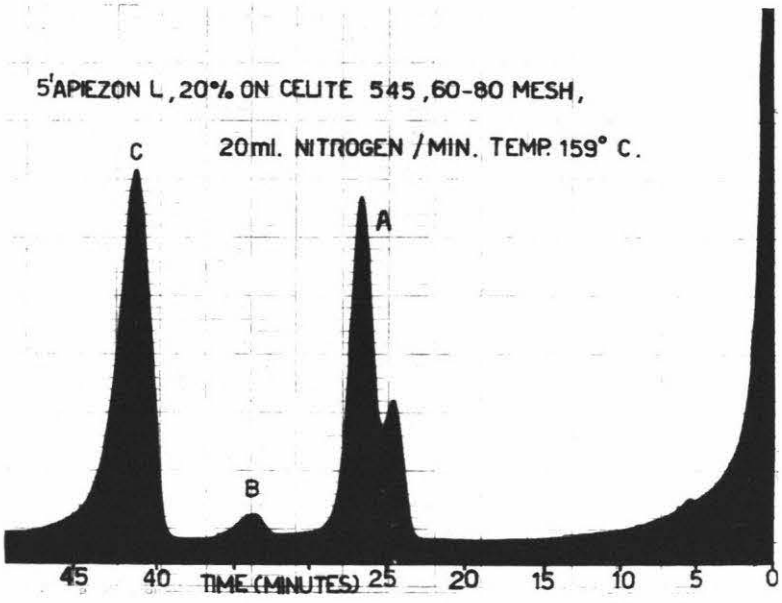
FIGURE 11

Gas-Liquid Chromatograph of Methyl Glycosides.

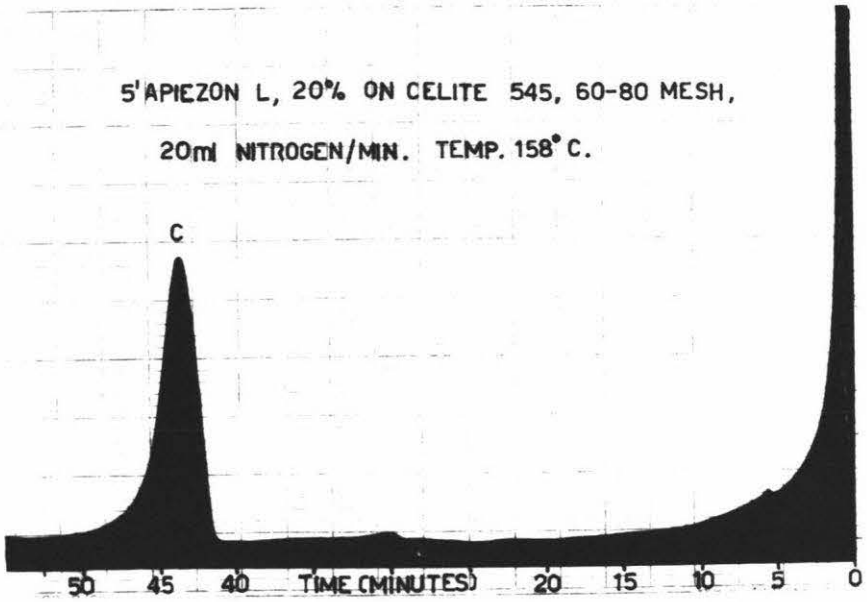
(1) Hydrolysis Products from Methylated Galactomannan plus methyl 2, 3 - di - O - methyl - $\alpha\beta$ - D-mannopyranose.

(2) Methyl 2, 3 - di - O - methyl - $\alpha\beta$ - D-mannopyranose.

(1)



(2)



D-mannopyranose (?) : methyl 2, 3, 4, 6 - tetra - O - methyl - D-galactopyranose was 7.7 : 1.0 : 16.1.

2.5.1 PERIODATE OXIDATION OF GALACTOMANNAN:

A sample of the polysaccharide (250 mgs) was dissolved in water (50 ml) and made up to 200 ml with a sodium metaperiodate solution so that the final molarity was 0.048. At the same time a blank solution was prepared as above, without the polysaccharide. The reaction was carried out in the dark at 20°C.

The determination of periodate consumption was made by the usual method due to Fleury and Lange (84) as described in (85).

To a sample (5 ml) withdrawn at intervals (see Table 6) were added aliquots of saturated $NaHCO_3$ (20 ml), excess 0.05 $NaAs_2O_3$ (20 ml), 20% KI (2 ml) and starch indicator solution (1 ml). A blank was run at the same time. The mixture was allowed to stand for one hour before the excess sodium arsenite was titrated with 0.01N Iodine.

The liberated formic acid was determined directly by titration using standard sodium hydroxide (86). To an aliquot (5 ml) of the periodate oxidation reaction mixture was added ethylene glycol (2 ml) to destroy the excess periodate. Phenolphthalein (2 drops) was added and the formic acid was then titrated with 0.01N NaOH (CO_2 free). A blank was run at the same time. The amount of periodate consumed and formic acid released was expressed in terms of moles per anhydrohexose unit. (See Table 6).

TABLE 6

PERIODATE OXIDATION OF GALACTOMANNAN

Time (hours)	1	4	12	27	51	75	99	123
Periodate Consumed per Hexose Unit	0.78	0.89	0.93	0.99	1.06	1.15	1.20	1.14
Formic Acid Released per Hexose Unit	0.27	0.36	0.43	0.47	0.48	0.49	0.49	0.49

Thus the consumption of periodate reached a maximum of 1.20 moles per hexose unit after 99 hours and the release of formic acid reached a constant value of 0.49 moles per hexose unit after 75 hours.

2.5.2. REDUCTION OF POLYALDEHYDE AND SUBSEQUENT MINERAL ACID HYDROLYSIS (95, 96)

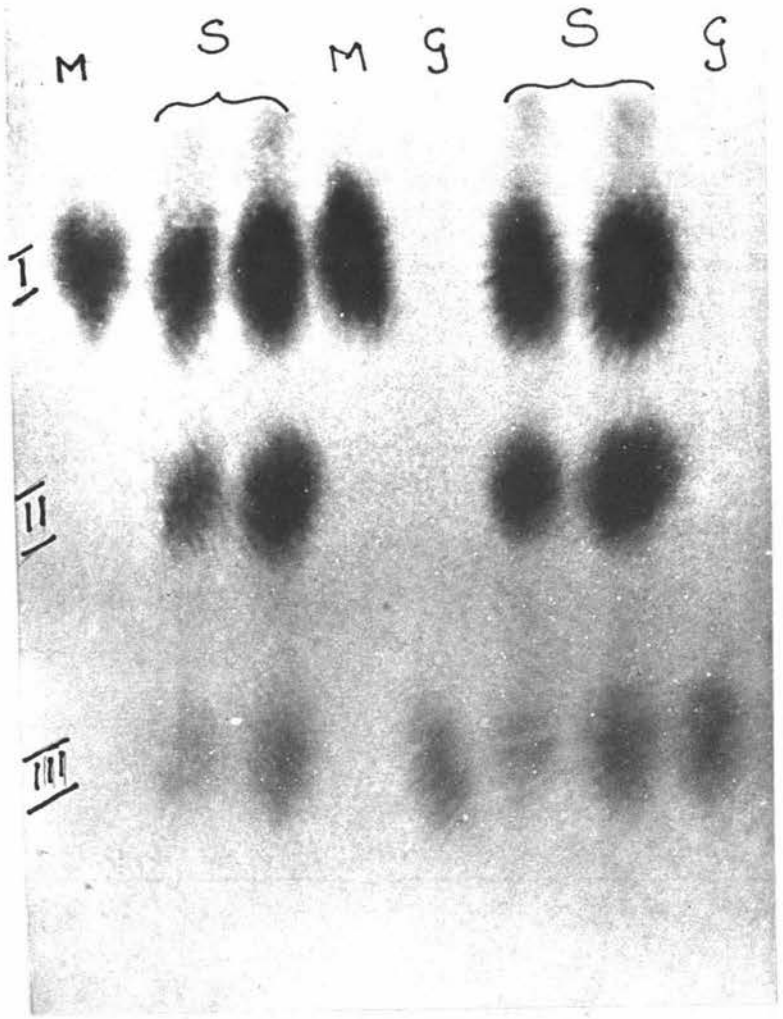
The polysaccharide (400 mgs) was dissolved in water (50 ml) and oxidised with sodium metaperiodate (final molarity 0.07M). After the reaction mixture (200 ml) had stood in a cool dark place for ten days the excess periodate was destroyed by the addition of ethylene glycol (20 ml) and dialysed against running tap water for 24 hours. This step removed ethylene glycol, periodate breakdown products, formic acid, inorganic ions, etc. To the dialyzed solution (240 ml) containing the oxidised polysaccharide (polyaldehyde) was added sodium borohydride (0.5 g) over a period of nine hours. Glacial acetic acid was then added to decompose the excess

