STRUCTURAL STUDIES ON POLYURONIDES

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CONTENTS

GENERAL INTRODUCTION.

POLYSACCHARIDES. The polysaccharides have been defined as "polymerised sugars with glycosidic intermolecular linkages and with more than nine monosaccharide residues in each molecule" (1). It is, however, impossible to give a rigid definition, as the number of anhydro sugar units varies greatly in different types of polysaccharide. Furthermore, as the molecular weight decreases, the properties usually associated with polysaccharides, such as negligible reducing power, and insolubility, gradually revert to those of simple sugars.

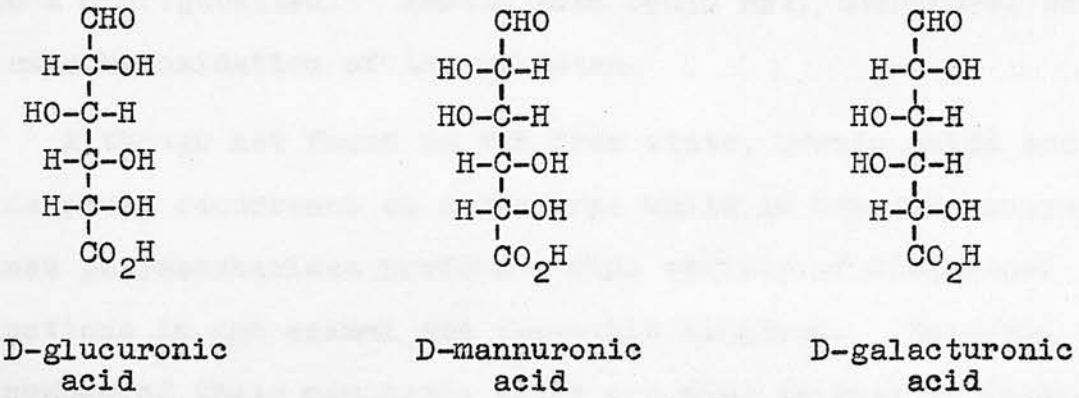
Polysaccharides occur in nearly all living organisms, but to a greater extent in the vegetable kingdom. If classification of these compounds is based on the physiological function they perform, two main groups are apparent. There are structural materials such as cellulose, alginic acid and pectins, which occur in the cell-walls of the organisms and there are polysaccharides which act as reserve food sources. Examples of this type are starch, glycogen, and inulin. There exist, in addition, polysaccharides such as plant gums and bacterial products, whose function is still unknown.

Classification of polysaccharides is also possible on the basis of their chemical constitution. Homopolysaccharides (or homoglycans) contain only one polymerised monosaccharide species,

while heteropolysaccharides (or heteroglycans) contain more than a single individual sugar. Members of both classes may be linear or branched. D-Glucose is the most common constituent of homopolysaccharides, in, for example, cellulose, starch, glycogen and laminarin, but polymers of D-mannose, D-galactose, D-xylose and L-arabinose also exist. Heteropolysaccharides are known with as many as five or six different monosaccharide species in the molecule. Such compounds are the plant gums, which have a highly-complex branched structure. Many bacterial polysaccharides contain two or three different monosaccharides.

Of the known polysaccharides, approximately one third to one half contain uronic acid units (2). This uronic acid may be present in small amount, or the entire polymer may be composed of hexuronic acid units. The term "polyuronide" has been used to describe any polysaccharide containing some proportion of uronic acid, while polymers consisting entirely of uronic acid units have been called "glycuronans" (3). A characteristic feature of polyuronides is the resistance they exhibit towards complete acid hydrolysis. This is due to the high acid stability of the uronosyl link which joins the uronic acid unit to the rest of the molecule. The conditions required to break this link are often so vigorous as to result in extensive degradation of the liberated uronic acids and monosaccharides.

URONIC ACIDS. The uronic acids may be defined as "carbohydrate derivatives possessing both aldehydic (or hemiacetal) and carboxyl groups" (4). The most commonly occurring uronic acids are D-glucuronic, D-mannuronic and D-galacturonic acids (I), and it is seen that they may be derived from the corresponding D-hexose by oxidation of the primary alcoholic grouping to a carboxyl group.



I

It has been suggested (5) that the presence of pentoses in natural carbohydrate material could be explained by oxidation of hexoses to uronic acids and subsequent decarboxylation, and that in polysaccharides containing pentoses and hexoses as integral parts of the molecule, uronic acids are intermediate compounds. D-galactose, for example, would be converted to L-arabinose via D-galacturonic acid, and in fact, plant gums containing these three sugars have been investigated (6, 7).

Further researches by Hirst and Jones (8), however, prove that the direct decarboxylation theory is untenable. Sugar beet and peanut pectins contain L-arabofuranose polymers which could not have arisen in this manner from the D-galacturonic acid units of pectic acid. Similarly, the D-galactopyranose units in the associated galactan are joined by β-1,4' links, while in pectic acid the D-galacturonic acid units, although joined 1,4', are of the α configuration. Pectic acid could not, therefore, be formed by oxidation of the galactan.

Although not found in the free state, uronic acids are of widespread occurrence as structural units in numerous polyuronides. These polysaccharides perform a wide variety of biological functions in the animal and vegetable kingdoms. Bacteria produce a number of these compounds which are used as reserve foods and protective materials or may be merely by-products of metabolism. Animal polysaccharides containing D-glucuronic acid have been isolated from blood and cartilage; these are known as heparin and chondroitin respectively.

Most polyuronides, however, are found in plants. Some trees, under certain conditions, exude tears of "gum", which usually contain a large proportion of polyuronide. Similar materials, known as mucilages, occur as intercellular constituents of certain plants. An important polyuronide is pectic acid, which consists entirely of α-1,4' linked D-galacturonic acid residues. This substance, together with an araban and a galactan, is a

major component of primary cell-walls in the higher plants, especially in citrous fruits and in wood (9, 10). Polysaccharides containing ca. 20% of D-glucuronic acid have been isolated from species of green algae, such as Acrosiphonia centralis (11) and Ulva lactuca (12). By far the most important seaweed polyuronide, however, is alginic acid. This polymer, consisting entirely of uronic acid residues, plays an important structural role in the cell-walls of most of the brown algae (13).

PART ONE.

THE GUM EXUDATE OF ALBIZZIA ZYGIA.

INTRODUCTION.

PLANT GUMS. Certain trees, in response to injury to their bark, exude sticky materials, which on exposure to air harden into nodules. These nodules of gum are basically polysaccharide in nature, but contain impurities such as lignin, protein, resinous materials and perhaps small pieces of bark.

The cause of formation of gums is still in doubt. Although they seem to be exuded exclusively to seal off an attacked or invaded area, it is unknown if they are intended as protection against bacteria or whether they are natural secretions of the tree. It seems unlikely that they arise from bacterial action as some gums, such as gum tragacanth, are formed immediately the bark is cut. It is interesting to note that the major sources of gums are trees which are old, desiccated and in poor health. Healthy specimens with a plentiful supply of water yield little gum.

