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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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A C K N O W L E D G E M E N T S

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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-galactopyranose) 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 β -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 C₆ of most of these mannose units are attached as single non-reducing D-galactopyranose residues.

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C H A P T E R I

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 not known 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 (Cinnamomum), 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, Carrageen), 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

<u>ACIDIC COMPONENT</u>	<u>NEUTRAL COMPONENT</u>
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

Hexoses

Amino alkyl ethers

Pentoses

Amino acids polypeptides
or proteins

6-Deoxyhexoses

Sugar alcohols

O - and N - Alkyl derivs.
of the above

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

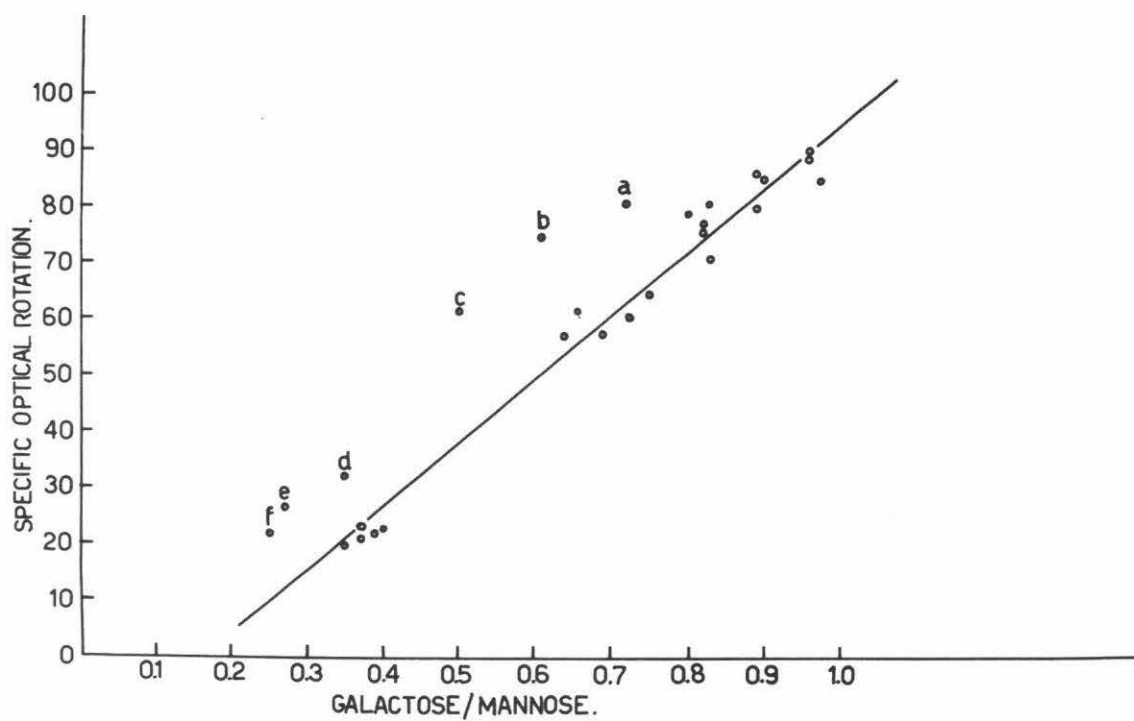
TABLE 1

COMPOSITION AND OPTICAL ROTATION OF GALACTOMANNANS (10)

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

FIGURE 1

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



- a. *Astragalus tenellus*.
- b. *Astragalus sinicus*.
- c. Guar gum (*Cyamopsis tetragonolobus*).
- d. *Crotalaria retuse*.
- e. *Cassia marilandica*.
- f. *Gymnocladus dioica*.