reductant and the acidic solution was dialysed against tap water (24 hours). The solution was evaporated *in vacuo* (35°C) to a syrup, taken up in IN H_2SO_4 (15 ml) and hydrolysed in a series of sealed tubes at 100°C for twelve hours. The hydrolysate was removed from the tubes, neutralised with barium carbonate and deionised by passing it through the ion exchange resins IR-45 and IR-120 respectively. The effluent was finally evaporated *in vacuo* (35°C) to a small volume. This was examined by paper chromatography on Whatman No. 1 filter paper (Solvent: n-butanol : ethanol : water, 4 : 1 : 5 (upper phase)). The air-dried chromatogram was sprayed with Tollens reagent (40) (or ammonical silver nitrate). (To 0.1N $AgNO_3$ (10 ml) was added concentrated NH_4OH (10 drops) and 10% NaOH (5 ml).) On maximum colour development (10-15 minutes) the paper was sprayed with $Na_2S_2O_3$ solution which eliminated background colour (see Figure 12). Of the three distinct black spots which were present, two corresponded to D-mannopyranose and glycerol markers. The third spot was assumed to be D-erythritol but no marker was available.

In order to identify these components the hydrolysate was resolved by paper chromatography as described in Section 2.4.3 (p52) (Solvent: n-butanol : ethanol : water, 4 : 1 : 5 (upper phase)). The appropriate strips were eluted with water and the filtered eluent was evaporated *in vacuo* (35°C) to a syrup. Each fraction ran as a single spot when examined by paper chromatography. (Tollen reagent spray).

FIGURE 12

Unidimensional Chromatogram of Hydrolysis Products of Polyalcohol and Markers.



S Sample of hydrolysis products of the polyalcohol.

M D-mannopyranose marker.

G Glycerol marker

I D-mannopyranose

II D-erythritol

III Glycerol

(Solvent): n-butanol : ethanol : water, 4 : 1 : 5 (upper phase).

Fraction 1. Identification of D-mannopyranose:

(12.4 mgs) D-mannose phenylhydrazone (5.1 mgs) was prepared as described in Section 2.3.1. (see p39). The compound had a melting point 188° undepressed in a mixed melting point determination with authentic D-mannose phenylhydrazone.

Fraction 2. Identification of D-erythritol:

(10.5 mgs) This fraction was converted to erythritol tetra-p-nitrobenzoate (56, 93). The syrup was dissolved in dry pyridine (3 ml) and p-nitrobenzoyl chloride (200 mgs) added. After heating for two hours at 70° - 80°C and standing for eight hours, saturated sodium bicarbonate (15 ml) was added to destroy the excess p-nitrobenzoyl chloride. The precipitate was collected by filtration, washed with water, recrystallised from acetone/methanol and chloroform/methanol (18.0 mgs). Melting point 247 - 249°C, lit., 249 - 252° (87), 252 - 254° (88).

(Found: C, 53.6; H, 3.5; N, 8.1; Calc. for $C_{30}H_{21}O_{16}H_4$: C, 53.6; H, 3.1; N, 7.8).

D-erythritol was also identified as tetra - O - tosyl - erythritol (89). To the syrup (12.6 mgs) was added dry pyridine (2 ml) and p-toluene sulphonyl chloride (0.780 g) and the mixture was stored at room temperature for 24 hours. The resulting solution was poured into distilled water (10 ml) and the crystals were collected by filtration, washed with water, with ethanol,

and then dried *in vide*. The crystals (17.0 mgs) were recrystallised from acetone/ethanol and had a melting point 164°C, lit., 165 - 166° (89).

Fraction 3. Identification of glycerol:

(13.6 mgs) This fraction was converted to glycerol tri - O - p-nitrobenzoate as described for Fraction 2 above. The crystals had a melting point 191° undepressed in a mixed melting point determination, lit., 194 - 196° (87), 188 - 190° (90), 189 - 191° (91), 189 - 192° (88)

(Found: C, 53.0; H, 3.5; N, 7.8. Calc. for $C_{24}H_{17}O_{12}N_3$: C, 53.4; H, 3.2; N, 7.8).

2.5.3 GAS LIQUID CHROMATOGRAPHY OF ACETYLATED DERIVATIVES:

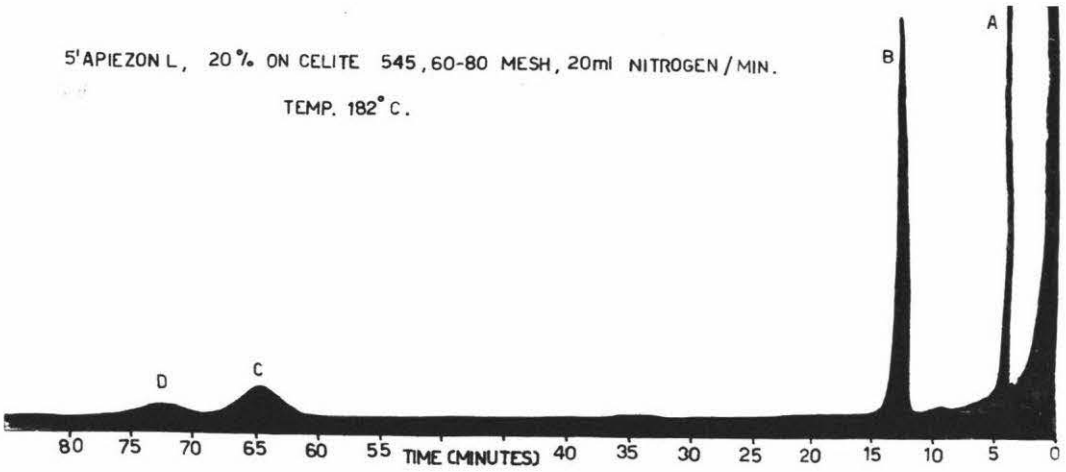
The column was the same as used in Section 2.4.4. (see p54). Before examining the hydrolysate or a sample they were acetylated in the usual way (5). Samples (the hydrolysate, D-mannopyranose, D-galactopyranose, D-glycerol and D-mannitol) were taken up in equal volumes (5 ml) of acetic anhydride and dry pyridine and allowed to stand at room temperature (12 hours) with occasional shaking. The reaction mixture of the hydrolysate was examined directly on the gas liquid chromatograph but the results were inconclusive as the solvent peak masked the first component, glyceryl triacetate. To the reaction mixture was added an equal volume of chloroform and the whole was washed (3x) with equal volumes of

water. The chloroform solution was dried over anhydrous Na_2SO_4 and concentrated to a small volume in vacuo (35°C). It was assumed that the washing with water resulted in the removal of acetic anhydride and pyridine only.

A typical chromatogram (see Figure 13)was interpreted as follows: Peak A was identified as tri - O - acetyl - glycerol by running a mixture of the acetylated hydrolysate with authentic tri - O - acetyl - glycerol (see Figure 14). In the same manner peak C was identified as penta - O - acetyl - D-mannopyranose and penta - O - acetyl - D-galactopyranose. Using this column authentic penta - O - acetyl - D-mannopyranose and penta - O - acetyl - D-galactopyranose were not differentiated. As only D-mannopyranose was identified by paper chromatography and as its crystalline derivative D-mannose phenylhydrazone (see Section 2.5.2. p 56) it is probable that this peak represents penta - O - acetyl - D-mannopyranose and not penta - O - acetyl - D-galactopyranose. Peak B was not identified but from its position and the identification of D-erythritol as the crystalline derivatives, D-erythritol - tetra - O - p - nitrobenzoate and tetra - O - tosyl - erythritol, it is probably that peak B could represent tetra - O - acetyl - D-erythritol. The compound giving rise to peak D was not identified.