The plant gums are to be distinguished from the mucilages, which are normal products of metabolism. These intercellular and membrane-thickening materials can occur in almost any part of the plant, where they perform a wide variety of biological functions. However, as the gums and mucilages are very similar in properties and molecular structure, they are usually reviewed together (14-19).

STRUCTURAL INVESTIGATION OF PLANT GUMS. Gums exist in the tree as calcium, potassium or magnesium salts of complex acidic polysaccharides. Addition of excess acidified ethanol to an aqueous or dilute alkaline solution of the crude nodules usually results in precipitation of the free acid. Purification of the polysaccharide may be effected by repeated dissolution in water and reprecipitation. Electrodialysis has also been used to remove ions and small molecular species (20).

After the polyuronide has been obtained in a pure condition, the next consideration is the homogeneity of the product. If a mixture, the material may be composed of polysaccharides comprising different monosaccharide units, or the same units combined in different ways or proportions. A further possibility is that the polysaccharide, although of only one molecular species, exists in a wide range of molecular weights. Resolution of some polysaccharides into two or more components has been achieved by a variety of methods. Graded precipitation with organic solvents may yield two or more fractions as in the case of gum tragacanth (21) and the gum component of olibanum (22). Certain polysaccharides may be made to complex with reagents such as copper salts (23) and cetyltrimethylammonium bromide (24, 25, 26) to give precipitates while other components may be left in solution. An immunological method, based on the fact that antipneumococcus sera give specific precipitates with certain polysaccharides, has also

yielded results (27). While a given polysaccharide may appear homogeneous as far as chemical fractionation methods are concerned, its heterogeneity may be established by utilisation of its physical properties. Thus, by using the technique of electrophoresis on glass-fibre paper, two components have been reported in gum from Acacia pycnantha (28). Most gums, however, have not so far yielded to any fractionation procedure.

Once the homogeneity of the material has been established as far as possible, preliminary examination is carried out. This involves the determination of optical rotation, acetyl, methoxyl and uronic acid content and the equivalent weight. Complete acid hydrolysis of the polysaccharide and subsequent chromatographic examination reveals the nature of the constituent monosaccharide units. These may be separated on cellulose and characterised by comparison of their crystals or crystalline derivatives with authentic samples. The relative proportions of the different sugars may be estimated by use of oxidative (29) or colorimetric micro-methods (30, 31, 32) after separation on paper chromatograms.

Hydrolysis under milder conditions, although degrading the macro-molecule to some extent, leaves certain linkages unattacked. This makes possible the isolation and characterisation of oligosaccharides and oligo-uronic acids. From the constitution of these fragments, valuable information about the structure of the macro-molecule can be deduced. If the polysaccharide contains

arabinose, autohydrolysis of an aqueous solution may result in the scission of arabofuranose residues. The main polysaccharide nucleus may then be reprecipitated. Thus by changing the conditions, hydrolysis of the polymer may be made selective, although no one type of linkage is affected exclusively.

Another method employed in breaking down polysaccharides is that of acetolysis. Treatment of the material with acetic acid, acetic anhydride and concentrated sulphuric acid at 0° and then at 25°, yields a series of acetylated fragments from which oligosaccharides may be obtained by deacetylation (33). This procedure has been applied with success to cellulose (34). A similar technique is that of mercaptolysis. Concurrent mercaptylation and hydrolysis of polysaccharides takes place in concentrated hydrochloric acid solution in the presence of ethyl mercaptan. The degraded fragments are isolated as crystalline mercaptal derivatives. Similarly, if the polysaccharide is heated with anhydrous methanolic hydrogen chloride, glycosidic bonds are broken. Methyl glycosides are formed and any carboxylic acid groups are esterified. This process has been used in the determination of the constitution of alginic acid (35).

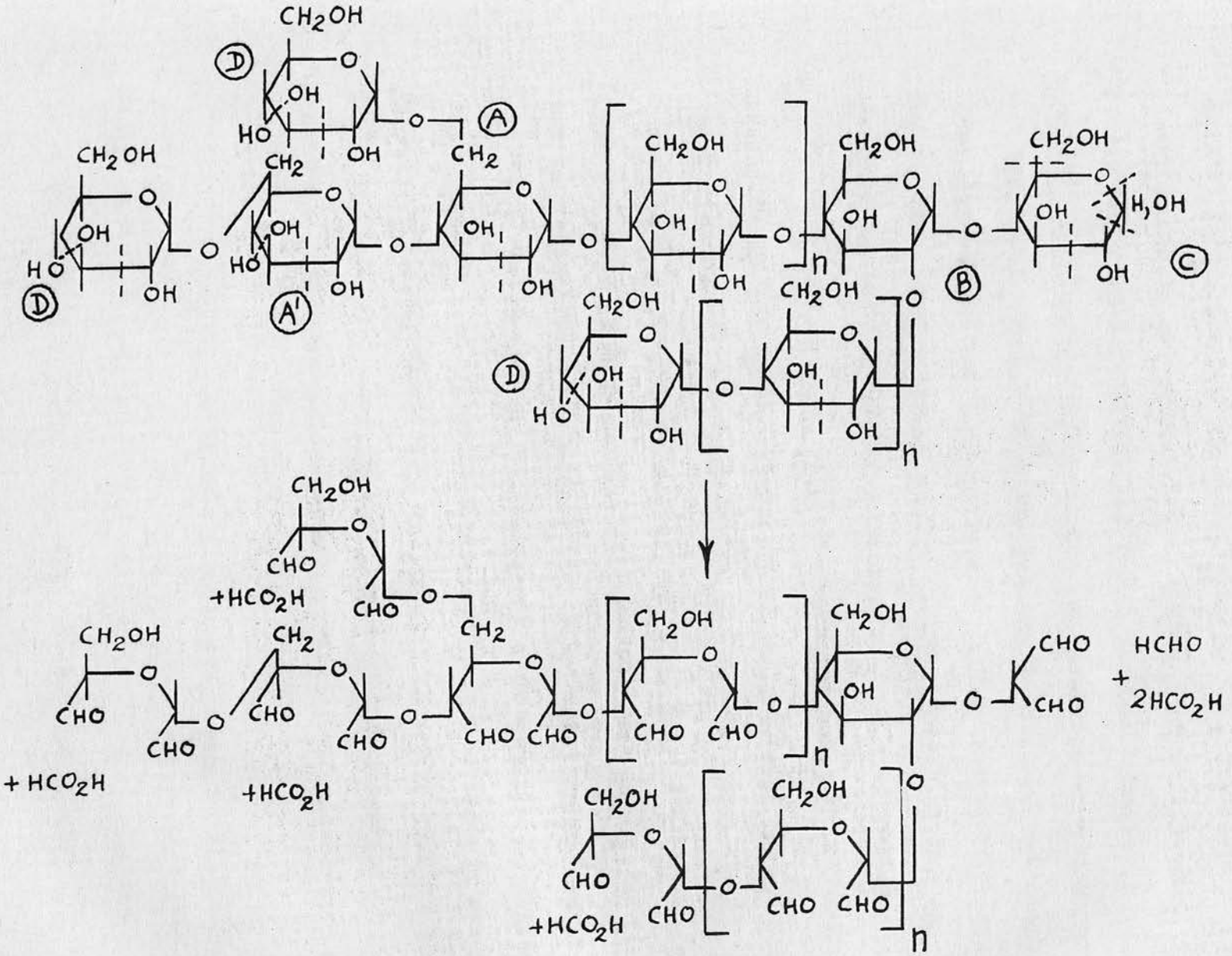
A major technique employed in the structural analysis of polysaccharides is that of methylation. All free hydroxyl groups are converted to methoxyl groupings and subsequent hydrolysis yields partially methylated sugars. The position of the free