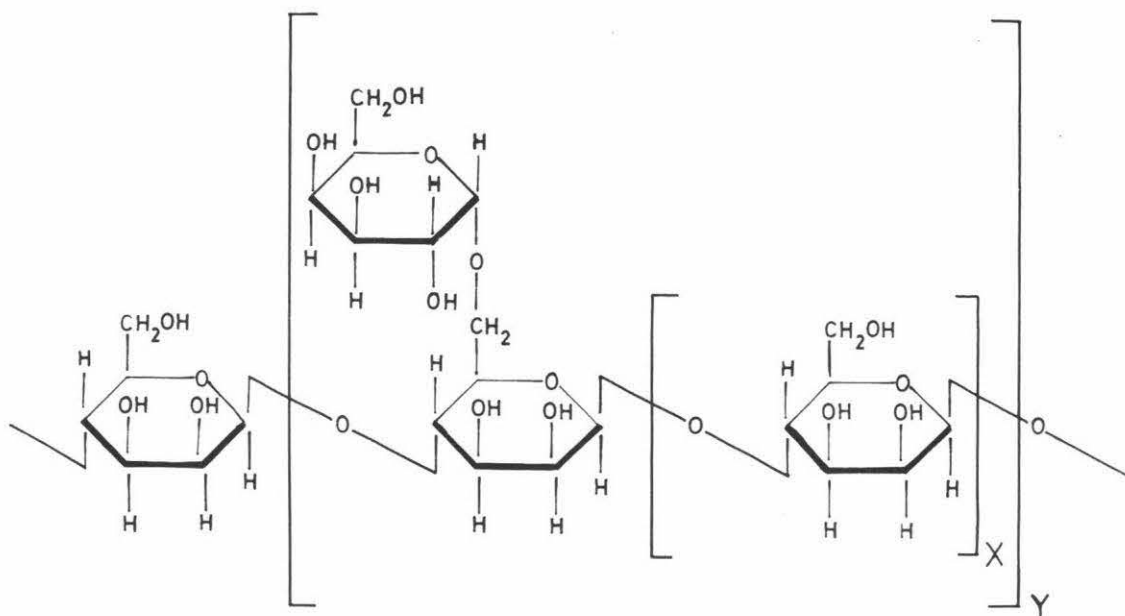
galactomannan mucilage. An exception to this is Circis canadensis (Red bud) which has an endosperm but is not mucilaginous.

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 \rightarrow 4) glycosidic linkages to which the D-galactopyranose units are attached as single non-reducing side chains by α (1 \rightarrow 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 C₂ and C₃ 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 $[\alpha]_D + 89^\circ$ (Water). The results of methylation studies indicated a structure with (1 \rightarrow 3) linked D-galactose units and (1 \rightarrow 6) and (1 \rightarrow 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	<i>Ceratonia siliqua</i> L. (Carob or Locust Bean)	1 : 5.25	+ 9° (N NaOH)	0.25	1.00
Leguminosae	<i>Ceratonia siliqua</i> L. (Carob or Locust Bean)	1 : 4.00		1.0	2.0 - 3.0
Leguminosae	<i>Gymnocladus dioica</i> (Kentucky Coffee Bean)	1 : 4.00	+ 29° (H ₂ O)	0.33	1.00
Leguminosae	<i>Gleditsia amorphoides</i>	1 : 2.50	+ 22.4° (H ₂ O)	0.48	1.00
Palmaceae	<i>Borassus Flabellifer</i> Limn. (Palmyra Palm Nut)	1 : 2.40	+ 8.5° (4% NaOH)	0.71	1.00
Leguminosae	<i>Cyamopsis tetragonolobus</i> (Guar Gum)	1 : 2.00	+ 60° (.6N NaOH)	1.00	1.00
Leguminosae	<i>Glycine hispida</i> Soybean Hulls	1 : 1.50	+ 65° (H ₂ O)	2.10	1.00
Leguminosae	<i>Trifolium repens</i> L. (White Clover)	1 : 1.30	+ 77.8° (H ₂ O)	3.38	1.00
Leguminosae	<i>Trifolium pratense</i>	1 : 1.28	+ 78 ± 11° (H ₂ O)	3.36	1.00
Leguminosae	<i>Medicago sativa</i> (Lucerne)	1 : 1.25	+ 118 ± 11° (H ₂ O)	3.80	1.00
Leguminosae	<i>Trigonella Foecum-Graenum</i> (Fenugreek)	1 : 1.20	+ 70 ± 10° (2N NaOH)	5.25	1.00