FIGURE 13

Gas-Liquid Chromatograph of Polyalcohol Acetate Products from Galactomannan Polyalcohol Hydrolysate.



A. Tri - O - acetyl - glycerol.

B. Tetra - O - acetyl - D - erythritol.

C. Penta - O - acetyl - D - mannopyranose.

D. Unknown.

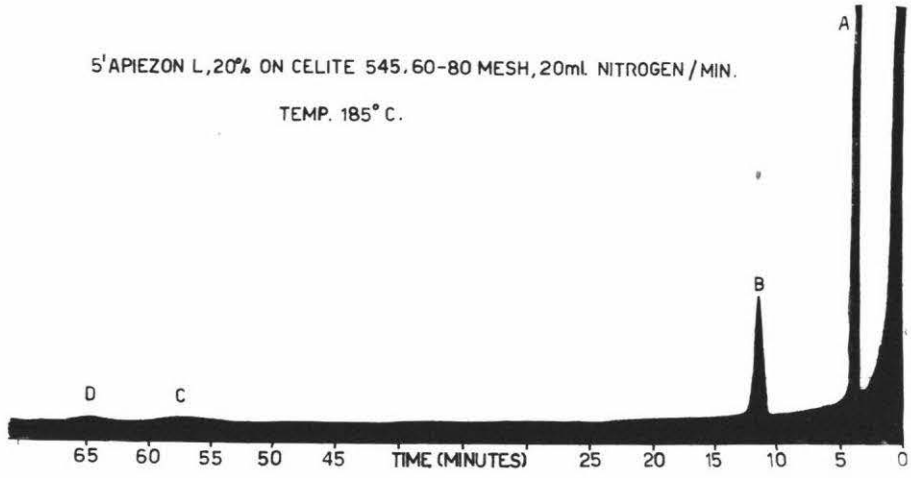
FIGURE 14

Gas-Liquid Chromatograph of Polyalcohol Acetates from Galactomannan Polyalcohol Hydrolysate.

(1) Polyalcohol Acetate Products from Galactomannan Polyalcohol Hydrolysate plus Tri - O - acetyl - glycerol.

(2) Tri - O - acetyl - glycerol.

(1)



(2)

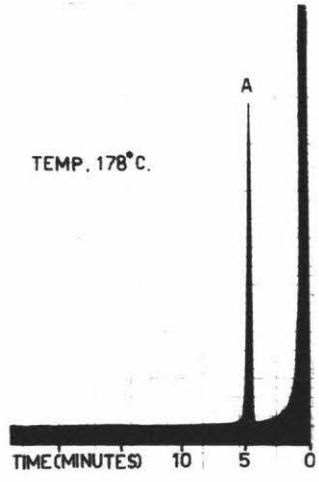


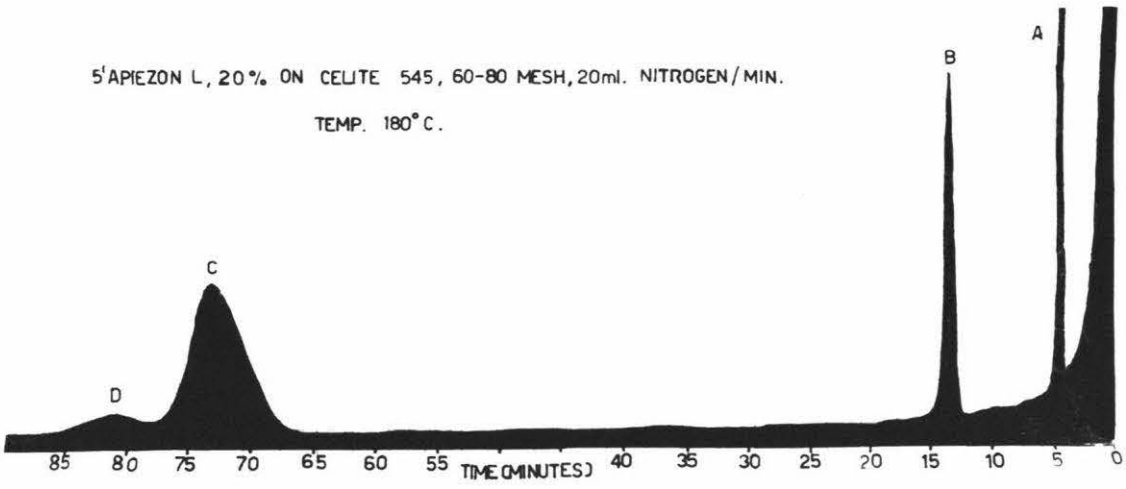
FIGURE 15

Gas-Liquid Chromatograph of Polyalcohol Acetates from Galactomannan Polyalcohol Hydrolysate.

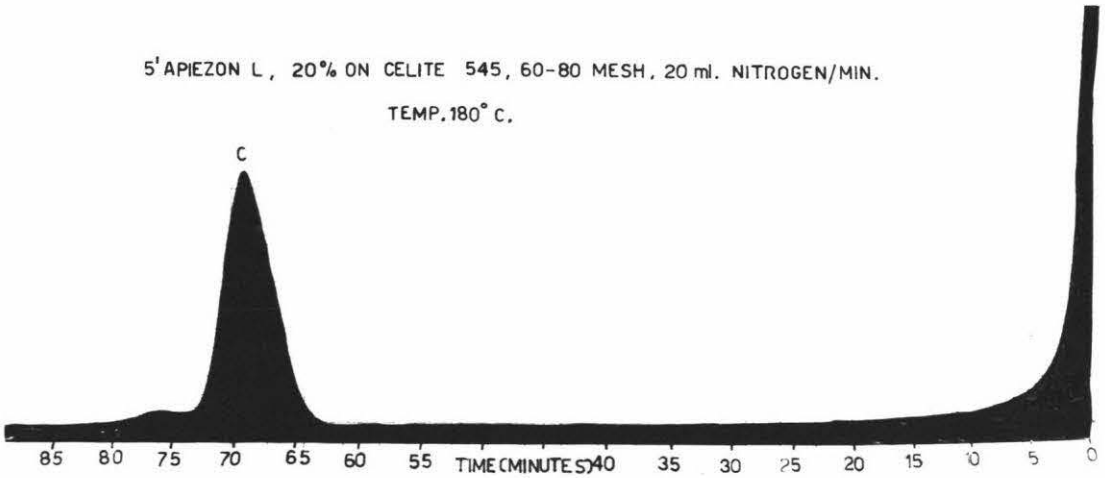
(1) Polyalcohol Acetate Products from Galactomannan Polyalcohol Hydrolysate plus Penta - O - acetyl - D-mannopyranose.

(2) Penta - O - acetyl - D-mannopyranose.

(1)



(2)



2.6.1 PARTIAL HYDROLYSIS OF THE GALACTOMANNAN IN DILUTE SULPHURIC ACID (0.02N):

A sample of the polysaccharide (5.0 g) was dissolved in 0.02 N H_2SO_4 (500 ml) and hydrolysed (6 hours) at 100°C. The hot solution was filtered through glass wool into 1 - 1¼ volumes of 95% ethanol and the precipitate was collected by centrifugation (2500 r.p.m.). A sample of the precipitate (0.20 g) was washed firstly in 95% ethanol and then with diethyl ether, taken up in water and freeze dried. The remaining precipitated polysaccharide was taken up in 0.02 N H_2SO_4 (500 ml) and hydrolysed as above. Fractions were collected in this way until no further precipitation occurred on adding the hydrolysate to 95% ethanol (30 hours). The supernatant was collected every six hours, evaporated in vacuo (35°C) to remove the ethanol, neutralised with barium carbonate and the filtered solution was evaporated in vacuo (35°C) to a small volume. Each supernatant fraction (six) was examined by paper chromatography using Whatman No. 1 filter paper. (Solvents: (a) n-butanol : ethanol : water, 4: 1 : 5 (upper phase); and (b) ethyl acetate : pyridine : water, 2 : 1 : 2). The paper developed in solvent (a) (36 hours) was air-dried, and sprayed with aniline - phosphoric acid. (See Section 2.3.2. p42) D-galactopyranose was present in large amounts with only traces of D-mannopyranose. The paper, developed in solvent (b) (24 hours), was air-dried, and sprayed with Tollens reagent (see Section 2.5.2. p56) permitting identification of D-galactopyranose.