hydroxyl groups on these sugars indicates the points of linkage in the macro-molecule. Owing to the complexity of plant gum structures, separation and identification of the large number of methylated monosaccharides present formidable problems. Methods of methylation in general use are those of Haworth (dimethyl sulphate and aqueous sodium hydroxide) (36) and Purdie (silver oxide and methyl iodide) (37). Where resistance to methylation is encountered, success has been achieved by the use of thallium derivatives of the polysaccharides (38).

Further information about the structure of a polysaccharide may be derived from a study of its oxidation with sodium meta-periodate. The oxidation of α-glycols by periodate was first noted by Malaprade (39, 40) in 1928 and in recent years the use of periodic acid and its salts has become a standard tool in the elucidation of carbohydrate structures. The reaction of a polyhydric alcohol may be represented as in (II). Carbon atoms carrying primary hydroxyl groups give rise to formaldehyde, while those carrying secondary hydroxyl groups yield formic acid. For each carbon-carbon bond broken, one mole of oxidant is consumed.

$$\begin{array}{l} CH_2OH \\ (CHOH)_n \\ CH_2OH \end{array} + (n+1)H_5IO_6 \longrightarrow nHCO_2H + 2CH_2O + (n+1)HIO_3 + (2n+3)H_2O$$

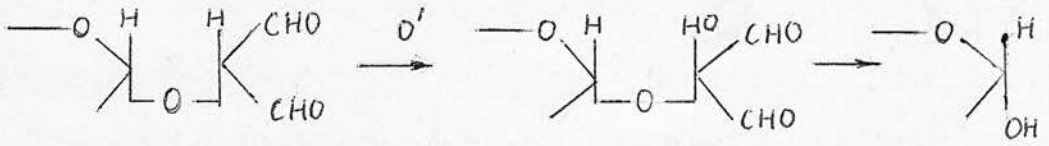
II


CH2OH
D
A
A'
B
C
OH
HO
O
n
+HCO2H
CHO
HCHO
+2HCO2H

The precise mechanism of the reaction is uncertain, but a cyclic periodate ester of the glycol has been postulated as an intermediate (41, 42). If the oxidation of hexoses by periodate is carried out at pH 7, the sugar is completely degraded with the consumption of 5 moles of periodate and the liberation of 5 moles of formic acid plus 1 mole of formaldehyde. On the other hand, the glycosidic link is stable to periodate attack and α-methyl-glucopyranoside is cleaved only through C_2, C_3 and C_4 with the liberation of one mole of formic acid.

Oxidation of polysaccharides occurs in a similar way. In a 1,4'-linked straight chain polysaccharide, the interchain linkages are unattacked but each unit in the chain reduces 1 mole of periodate and is cleaved through C_2 and C_3 (III). Branch points at C_6 have no effect on the reaction (III unit A), but if the branch point is at C_2, the sugar residue at this point is linked through C_1, C_2 and C_4 and is not cleaved by the periodate (III unit B). If the polysaccharide consists entirely of 1,3'-linked hexose units, no free adjacent hydroxyl groups are present in the interior of the molecule and oxidation occurs only on the terminal units. In contrast, oxidation of 1,6'-linked polymers (III unit A^1) results in liberation of one mole of formic acid for every anhydro sugar residue. The reducing end unit in a 1,4'-linked chain (III unit C) reduces four moles of periodate liberating 2 moles of formic acid and 1 mole of formaldehyde. The non-reducing end units (III unit D) are cleaved between C_2,

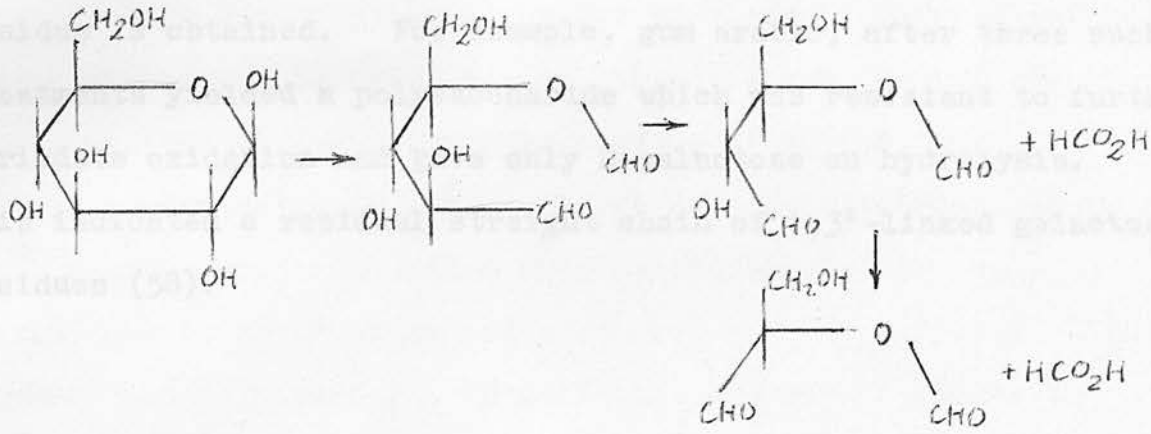
C_3 and C_4 with the release of 1 mole of formic acid. Formation and oxidation of reactive methylene groups at the reducing end unit can take place, resulting in a further uptake of periodate by the mechanism of over-oxidation (IV). The polymer may thus be degraded stepwise from this end as far as the branch-point.