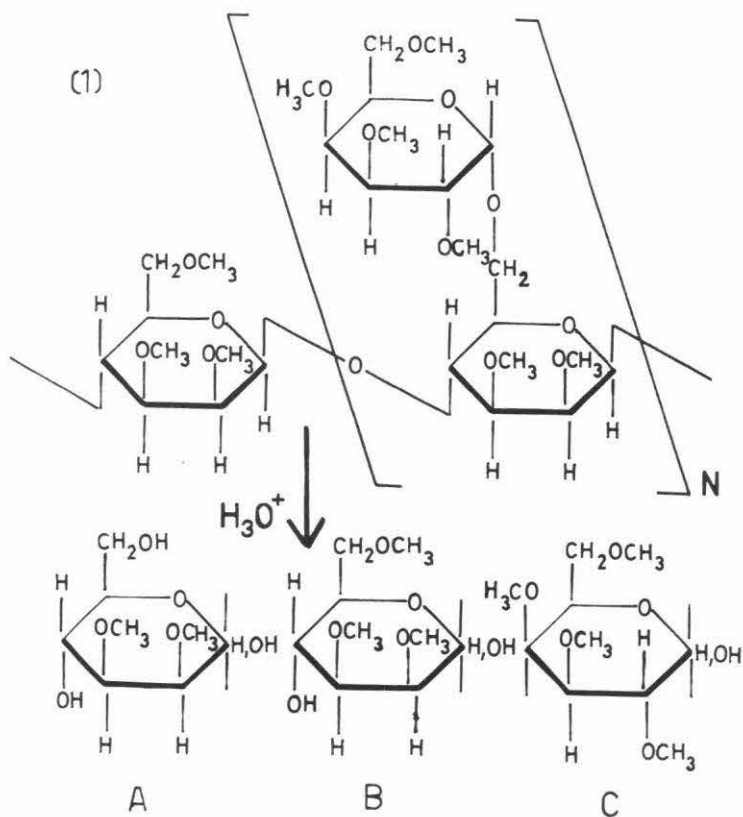
RELEASED PER HEXOSE UNIT (IN MOLES), PER CENT. END GROUPS (FROM COMPOSITION, METHYLATION AND PERIODATE RESULTS)
GALACTOMANNANS OF THE TYPE COMMONLY EXTRACTED, FROM THE FAMILY LEGUMINOSAE.

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° (Chloroform)	1.20	1.29	0.250	0.286	25.0	24.0	28.6	16
0.68	+ 28.7° (Chloroform)	1.30	1.30	0.270	0.294	27.0	29.8	29.4	17
1.00	+ 42° (Chloroform)	1.36	1.33	0.320	0.333	32.0	33.3	33.3	18
2.00	+ 58° (Chloroform)	1.15	1.40	0.368	0.400	36.8	41.1	40.0	19
3.00	+ 74.4° (Chloroform)	1.14	1.44	0.412	0.435	41.2	45.8	43.5	20
3.43	+ 76 ± 2° (Chloroform)		1.44	0.432	0.438	43.2	43.2	43.8	12
3.82	+ 66 ± 3° (Chloroform)		1.45	0.437	0.445	43.7	44.2	44.5	12
5.15	+ 50 ± 3° (Chloroform)	1.49	1.46	0.435	0.455	43.5	46.0	45.5	21

water the polymer which separated had a $[\alpha]_D^{19} + 118 \pm 11^\circ$ (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 $[\alpha]_D - 10^\circ$ (sodium hydroxide). This negative optical rotation probably indicated structural differences (10).

The principal evidence which confirmed the general chemical nature 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 4Methylated 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 \rightarrow 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 \rightarrow 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-galactopyranosyl) β - D-mannopyranose]. Whistler et al., (25) have also identified a mannotriose [(β - D-mannopyranosyl (1 \rightarrow 4) β - D-mannopyranosyl (1 \rightarrow 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 \rightarrow 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 granulorum, T. interdigitale, T. rubrum, T. schonleinii, and Microsporium 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 \rightarrow 6) linked α - D-mannopyranose units for approximately every 22 of which there is a (1 \rightarrow 3)linked α - D-mannopyranose residue. Branch points occur along the (1 \rightarrow 6) linked chain at C₂ positions of the D-mannopyranose units and once in every 45 units at the C₂ position of a (1 \rightarrow 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 phenomenon 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 distortion 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-charged 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 hydro-cellulose (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 phenolsulphuric acid, Somogyi reagent, hypiodate, alkaline ferricyanide and sodium metaperiodate. Methods involving the use of sprays and development 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 QUANTITATIVE 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 and has 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 $3400 - 3600 \text{ cm}^{-1}$

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

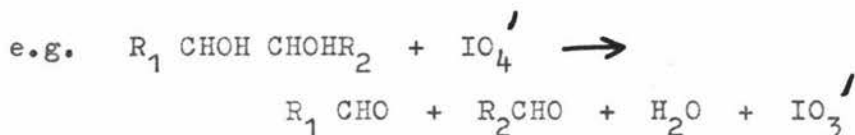
and co-workers (60) have found that columns of diatomaceous earth (e.g. celite) are as good as, or better than, cellulose columns. Separation can also be achieved by absorption chromatography on carbon-celite columns preferably using gradient elution. The capacity of carbon columns is high and a wide 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).