The fractions of precipitate collected were anlysed for their optical rotations and molar ratios of D-galactopyranose to D-mannopyranose (see Section 2.3.2, p42).

TABLE 7

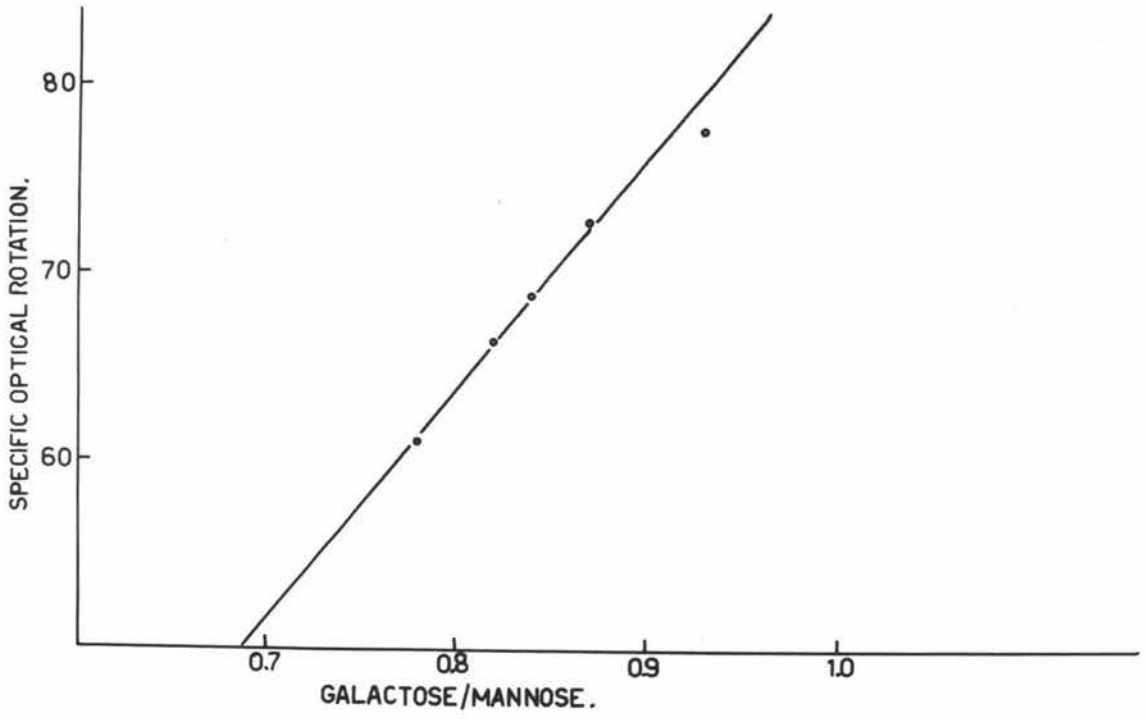
VARIATION OF SPECIFIC OPTICAL ROTATION WITH MOLAR RATIO OF D-GALACTOPYRANOSE AND D-MANNOPYRANOSE

Fraction Number	Hours of Hydrolysis	Specific Opt. Rotation $[\alpha]_D^{20}$ (H_2O)	D-galactose to D-mannose Ratio
1	6	77.3	0.93
2	12	72.5	0.87
3	18	68.7	0.84
4	24	66.3	0.82
5	30	61.1	0.78

The graph (see Figure 16) indicates a linear relationship between specific optical rotation and the molar ratio of D-galactopyranose and D-mannopyranose.

FIGURE 16

The Relationship Between the Optical Rotation and D-galactopyranose to D-mannopyranose Ratio of the Galactomannan in a Partial Acid Hydrolysis Experiment.

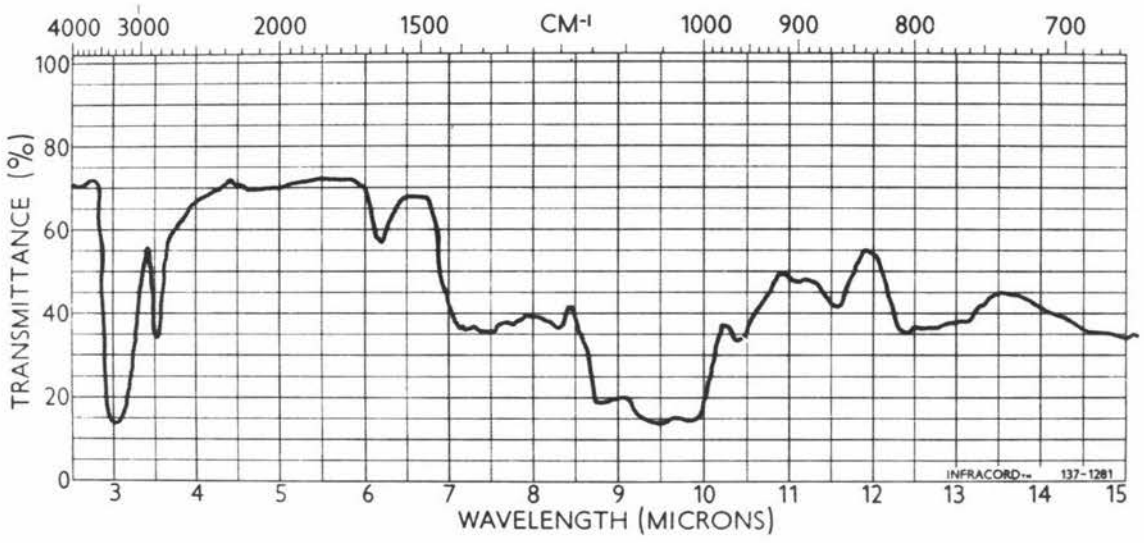


2.7.1 INFRA RED ABSORPTION SPECTRA PATTERN OF GALACTOMANNAN FILM:

A polysaccharide film was made by preparing a galactomannan solution (0.05%) which was dehydrated over mercury in a vacuum dessicator. The film was scanned, between NaCl windows in a Perkin-Elmer 137 NaCl I.R. Spectrophotometer and a typical trace may be seen in Figure 17. Major peaks occurred at 868, 966, 1610, 2850 and 3220 cm^{-1} while minor peaks occurred at 810, 1030, 1080 and 1150 cm^{-1}. Little is known of the relationship of these peaks to the structure of the galactomannan. A paper by Cerzo (16) observed that absorption bands at 817 and 874 cm^{-1} correspond to the α linkages of D-galactopyranose and the β linked mannopyranose residues respectively. In the Figure 17 it was noted that there were peaks at 810 and 868 cm^{-1}.

FIGURE 17

Infra Red Absorption Spectra Pattern of Polysaccharide Film.



CHAPTER III

DISCUSSION

The purpose of this project was to isolate the water soluble polysaccharide from Lotus pedunculatus (family : Leguminosae) and to study some aspects of its structure.

From the review of literature (see Chapter I) it was observed that galactomannans (heteroglycans composed of two monosaccharide types - D-galactopyranose and D-mannopyranose) are commonly found as constituents of ungerminated seeds of various species belonging to the family Leguminosae. They are usually found in the endosperm where they serve as a food reserve during germination. The isolation of these mucilages (so-called because of their ability to form thick, "slimy", highly-viscous solutions) can be achieved by extracting the freed endosperm or whole seed with water or alkali. These polysaccharides from the species so far studied (see Table 2, p11a) all appear to have the same basic structural features, i.e. a D-mannopyranose backbone joined by β (1 $\rightarrow$ 4) glycosidic linkages to which single, non-reducing α - D-galactopyranose units are linked through their reducing groups to the six-position of a mannose residue. This creates a highly branched polysaccharide where the degree of branching is a function of the molecular proportion of D-galactopyranose to D-mannopyranose (which varies approximately

between 0.25 and 1.00). One exception to these generalities is Medicago sativa (11) (see Chapter I, p11).