Malondialdehyde grouping

IV

The extent of the oxidation, however, is dependent on the experimental conditions (43-45). In the case of hexoses, 3 moles of oxidant are rapidly consumed with the cleavage of the C_1-C_2, C_2-C_3 and C_3-C_4 bonds. The resulting formyl ester is stable if the oxidation is conducted at 2° and pH 3.6 to 4.6 (V) and the oxidation is arrested at this point (46-48). By carrying out oxidation of polysaccharides under these conditions, over-oxidation and consequent degradation of the molecule is avoided.



stable ester at pH 3.6-4.6 and 2°

V

Measurement of periodate uptake and release of formaldehyde and formic acid may thus yield valuable information on the mode of linkage and extent of branching in a polysaccharide, and laboratory techniques have been devised for this purpose (48-53). Experimental methods and conditions of oxidation have been reviewed by Bobbit (54) and Dyer (55).

In the case of polysaccharides in which only the terminal units are attacked by periodate, additional information has been obtained by the use of Barry degradation (56, 57). Treatment of the oxidised polysaccharide with phenylhydrazine and glacial acetic acid at 70-80° removes all the cleaved monosaccharide units, exposing fresh units which are susceptible to attack by periodate.

This process may then be repeated until a periodate-resistant residue is obtained. For example, gum arabic, after three such treatments yielded a polysaccharide which was resistant to further periodate oxidation and gave only D-galactose on hydrolysis. This indicated a residual straight chain of 1,3'-linked galactose residues (58).

MOLECULAR STRUCTURE OF PLANT GUMS. In general, the molecules are large in size, usually with a stable inner core carrying numerous side-chains of various types. These side-chains are linked to the inner skeleton by glycosidic bonds and produce a highly-branched structure. It is probable that the overall shape of the molecule determines the characteristic properties of these polymers. While the large proportion of free hydroxyl groups must permit a considerable degree of hydrogen bonding, the geometry of the molecule is such that these groups cannot readily align themselves to form fibres as do the straight chain polysaccharides cellulose, pectic acid and alginic acid. Consequently, the molecules will remain heavily hydrated even in concentrated solutions (16). It is this property which accounts for the widespread application of gums in the food and other industries as thickeners and adhesives.

A skeletal chain of D-galactose residues is of widespread occurrence in gums from many species of trees. These residues may be linked 1 , 3' and 1 , 6', as in damson, cherry and egg-plum gums (59-61), while in some species of Acacia the 1 , 3' linkage predominates (62-64). Apart from D-galactose, the more usual neutral monosaccharides encountered are D-mannose, L-arabinose (both furanose and pyranose) and, to a lesser extent, D-xylose and L-rhamnose. In addition, however, L-fucose and D-tagatose have been reported in isolated examples (65, 66). Hydrolysis under mild conditions has yielded di- and trisaccharides, both from the galactan skeleton and the side-chains (67-71).

While the uronic acid normally present in plant gums is D-glucuronic acid, the mucilages tend to possess D-galacturonic acid as a constituent. This division is not absolute, however, as several gums, notably from Sterculia spp., contain D-galacturonic acid. A number of gums contain 4-O-methyl-D-glucuronic acid, while the Khaya gums have both this acid and D-galacturonic acid (7, 72). The proportion of uronic acid present in plant gums varies from 5 to 50%. Species containing D-galacturonic acid and 4-O-methyl-D-glucuronic acid tend to have a higher proportion than those containing D-glucuronic acid.

D-glucuronic acid is usually found linked to D-galactose or D-mannose units and, in fact, 6-O-β-D-glucopyranuronosyl-D-galactopyranose and 2-O-β-D-glucopyranuronosyl-D-mannopyranose

have been isolated from several species such as Acacia and Prunus spp. (73-76). Similar aldobiuronic acids have been obtained from gums containing the 4-O-methyl ether of D-glucuronic acid (77, 78). In addition, partial hydrolysis has yielded 4-O-(4-O-methyl-D-glucopyranuronosyl)-L-arabinose from lemon gum (79) and 3-O-(4-O-methyl-D-glucopyranosyl)-L-arabinose from golden apple gum (80). This would seem to indicate that uronic acid units occur in the side-chains of the molecule. Gums containing D-galacturonic acid appear to differ considerably from the others in that they do not contain arabofuranose residue and do not appear to possess a back-bone of D-galactopyranose units. Both 4-O-D-galactopyranuronosyl-D-galactose and 2-O-D-galactopyranuronosyl-L-rhamnose have been isolated from Sterculia setigera gum (66). This species has also yielded a D-galactopyranuronosyl-D-galactopyranuronic acid (81).

RELATIONSHIP OF CHEMICAL STRUCTURE TO BOTANICAL ORIGIN. The plant gums are remarkably specific in that each species of tree apparently produces a unique chemical entity. However, certain relationships are discernible between gums of similar genera, and it is feasible that detailed knowledge of the chemical structure of gums could be useful for the elucidation of taxonomic problems.

All the gums from species of Acacia, for example, contain the same constituent sugars (Table 1) although in different proportions.

All of these gums contain 6-O-β-D-glucopyranuronosyl-D-galactose, and similar neutral disaccharides have been isolated from a majority of the species. The proportion of uronic acid, although differing in the various species of Acacia, is nowhere as high as in gums from some other genera, e.g. Sterculia. The optical rotation of A.karroo is surprising in that it is positive, whereas all other Acacia gums show a negative rotation. This is probably due to the presence of a large number of α-links in the molecule, and, in fact, 4-O-α-D-glucopyranuronosyl-D-galactose has been isolated from this species. It seems evident that Acacia gums have essentially the same constitution but with differences in their fine structure.

Gums of the genus Prunus are more varied in their monosaccharide content. While all species so far examined contain D-glucuronic acid, D-galactose and L-arabinose, some contain D-mannose and the proportion of D-xylose varies considerably. Rhamnose has been reported in peach gum (P.persica) although not as a major constituent of the polysaccharide. In addition to the common aldobiuronic acid of the Acacia gums, 2-O-β-D-glucopyranuronosyl-D-mannose has been isolated from damson (P.insitia) and cherry (P.cerasus) gums. The equivalent weights of all the Prunus gums are similar and the specific rotations are of the same order (Table 2). Values for uronic acid content, so far reported, do not exceed 17%.

In contrast, the Sterculia gums are characterised by a positive rotation and a very high uronic acid content (Table 3). While in S.setigera, S.tormentosa and S.urens this is D-galacturonic acid, S.caudata contains D-glucuronic acid. All species so far examined contain D-galactose and L-rhamnose in approximately equimolecular proportions, while S.setigera is unusual in that it contains the rare sugar D-tagatose. Sterculia spp. have not been investigated as thoroughly as have some other genera, but aldobiuronic acids containing L-rhamnose appear to be usual.

PART ONE of this thesis describes a structural investigation of the gum exudate of the West African tree Albizzia zygia. This is the first recorded investigation of the gum from this species of tree.

TABLE 1. ACACIA GUMS.