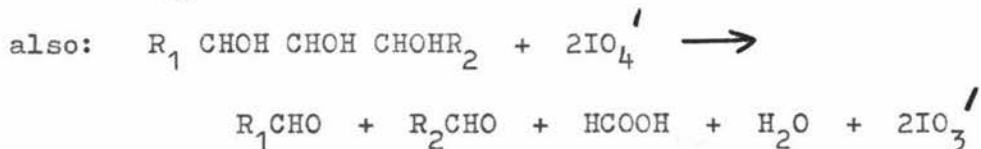
Quantitative determination of methyl derivatives involves either paper partition chromatography, elution and a micro-volumetric or micro-colorimetric procedure (41a), or gas-liquid partition chromatography (63, 64). The amounts of methyl sugars in the fractions eluted from the paper chromatogram can 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.



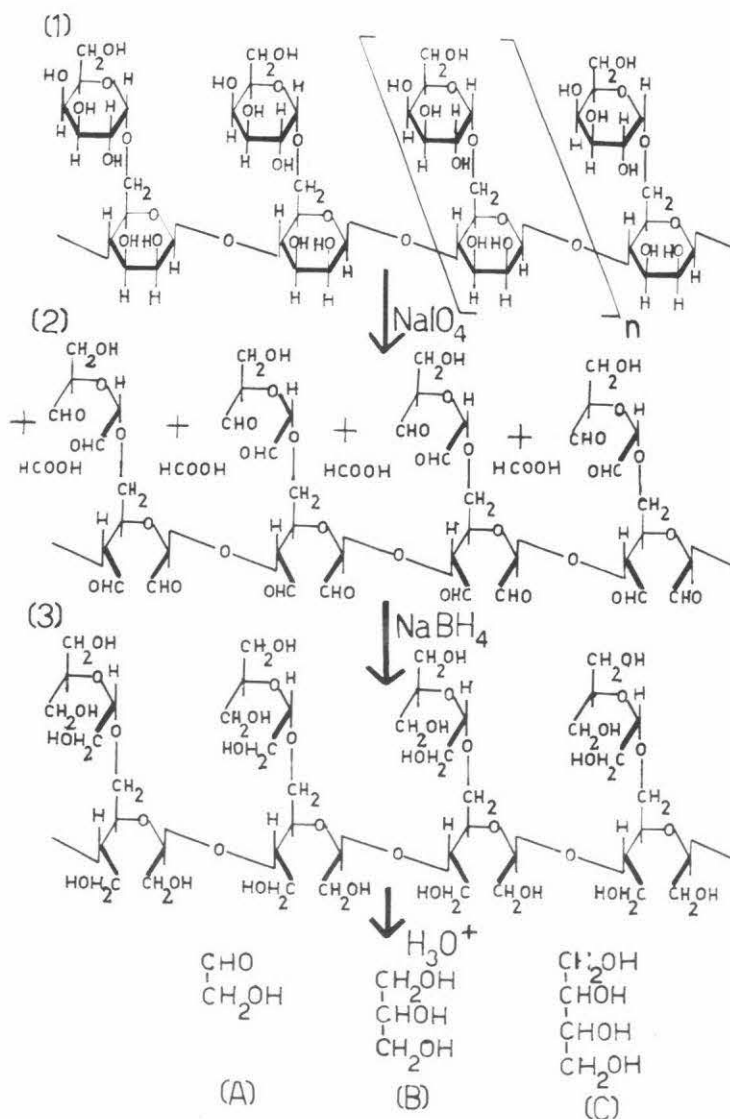
In this reaction there is a cleavage of the carbon chain, the consumption of one molecular proportion of periodate and the formation of two aldehyde groups. In the galactomannan under study this reaction involves the hydroxyl groups on the C₂ and C₃ of the D-mannopyranose units.



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, C₂, C₃, and C₄ 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 anhydrohexoses. 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 C₂ and C₄ 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).