The polysaccharide under study was extracted from the milled seeds of Lotus pedunculatus with two separate portions of water. The clarification of the aqueous extract (from fine dust, etc.) was an important step in obtaining an uncontaminated polymer. By allowing the viscous supernatant to stand for 24 - 36 hours much of the dust, etc., settled and was removed by centrifugation at 2500 r.p.m. A final clarification was carried out by centrifugation at 30,000 r.p.m. for one hour leaving a clear brown solution. The succeeding steps were aimed at obtaining a polymer free from colouring matter and other impurities (fats, proteins, etc.) Acidified ethanol (to facilitate the removal of proteins, fats and colouring matter) was used to precipitate the polysaccharide from the aqueous extract. Subsequent washings of the polymer using acidified ethanol and absolute ethanol removed the colouring matter and hydrochloric acid respectively. Freeze drying of this precipitate yielded a white, horny, and matted substance (6.7 g) representing a 2.2 percentage mucilage extract from the whole seeds. On treating an aqueous solution of this polymer with Fehlings solution a blue, gel-like precipitate was collected. This property of forming a water-insoluble copper complex with Fehlings solution is a characteristic reaction of all leguminous galactomannans so far studied and is attributed to the presence of "cis" hydroxyl groups on the C_2 and C_3 of the D-mannopyranose

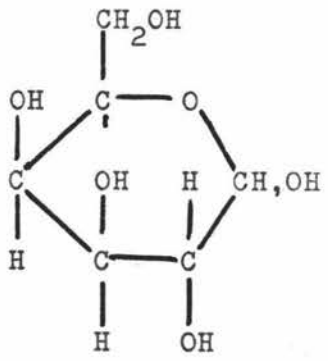
residues (1). The regeneration of the polysaccharide was carried out by the use of 2N HCl. Mukherjee et al., (17) in a study on the constitution of a galactomannan from the kernel of Palmyra Palm Nut suggested that the increase in the mannose content during purification via Fehlings solution could be attributed to the fact that some of the α linked galactose residues (which are more labile) were removed during the acid treatment. Whether or not this occurred in the regeneration of the polysaccharide under study, was not ascertained, but it cannot be ruled out as a possibility (17). An optical rotation determination was made in water and found $[\alpha]_D^{20}$ + 82.5°. This value was considered to reflect a large proportion of α glycosidic linkages (see Section 1.2. p.10) and (Figure 1, p.9) and a structural similarity to other leguminous galactomannans.

Fractional precipitation and ultracentrifuge studies indicated a highly-homogeneous polymer. In the fractional precipitation procedure (see Section 22, p37, and Table 3, p37a) five fractions were collected representing 93.0 per cent recovery of the starting material. Furthermore, 96.8 per cent of the total polysaccharide precipitated in the narrow range of 31.6 to 33.0 per cent ethanol by volume. The optical rotations, molar ratios of D-galactopyranose to D-mannopyranose and intrinsic viscosities for each fraction were ascertained and the similarities of these suggested that the starting material was a single

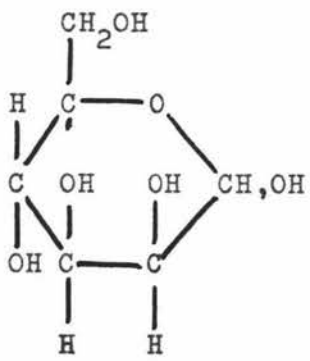
polysaccharide species. The presence of more than one polysaccharide species in the starting material could be accounted for if their respective solubilities under these experimental conditions were similar. Another possibility is that co-precipitation or occlusion of different polysaccharide species could not be differentiated by this method. Photographs of the sedimentation pattern (see Figure 7, p38a) at three concentrations (2.5, 5.0 and 10.0 mgs/ml buffer) showed a single peak and hence provided evidence for a homogeneous polymer. It was unlikely that this one peak represented in reality two components of closely overlapping molecular weight distributions. From the ultracentrifuge study an approximation of the molecular weight of the polymer was made according to the Archibold method (32). The average value was found to be 1.0×10^5 (Range: 1.14×10^5 to 9.75×10^4). Thus, as these two attempts to separate the starting material into more than one homogeneous species failed, any suggestion that this galactomannan could be a mixture of a mannan and a galactan (or other feasible possibilities) was not substantiated.

Mineral acid hydrolysis lead to the release and subsequent identification of only two monomers, D-galactopyranose and D-mannopyranose. D-galactopyranose was identified by melting point and optical rotation of the crystalline free sugar whereas D-mannopyranose was converted to its phenylhydrazone for identification. The values obtained for optical rotation and

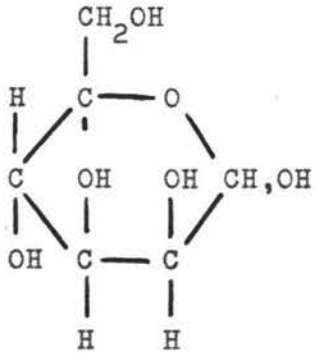
melting point and mixed melting point (see Section 2.3.1. p.40) were within the ranges of values obtained from the literature. On the basis of these results (and the homogeneity studies above) the polysaccharide was defined as a heteroglycan composed of the monoses D-galactose and D-mannose and hence given the usual title of galactomannan. A quantitative analysis of the sugars present, using a slight modification of a method devised by Curtis, C.M. (70), gave molar proportions of D-galactopyranose to D-mannopyranose of 49 to 51. The modifications of the Curtis method was merely in the use of aniline phosphate instead of aniline phthalate as the spray reagent. The results (see Table 4, p.44) illustrated that changes in optical density with concentration (over the range given) for the aniline derivatives of D-galactopyranose and D-mannopyranose obeyed Beer's law.