Species	Equiv. wt.	$[\alpha]_D$	Uronic acid (%)	Aldobiuronic acids	Approx. molar ratios			Neutral disaccharides	Ref.
					Gal	Ar	Rh		
Acacia catechu	1025	-32°	17.6	GA 1 $\xrightarrow{\beta}$ 6 Gal	9	4	3	-	82
A. cyanophylla	740	-20°	21.7	GA 1 $\xrightarrow{\beta}$ 6 Gal	11	2	5	Gal 1 $\xrightarrow{\alpha}$ 3 Ar	73
A. karroo	1660	+54°	12	GA 1 $\xrightarrow{\beta}$ 6 Gal GA 1 $\xrightarrow{\alpha}$ 4 Gal	6	5	tr.	Arp 1 $\xrightarrow{\beta}$ 3 Ar	67
A. mollissima	1880	-49°	7.7	GA 1 $\xrightarrow{\beta}$ 6 Gal	5	6	1	Arp 1 $\xrightarrow{\beta}$ 3 Ar	74
A. pycnantha	3700	-8°	5	GA 1 $\xrightarrow{\beta}$ 6 Gal	5	3	tr.	Arf 1 $\xrightarrow{\beta}$ 3 Ar Gal 1 $\xrightarrow{\beta}$ 3 Gal	68 64
A. senegal	1300	-30°	12.5	GA 1 $\xrightarrow{\beta}$ 6 Gal	3	3	1	Gal 1 $\xrightarrow{\alpha}$ 3 Ar Gal 1 $\xrightarrow{\beta}$ 3 Gal Arp 1 $\xrightarrow{\beta}$ 3 Ar	62,63 83,84
A. sundra	980	-29.6°	18	GA 1 $\xrightarrow{\beta}$ 6 Gal	3	2	2	-	85

TABLE 2. PRUNUS GUMS.

Species	Equiv. Wt.	$[\alpha]_D$	Uronic acid (%)	Aldobiuronic acids	Approx. molar ratios				Neutral Disaccharides	Ref.
					Gal	Ar	Mn	Xy		
P.insitia (Damson)	1100	-26°	16.4	GA 1 $\xrightarrow{\beta}$ 2 Mn	2	3	1	tr.	gal 1 → 3 gal gal 1 → 6 gal	75 86
P.domestica (Egg-plum)	1215	-25°	14.7	GA 1 $\xrightarrow{\beta}$ 6 Gal	3	3	-	1	gal 1 → 3 gal gal 1 → 6 gal	60, 87, 88
P.cerasus (Cherry)	1450	-27°	11.9	GA 1 $\xrightarrow{\beta}$ 2 Mn	2	6	1	tr.	Arp 1 $\xrightarrow{\beta}$ 3 Ar gal 1 → 3 gal	61,76 89,90
P.amygdalus (Almond)	1470	-44°	10	GA 1 → 6 Gal	3	4	-	2	-	91
P.persica (Peach)	2040	-47°	7	GA 1 → 6 Gal	5	6 + Rh (tr.)	-	2	Arp 1 $\xrightarrow{\beta}$ 3 Ar Xy 1 → Ar	89, 92

TABLE 3. STERCULIA GUMS.

Species	Equiv. Wt.	$[\alpha]_D$	Uronic acid (%)	Aldobiuronic acids	Approx. molar ratios		Neutral Disaccharides	Ref.
					Gal	Rh		
S.caudata	342	+69°	50.1 (D-GA)	GA 1 $\xrightarrow{\alpha}$ 2 Rh	1	1	-	93
S.setigera	370-400	+88° *	42.1 (D-Gal A)	Gal A1 → 2 Rh Gal A1 → 4 Gal	5 Ta(1)	5	-	66, 81
S. tormentosa			43 (D-Gal A)	-	1	1	-	94
S.urens		+53°	37.3 (D-Gal A)	-	6	4	-	95

* After 3 hours hydrolysis with 0.5N H_2SO_4.

Key to Tables.

GA = D-glucuronic acid
Gal A = D-galacturonic acid
Xy = D-xylose
Ar = L-arabinose
Ta = D-tagatose
Gal = D-galactose
Mn = D-mannose
Rh = L-rhamnose
p = pyranose
f = furanose
tr. = trace amount

EXPERIMENTAL

GENERAL METHODS OF INVESTIGATION

Paper Chromatography. Unless otherwise stated, chromatograms were run with control sugars at a constant temperature of 20° on Whatman No.1 or No.4 (fast-running) paper. Whatman No. 3MM and No. 17 papers were used for preparative separations. Chromatograms were dried at room temperature.

Principal chromatographic solvents

(1) benzene : butan-1-ol : pyridine : water (1 : 5 : 5 : 3)

(2) ethyl acetate : pyridine : water (10 : 4 : 3)

(3) ethyl acetate : acetic acid : water (3 : 1 : 3)

(4) ethyl acetate : acetic acid : formic acid : water (18 : 3 : 1:4)

(5) ethyl acetate : acetic acid : water (9 : 2 : 2)

(6) butan-1-ol : ethanol : water (4 : 1 : 5).

Developers

(a) Saturated aqueous aniline oxalate

(b) Aniline phthalate : aniline (1.86 g.) + phthalic acid (3.52 g.) in saturated aqueous butan-1-ol (200 ml.)

(c) Bromocresol green : 0.1% solution in 95% ethanol made alkaline with NaOH.

After spraying with (a) or (b), the chromatograms were dried at

120° and examined under ultra-violet light. After spraying with (c) the chromatograms were dried at room temperature.

The following abbreviations have been used to describe the distances travelled by sugars on paper chromatograms:

$$R_{Glu} = \frac{\text{Distance travelled by sugar}}{\text{Distance travelled by glucose}}$$

$$R_{Gal} = \frac{\text{Distance travelled by sugar}}{\text{Distance travelled by galactose}}$$

$$R_{G} = \frac{\text{Distance travelled by sugar}}{\text{Distance travelled by 2,3,4,6-tetra-O-methylglucose}}$$

$$R_{GA} = \frac{\text{Distance travelled by sugar}}{\text{Distance travelled by glucuronic acid}}$$

$$R_{F} = \frac{\text{Distance travelled by sugar}}{\text{Distance travelled by solvent front.}}$$

Ionophoresis, unless otherwise stated, was carried out on Whatman No.1 paper, at 750 volts, in borate buffer at pH 10 (5 hours). The chromatograms were sprayed with saturated aqueous aniline oxalate containing 25% of acetic acid (96). The distance travelled by the sugars was described by the following ratio:

$$M_{G} = \frac{\text{Distance between sugar spot and 2,3,4,6-tetra-O-methylglucose spot}}{\text{Distance between glucose spot and 2,3,4,6-tetra-O-methylglucose spot.}}$$

Cellulose columns were packed with dry cellulose powder. The powder was poured in to a depth of ca. 1 inch and packed down by tapping and pressing with a flattened glass rod. This process was repeated until sufficient cellulose had been added. The column was then washed with water (1-2 l.), followed by butan-1-ol (1 l.) and the required solvent (1 l.).