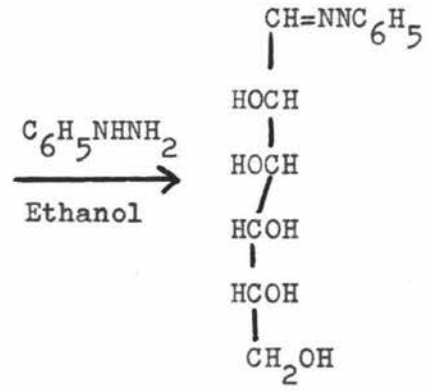
D-galactopyranose



D-mannopyranose



D-mannopyranose

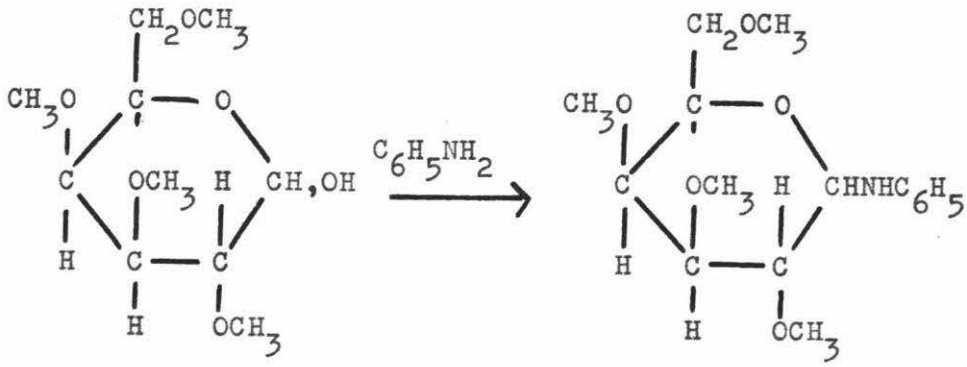


D-mannose

phenylhydrazone

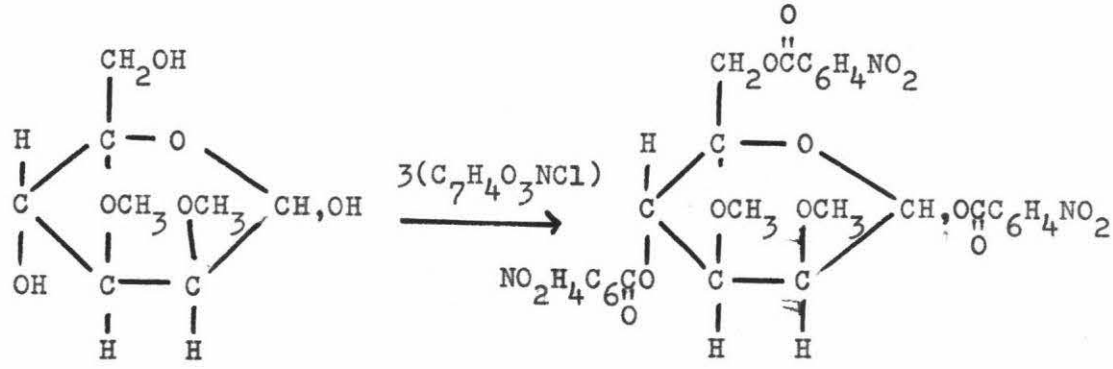
The galactomannan was methylated, first by dimethyl sulphate and sodium hydroxide (Haworth method (43)), then by Kuhn methods (49, 50, 51, 52, 53, 54) and finally by Purdies method (48). The final product was a clear, yellow-brown glassy syrup with an optical rotation in chloroform of $\left[\alpha\right]_D^{20}$ + 71°. (See Figure 4 p.12a). This value again indicates a probable high proportion of

α glycosidic linkages. After methanolysis of the methylated galactomannan and hydrolysis of the methyl glycosides the mixture of methylated sugars were resolved on a cellulose column using methyl ethyl ketone : water (89 : 11, $^{W}/_{W}$) as eluent. Four main fractions were collected and examined (together with the starting material) by paper chromatography using the solvent systems (a) n-butanol : ethanol : water, 4 : 1 : 5 (upper phase) and (b) methyl ethyl ketone : water, 89 : 11 ($^{W}/_{W}$). (See Table 5, p47a). With the exception of Fraction 3, the fractions each contained a single compound. (See Figure 8 p48a) Fraction 1 was identified as 2, 3, 4, 6 - tetra - O - methyl - D-galactopyranose, via its aniline derivative while Fractions 2 and 4 were identified via their nitrobenzoate derivatives as 2, 3, 6 - tri - O - methyl - D-mannopyranose and 2, 3 - di - O - methyl - D-mannopyranose and 2, 3 - di - O - methyl - D-mannopyranose respectively.



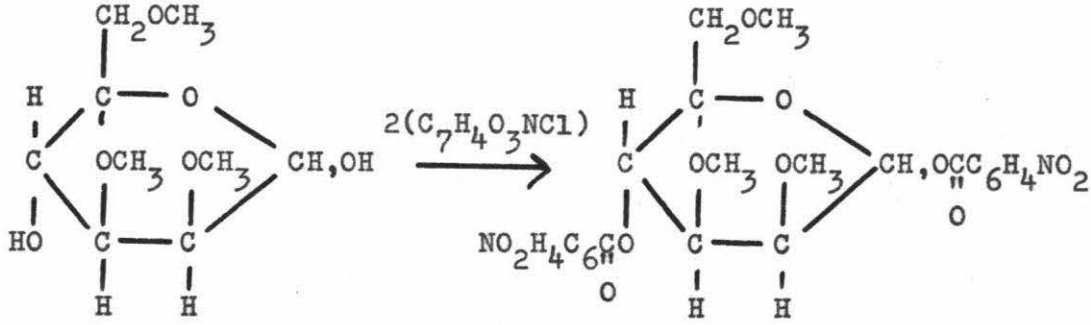
2, 3, 4, 6 - tetra - O - methyl - D-galactopyranose

2, 3, 4, 6 - tetra - O - N-phenylglycosylamine



2, 3, - di - O - methyl - D-mannopyranose

2, 3, - di - O - methyl - D-mannopyranose 1, 4, 6 - tris - p-nitrobenzoate

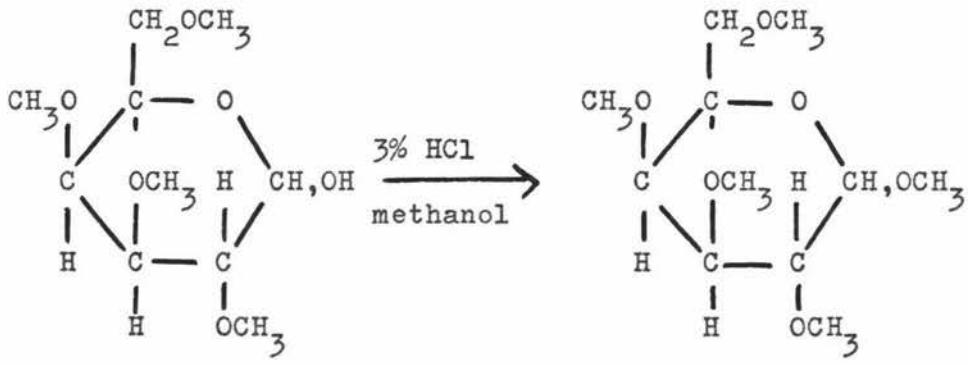


2, 3, 6 - tri - O - D-mannopyranose

2, 3, 6, - tri - O - methyl - D-mannopyranose 1, 4 - bis - p-nitrobenzoate

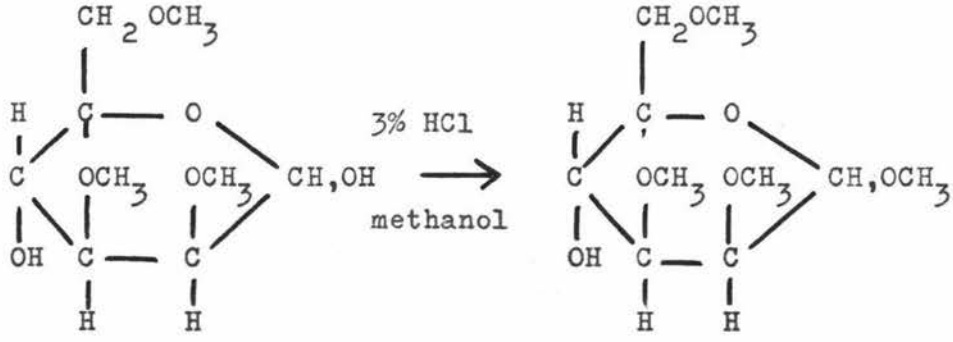
Fraction 3 appeared to be a mixture of two components (see Figure 8, P.48a) one of which corresponded to Fraction 1. (2, 3, 6 - tri - O - methyl - D-mannopyranose) while the other was suggested to be a tri - O - methyl - D-galactopyranose. This latter residue (only present in trace amounts) is considered to be a degradation product and therefore of no structural significance. Demethylation of samples from Fractions 1 and 4 according to the method of Hough et al., (61) showed the parent sugars to be D-galactopyranose and D-mannopyranose respectively. The latter results, together with C,H,N, and OCH_3 microanalyses, and melting-point and optical rotation determinations for these fractions confirmed their chemical identities. Comparison of R_F and R_S values with those from the literature did not give a very good guide for identification. The proportions of the above methyl sugars were determined in a separate experiment using the alkaline hypoiodate method developed by Hirst et al., (80). A solution of the methylated hydrolysate was separated by paper chromatography using n-butanol : ethanol : water, 4 : 1 : 5 (upper phase) as solvent. Two major spots were recognised with R_F and R_S values corresponding to 2, 3, 4, 6 - tetra - O - methyl - D-galactopyranose and 2, 3 - di - O - methyl - D-mannopyranose respectively. The minor third component 2, 3, 6 - tri - O - methyl - D-mannopyranose was not obvious on the marker strips. This component corresponded to Fraction 2 (see Figure 8, p.48a) and from its R_F and R_S values it was assumed to run close to 2, 3, 4, 6 - tetra - O - methyl - D-galactopyranose.