Charcoal columns. Equal quantities (250 g.) of animal charcoal and Celite were mixed and washed successively with hot concentrated hydrochloric acid : water (1 : 1) (2 l.), water (5 l.), 1% aqueous sodium bicarbonate (2 l.) and water (10 l.). The charcoal-Celite mixture was packed as a slurry into a column of the required dimensions and washed with water (5 l.).

Purification of column solvent. Butan-1-ol was refluxed with 1% (w/v) potassium hydroxide for one hour, and then redistilled.

Evaporations were carried out at 40-50° (bath temperature) and 10 mm.

Specific rotations, unless otherwise stated, were measured in aqueous solution in a 1 dm. tube at room temperature.

Ash determinations were carried out by ignition of samples (50 mg.) in a platinum crucible until constant weight was attained.

Hydrolysis of a Polysaccharide. A sample (ca. 50 mg.) was heated under reflux at 100° with N sulphuric acid (2 ml.) for 7 hours or 2N sulphuric acid (2 ml.) for 18 hours. The resulting solution was cooled, and neutralised with solid barium carbonate. After filtration, and treatment with IR 120(H) resin, the solution was again filtered and concentrated to a syrup.

Dialysis of polysaccharide solutions was carried out in cellophane tubes against running water with a layer of toluene to prevent bacterial action.

Conversion to methyl ester methyl glycoside. The sugar (10 mg.) was refluxed with 4% anhydrous methanolic hydrogen chloride (1 ml.) for 7 hours. The cooled solution was then neutralised with dry solid silver carbonate, filtered and concentrated to a syrup.

Reduction with sodium borohydride. The sugar (10 mg.) was dissolved in water (0.5 ml.) and solid sodium borohydride (10 mg.) added. After allowing to stand for 12 to 18 hours, the solution was neutralised with acetic acid, treated with IR 120(H) and

IR 4B(OH) resins and concentrated to a syrup. Removal of a glycosidic group was effected by hydrolysis with N sulphuric acid for 4 hours at 100°.

Equivalent weights. The equivalent weights of acidic oligosaccharides were determined by titration with 0.01 N sodium hydroxide in presence of phenolphthalein in absence of carbon dioxide.

ALBIZZIA ZYGIA: THE CRUDE GUM

Appearance. The gum consisted of dark brown nodules of various sizes and contaminated in some cases with bark. An odour of acetic acid was apparent. In contact with water, swelling took place with formation of a thick pale-brown gel and an insoluble residue.

Properties. The crude gum had moisture 17%; ash 5.8%; acetyl 3.6% (97); methoxyl (trace) (98) and was devoid of nitrogen, sulphur and halogens. It was non-reducing to Fehlings solution and formed no insoluble complex with this reagent.

EXPT. 1. COMPLETE HYDROLYSIS AND IDENTIFICATION OF MONOSACCHARIDES

Powdered crude gum (100 mg.) was refluxed at 100° with 2N sulphuric acid (4 ml.) for 18 hours. Neutralisation with solid barium carbonate, filtration and evaporation yielded a syrup which was extracted with ethanol (4 x 5 ml.) to separate neutral sugars from barium salts of uronic acids. The latter were treated with IR 120(H) resin to convert them to the free acids. Chromatographic examination in solvents (1) and (3) revealed the presence of the following monosaccharides: galactose, mannose, arabinose, rhamnose, glucurone and 4-O-methyl-glucuronic acid.

EXPT. 2. EXTRACTION OF THE POLYSACCHARIDE

The crude gum (10 g.) was broken up into small pieces and dispersed with stirring in cold water (500 ml.) for 30 hours. Concentrated aqueous sodium hydroxide was then added to give a N solution and stirring was continued at room temperature under nitrogen for 20 hours. The pale brown mixture was then centrifuged and the residue (B) washed with cold water (2 x 250 ml.). After cooling to 0°, the centrifugate was acidified to pH 2 by addition of hydrochloric acid (50% v/v). Precipitation of the polysaccharide from this solution was effected by addition, with rapid stirring, of ethanol (4 volumes). The precipitate was removed on the centrifuge, washed free from chloride ions with

ethanol and then dried to constant wt. over phosphorus pentoxide at 61°/10 mm. The product (A) (4.8 g.; 58% yield from dried crude gum) was a fine off-white powder.

The residue (B) was stirred with water (1500 ml.) at 100° for 24 hours. After cooling, an insoluble residue was removed on the centrifuge, and the solution was acidified to pH 2 with hydrochloric acid. Addition of ethanol (5 volumes) to the solution resulted in the precipitation of a further quantity of polysaccharide (B) (1.3 g.; total yield from dried crude gum 74%).

EXPT. 3. ATTEMPTED FRACTIONATION OF THE POLYSACCHARIDE

a) Ethanol precipitation. The polysaccharide (A) (1.3 g.) was dissolved in 0.1 N sodium hydroxide (300 ml.). After acidification to pH 2 with hydrochloric acid, graded amounts of ethanol were added with constant stirring. Each precipitate was removed on the centrifuge, washed with ethanol, dried and weighed.

Volumes of ethanol added:	0.5	0.75	1.0	1.5	1.75	2.0	3.0
Wt. precipitated (g.):	-	-	-	0.750	0.500	0.020	-

The fractions precipitated by 1.5 and 1.75 volumes of ethanol were hydrolysed with acid under identical conditons. Examination of the products in a variety of solvents gave identical chromatograms from the two fractions. Polysaccharide (B) yielded similar results, the whole of the material being precipitated upon addition of 1.5 and 1.75 volumes of ethanol.

b) "Cetavlon" complex. The polysaccharide (A) (0.5 g.) was dissolved in 0.5 N sodium hydroxide (100 ml.) and a 10% aqueous solution of cetyltrimethylammonium bromide ("Cetavlon") (100 ml.) added with stirring. The precipitated polysaccharide complex, after centrifugation, was dissolved in 5% aqueous acetic acid and the free polysaccharide (0.47 g.) precipitated by addition of excess ethanol. Addition of ethanol to the supernatant "Cetavlon" solution did not result in any precipitation of polysaccharide showing that no fractionation had occurred. The process was repeated at pH 9, 7 and 4 using appropriate buffer solutions (99) with similar negative results.

EXPT. 4. PROPERTIES OF THE PURIFIED POLYSACCHARIDE

Polysaccharide (A) had the following properties:

$[\alpha]_D$ + 21° (c, 0.49); ash 0.25%; acetyl nil; methoxyl 1.3%; ethoxyl approx. 1.3%; uronic anhydride, calculated from the yield of carbon dioxide on decarboxylation with 19% hydrochloric acid, 23.5% (100). Titration with standard alkali in presence of phenolphthalein (corrected for blank) gave 24.3% (equivalent weight 723).

Complete acid hydrolysis. Hydrolysis of the polysaccharide under the same conditions as used for the crude gum (EXPT. 1) indicated the presence of the same monosaccharides. In addition, a faint, unidentified spot was detected, with R_{Gal} 4.8 in solvent (3).

This sugar is further investigated in EXPT. 14 (page 52). The acid fraction of the hydrolysate, after conversion to methyl ester methyl glycosides, was reduced with sodium borohydride. Hydrolysis with N sulphuric acid and chromatographic examination in solvents (1) and (2) showed the presence of glucose and 4-O-methylglucose in addition to the original neutral monosaccharides.

Polysaccharide (B) had the following properties:

An off-white powder, insoluble in cold water and sparingly soluble in 0.1N sodium hydroxide; $[\alpha]_D$ +38.7° (c, 0.32, 0.1N NaOH); ash 0.22%; acetyl nil; uronic anhydride by decarboxylation 32.6%.

Complete acid hydrolysis. Hydrolysis under identical conditions as used for polysaccharide (A) (EXPT. 1), followed by chromatographic examination of the hydrolysate, showed that the same sugars were present in the same relative amounts (visual examination) as found in polysaccharide (A).

EXPT. 5. QUANTITATIVE ESTIMATION OF THE RELATIVE PROPORTIONS OF THE NEUTRAL SUGARS

I. In a complete acid hydrolysate.

a) By periodate oxidation (101). The polysaccharide (A) (ca. 230 mg.) was hydrolysed at 100° for 18 hours with 2N sulphuric acid. After cooling, the solution was neutralised with barium

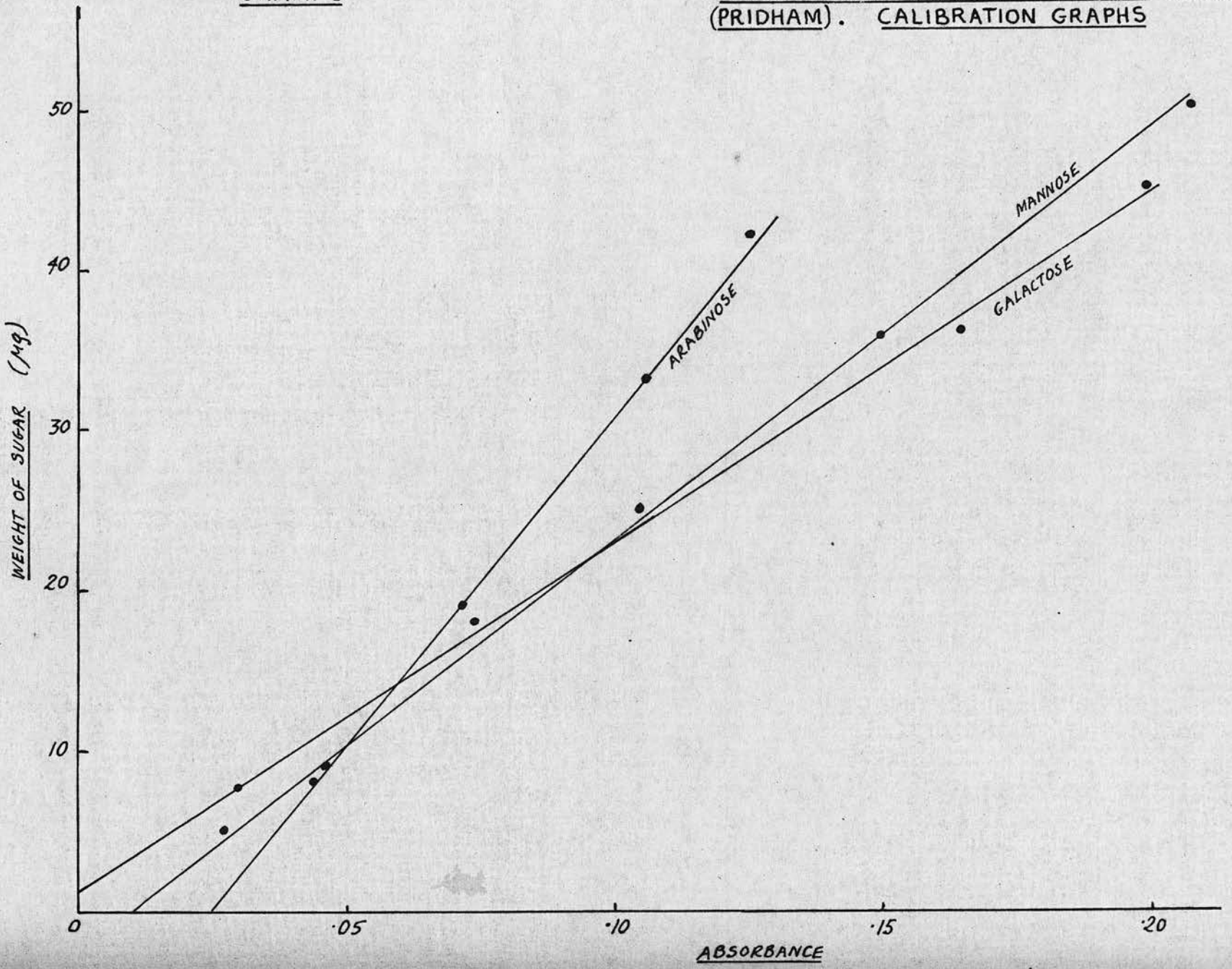
carbonate, filtered and evaporated to dryness. The residue was extracted with cold water, concentrated and made up to 10 ml. in a standard flask. An aliquot (0.2 ml.) was accurately placed on Whatman No.1 paper with an Agla micro-syringe, and the chromatograms were irrigated with solvent (3) for 48 hours at 25°. The papers were then dried in warm air and in vacuo until free from acid. The positions of the sugars were located by spraying side-strips with aniline oxalate and by examination under ultra-violet light. Sections of the papers containing the respective sugars and a blank were cut out, after which the paper strips were extracted under reflux with water (5 ml.) for 45 minutes. The extracts were then heated at 100° with sodium meta-periodate (0.25M, 2 ml.) for 20 minutes in stoppered tubes (8" x 1"), the upper parts of which were cooled by condenser coils. After cooling, excess periodate was destroyed by addition of neutral ethylene glycol (0.3 ml.) and after 30 mins. the formic acid was titrated with carbonate-free sodium hydroxide (.0087N) in presence of methyl red indicator. The values were corrected for the paper blank.

Sugar	Titre (ml.)	Wt. of sugar in 10 ml. (mg.)	Approximate molar proportions
galactose	4.142	64.8	1
mannose	1.570	24.6	0.36
arabinose	2.865	46.7	0.86

The proportion of rhamnose was too small to be accurately estimated.