The molar ratio of 2, 3 - di - O - methyl - D-mannopyranose to 2, 3, 4, 6 - tetra - O - methyl - D-galactopyranose (plus 2, 3, 6 - tri - O - methyl - D-mannopyranose) was 49 to 51 (or 0.98). Theoretically the molar ratio of "di - O - methyl" sugar : "tetra - O - methyl" sugar should be unity but the slight discrepancy can be accounted for by experimental error and the presence of trace amounts of 2, 3, 6 - tri - O - methyl - D-mannopyranose. The hydrolysate was also successfully examined by gas-liquid chromatography using a column of Apiezon L 20% on Celite 545 packed into a 5' spiral column. A typical gas-liquid chromatogram is shown in Figure 9 (see p54a). Four peaks were observed, three of which were identified. Peaks A and C were shown to correspond to methyl 2, 3, 4, 6 - tetra - O - methyl - D-galactopyranose (α and β forms) and methyl 2, 3 - di - O - methyl - $\alpha\beta$ - D-mannopyranose respectively, by running the methylated hydrolysate mixture of methyl ethyl sugars with the methyl ethyl sugars corresponding to Fractions 1 and 2 (see Section 2.4.2, p47). Thus peak A (see Figures 9 and 10, p54a and 54b) appears to differentiate very well the α and β anomers of methyl 2, 3, 4,6 - tetra - O - methyl - D-galactopyranose and it is thought unlikely that these two peaks could represent different sugars, e.g. pyranose and furanose forms of the above.



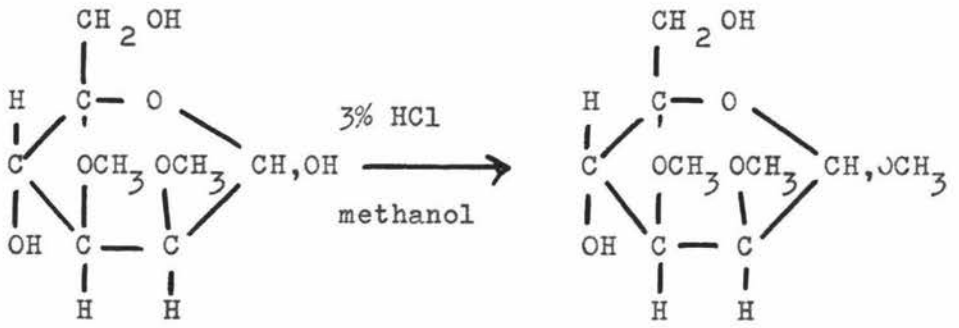
2, 3, 4, 6 - tetra - O - methyl - D-galactopyranose

Methyl 2, 3, 4, 6 - tetra - O - methyl - D-galactopyranose



2, 3, 6 - tri - O - methyl - D-mannopyranose

Methyl 2, 3, 6 - tri - O - methyl - D-mannopyranose



2, 3, - di - O - methyl - D-mannopyranose

Methyl 2, 3 - di - O - methyl - D-mannopyranose

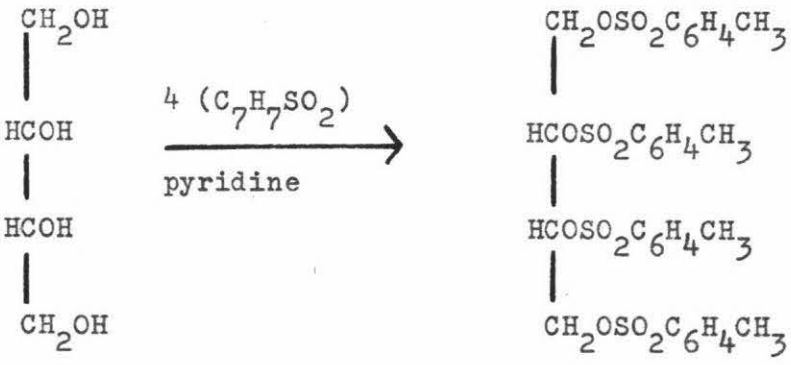
Peak B is suggested as representing methyl 2, 3, 6 - tri - O - methyl - $\alpha\beta$ - D-mannopyranose. The relative amounts of the separated components, estimated by triangulation was not in agreement with the results obtained by the alkaline hypoiodate method or the molar ratio of methyl sugars collected by the column separation (see Table 5 and p47a). The molar ratio was estimated as 16.1 : 1.0 : 7.7 (methyl tetra - methyl sugar : methyl tri - methyl sugar : methyl di - methyl sugar). Whether or not there is any difference in response of the ionisation detector to the different methyl hexosides was not ascertained. Bishop et al., (7) state that, the response of the β - ionisation detector was the same for methyl tetra - O - and methyl tri - O - methyl hexosides. But, however, gave 1.3 times greater response than the methyl di - O - methyl hexosides on a molar basis. In this experiment a H detector was used and it is quite possible that its response is not quantitative (on a molar basis). A correction

factor would have to be determined using standard mixtures. The results of these methylation studies (apart from the quantitative aspects of gas-liquid chromatography) is consistent with the structure (see Figure 2, p.10a) attributed to galactomannans from leguminous seeds. All the D-galactopyranose was present in the methyl hydrolysate as 2, 3, 4, 6 - tetra - O - methyl - D-galactopyranose and therefore would represent single non-reducing D-galactopyranose units in the natural polymer. The mannose in the methyl hydrolysate was present in two fractions. The 2, 3 - di - O - methyl - D-mannopyranose (present in approximately equimolar quantities to 2, 3, 4, 6 - tetra - O - methyl - D-galactopyranose) would be formed from those D-mannose units in the mannose chain to which are attached the D-galactopyranose side chains. Finally the trace amounts of 2, 3, 6 - tri - O - methyl - D-mannopyranose would represent D-mannose units in the main chain to which there are no side units attached.

On periodate oxidation 1.2 moles of periodate are consumed per anhydrohexose sugar and 0.49 moles of formic acid are released per anhydro sugar. Theoretically (assuming a D-galactopyranose to D-mannopyranose molar ratio of 49 to 51 and a chemical structure as given in Figure 3 (p.30a)) the consumption of approximately 1.5 moles of periodate together with the release of 0.5 moles of formic acid per anhydrose unit would be expected. Thus in this experiment the release of 0.49 moles of formic acid per anhydrose unit is in agreement with that theoretically expected. The consumption of 1.2 moles of periodate per anhydrose unit is less than theoretically expected.

This could be accounted for by the resistance of a proportion of the D-mannopyranose residues to oxidation.

Examination of the periodate - oxidised galactomannan by Smith's degradation procedure (95, 96) yielded D-mannopyranose, D-erythritol and glycerol. D-mannopyranose was identified as its phenylhydrazone derivative; D-erythritol as its tetra - O - tosyl and tetra - p - nitrobenzoate derivatives; and glycerol as its tri - p - nitrobenzoate derivative.

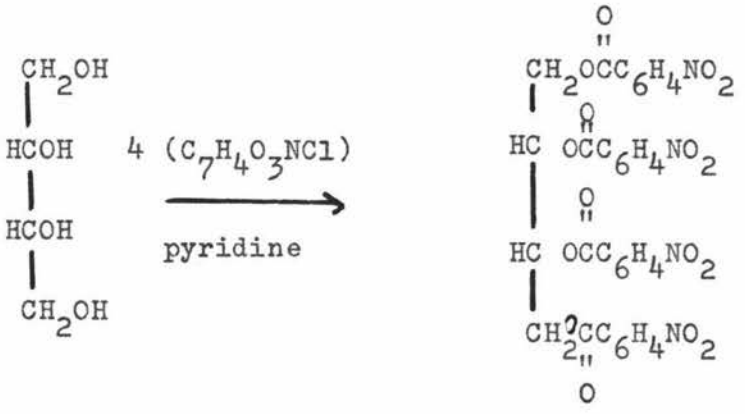


D-erythritol

Tetra - O - tosyl - erythritol

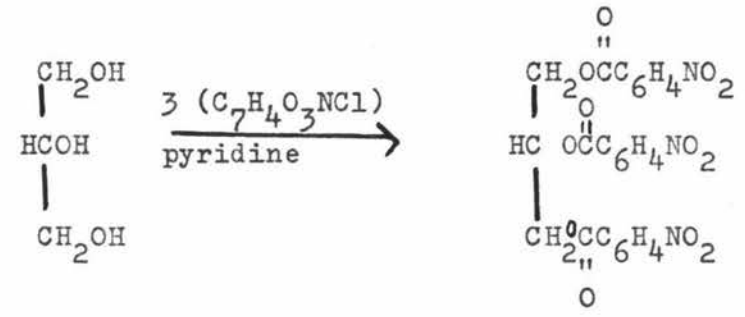
p-toluene sulphonyl chloride

$(C_7H_7SO_2)$



D-erythritol

Erythritol tetra - O - p - nitrobenzoate



Glycerol

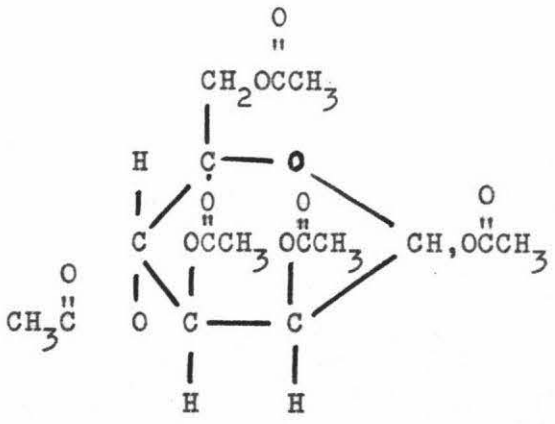
Glycerol tri - O - p - nitrobenzoate

p - nitrobenzoyl chloride

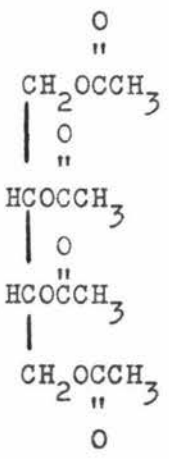
$(C_7H_4O_3NCl)$

Theoretically the presence of D-mannopyranose in the hydrolysate suggests its resistance to periodate oxidation because of the lack of two or more contiguous hydroxyl groups. However, it is thought that the presence of this sugar residue is due to cyclic acetal formation (16) and not to the presence of other glycosidic linkages, e.g. (1→3). Two other explanations have been suggested to explain this phenomenon. One attributes it to a steric effect resulting from the highly ramified structures of galactomannans (12) and the other to the use of low concentrations of periodic acid (12, 16, 21). The absence of D-galactopyranose in the hydrolysate is probably due to its greater accessability to the oxidising agent because these residues appear to be present in the galactomannan molecule as single non-reducing residues, glycosidically linked to the mannose backbone (see Figure 18, p84). The acetylated hydrolysate was examined by gas-liquid chromatography using the same column as for the methyl derivatives. Four peaks were observed (see Figure 13, p 30a). By running the acetylated hydrolysate with authentic samples, peaks A and C were identified as tri - O - acetyl - glycerol and penta - O - acetyl - D-mannopyranose respectively. However, gas chromatography of a sample of penta - O - acetyl - D-galactopyranose gave a peak with the same retention time as peak C. In spite of this result, as no D-galactopyranose was identified in the above hydrolysate, it is probable that this peak represents only penta - O - acetyl - D-mannopyranose. Peak B was

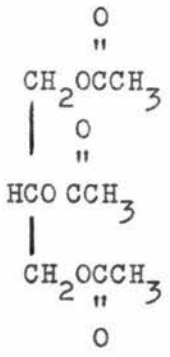
not identified but from its position and the identification of D-erythritol (see Section 2.5.2, p56) it could correspond to tetra - O - acetyl - D-erythritol (for which no marker was available). Peak D remained unidentified but peaks C and D might possibly be classified together as α and β anomers of penta - O - acetyl - D-mannopyranose. This peak (D) did not have the same retention time as penta - O - acetyl - D-mannitol or as stated above, penta - O - acetyl - D-galactopyranose. The results of this periodate study are consistent with the structure normally possessed by galactomannans from leguminous seed (see Figure 2, p10a). The erythritol released on acid hydrolysis of the polyalcohol indicated the presence of a β (1→4) mannopyranose backbone while the glycerol must have come from the non-reducing D-galactopyranose units.



penta - O - acetyl - D-mannopyranose



tetra - O - acetyl - D-erythritol



tri - O - acetyl - glycerol

In another experiment the galactomannan underwent a controlled partial hydrolysis in dilute sulphuric acid (0.02N). (See Section 2.6.1, p61). From paper chromatographic work it was noted that D-galactopyranose was released in large quantities with only traces of D-mannopyranose. Table 7 (see p62) and Figure 16 (see p62a) illustrate the variation of specific optical rotation (in water) with the molar ratio of D-galactopyranose to

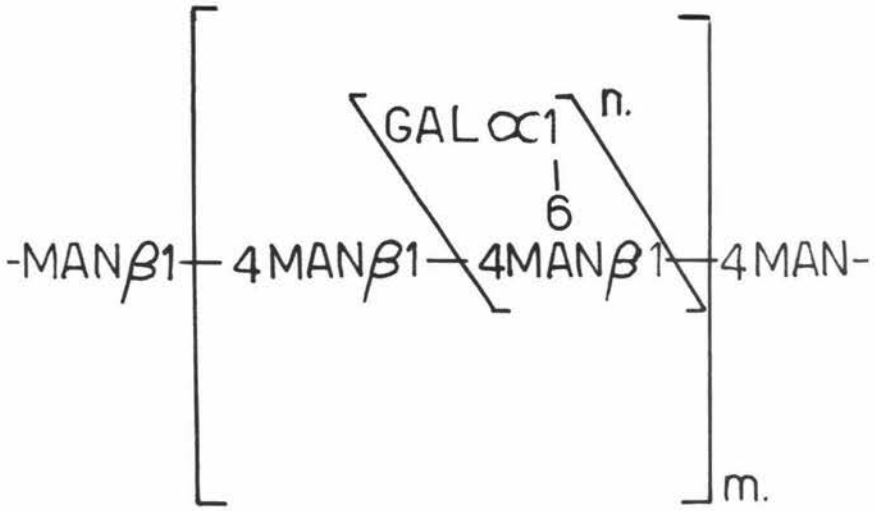
D-mannopyranose. The linear relationship indirectly supports the theory that the specific rotation of mucilages increases (or decreases) regularly as the molar ratio of D-galactopyranose to D-mannopyranose increases (or decreases) (see Tables 1 and 2, p9a and 11a; and Figure 1, p9b). This phenomenon is attributed to a similarity in the mode of linkage of various galactomannans and in particular reflects an increasing number of α glycosidic linkages as the D-galactopyranose to D-mannopyranose ratio increases. If in this experiment the D-galactopyranose units are linked α (1 $\rightarrow$ 6) to a β (1 $\rightarrow$ 4) D-mannopyranose backbone, hydrolysis (as given, see Section 2.6.1, p 61) would lead to the removal of D-galactopyranose residues as the energy of hydrolysis of α (1 $\rightarrow$ 6) linkages in less than β (1 $\rightarrow$ 4) linkages. Thus, the removal of D-galactopyranose residues reduced the proportion of α (1 $\rightarrow$ 6) linkages per molecule and hence resulted in a concomitant decrease in specific optical rotation.

The infrared spectrum of the polysaccharide showed a number of major and minor absorption bands. Little, however, is known of the relationship of these peaks to the structural features of the galactomannan. Cerezo (16) observed that absorption bands at 817 and 874 cm^{-1} indicated the presence of α linked D-galactopyranose units and β linked D-mannopyranose units, respectively. In Figure 17 (see p63a) it may be noted that peaks are present at 810 and 868 ^{-1}cm.

From the above results and by analogy with the structures of other galactomannans (1), it is postulated that the galactomannan extracted from Lotus pedunculatus consists of a linear backbone of (1 → 4) linked D-mannopyranose residues, and to position 6 of each, a D-galactopyranose residue was attached by an (1 → 6) linkage. This postulate is in agreement with the specific rotation of the polysaccharide the molar ratio of sugars present, the identification of the methylated products and periodate studies carried out.

FIGURE 18

Proposed Fine Structure of Galactomannan Extracted from Lotus Pedunculatus.



GAL. = D-Galactopyranose.

MAN. = D-Mannopyranose.

Assuming a molar ratio of D-galactopyranose to D-mannopyranose of 49 to 51 and a molecular weight of 1×10^5

M = 20 per molecule

N = 15

R E F E R E N C E S

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