GRAPH 1

COLORIMETRIC ESTIMATION OF SUGARS (PRIDHAM). CALIBRATION GRAPHS



b) By a colorimetric method (102). Standard solutions of sugars were prepared by dissolving millimolar amounts in water, followed by dilution to 25 ml. in standard flasks. Accurately measured volumes (containing 10 to 50 ug. of sugar) were spotted onto paper chromatograms together with suitable volumes of the polysaccharide hydrolysate. The chromatograms were irrigated with solvent (3) for 48 hours at 25° and then dried in air and in vacuo. The papers were sprayed with a freshly prepared p-anisidine hydrochloride solution [purified p-anisidine hydrochloride (1 g.) in methanol (10 ml.) diluted to 100 ml. with butan-1-ol) containing sodium hydrosulphite (0.1 g.)]. Development was carried out at 130° for 5 minutes.

Each coloured spot was cut out and eluted by shaking with 95% aqueous methanol (3.5 ml.) for 5 minutes. The absorbance of each sugar solution was measured within 30 minutes in a Unicam SP 500 Spectrophotometer, at the wavelength of maximum absorbance for the particular sugar under examination (510 mu for pentoses and 400 mu for hexoses). The absorbance was plotted against the weight of each sugar and the linear relationships so obtained enabled the quantities of sugars in the hydrolysate to be determined (Graph 1). In all cases the absorbance of a sugar solution was measured against that of a blank.

Sugar	Absorbance	Wt. of sugar (µg)	Approximate molar proportions
galactose	.170	40	1
mannose	.065	14	0.36
arabinose	.096	29	0.90

II. In a partial acid hydrolysate. Polysaccharide (A) (ca. 200 mg.) was heated at 100° for 7 hours with N sulphuric acid (10 ml.). After working up in the usual way, the hydrolysate was made up to 10 ml. and the relative proportions of the sugars present determined by the colorimetric method. The quantities of mannose and rhamnose were very small and could not be accurately measured.

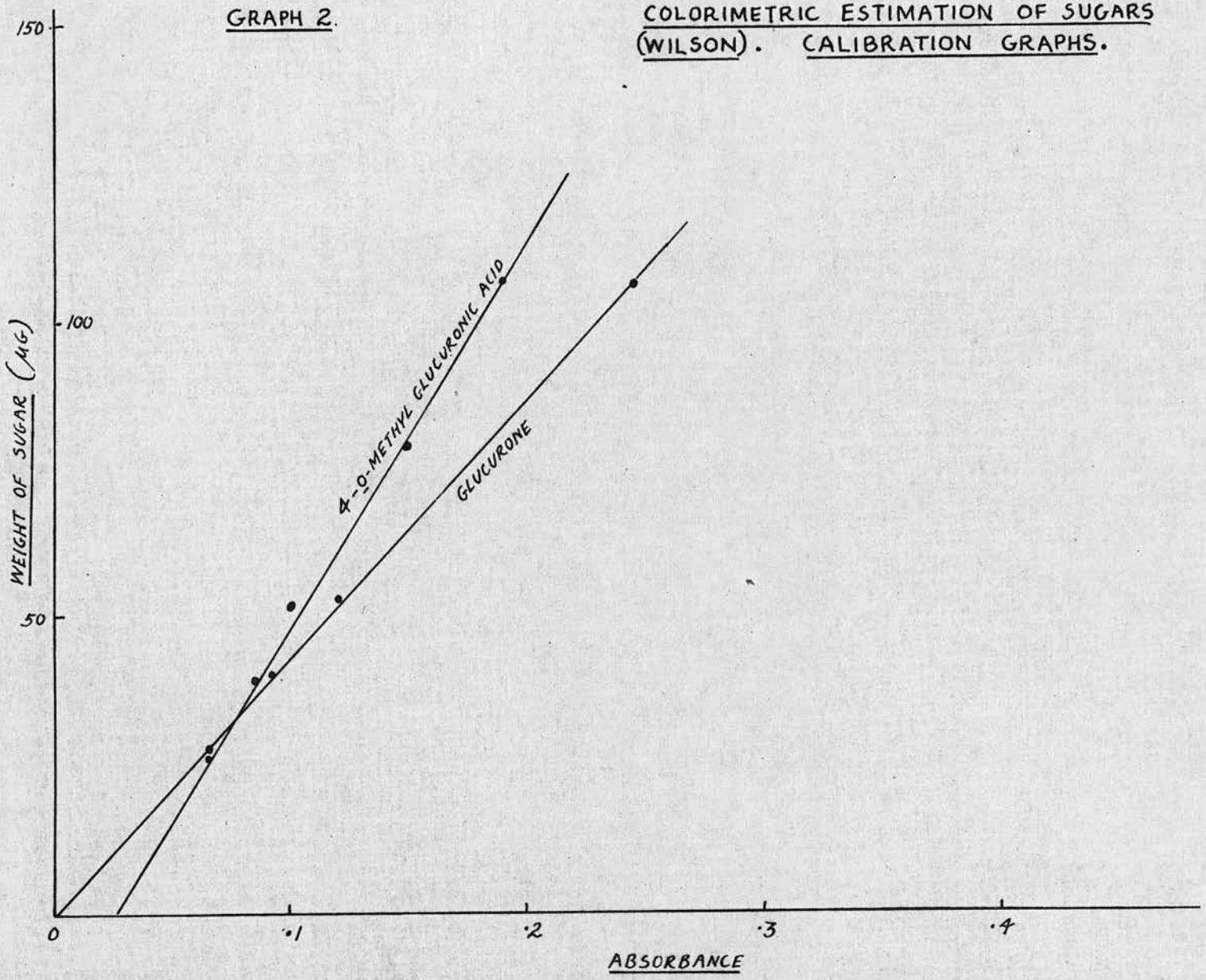
Sugar	Absorbance	Wt. of sugar (µg)	Approximate molar proportions
galactose	.144	32	1
arabinose	.166	37	1.4

EXPT. 6. QUANTITATIVE ESTIMATION OF RHAMNOSE

Determination of the proportion of rhamnose in the unhydrolysed polysaccharide (A) was attempted by the colorimetric method of Dische and Shettles (103). Polysaccharide (A) (5 mg.) in distilled

GRAPH 2.

COLORIMETRIC ESTIMATION OF SUGARS (WILSON). CALIBRATION GRAPHS.



water (1 ml.) and concentrated sulphuric acid (6 ml.) was heated with 3% aqueous cysteine hydrochloride (0.15 ml.) for 3 minutes at 100°. The resulting solution, when cooled, showed none of the greenish-yellow colour characteristic of methyl pentose, and the value of ($E_{396\ mu} - E_{430\ mu}$) measured in a Unicam SP 500 spectrophotometer was indistinguishable from that of a blank. Greater concentrations of polysaccharide resulted in the production of pink colours due to large excess of hexose, and anomalous readings were obtained on the spectrophotometer.

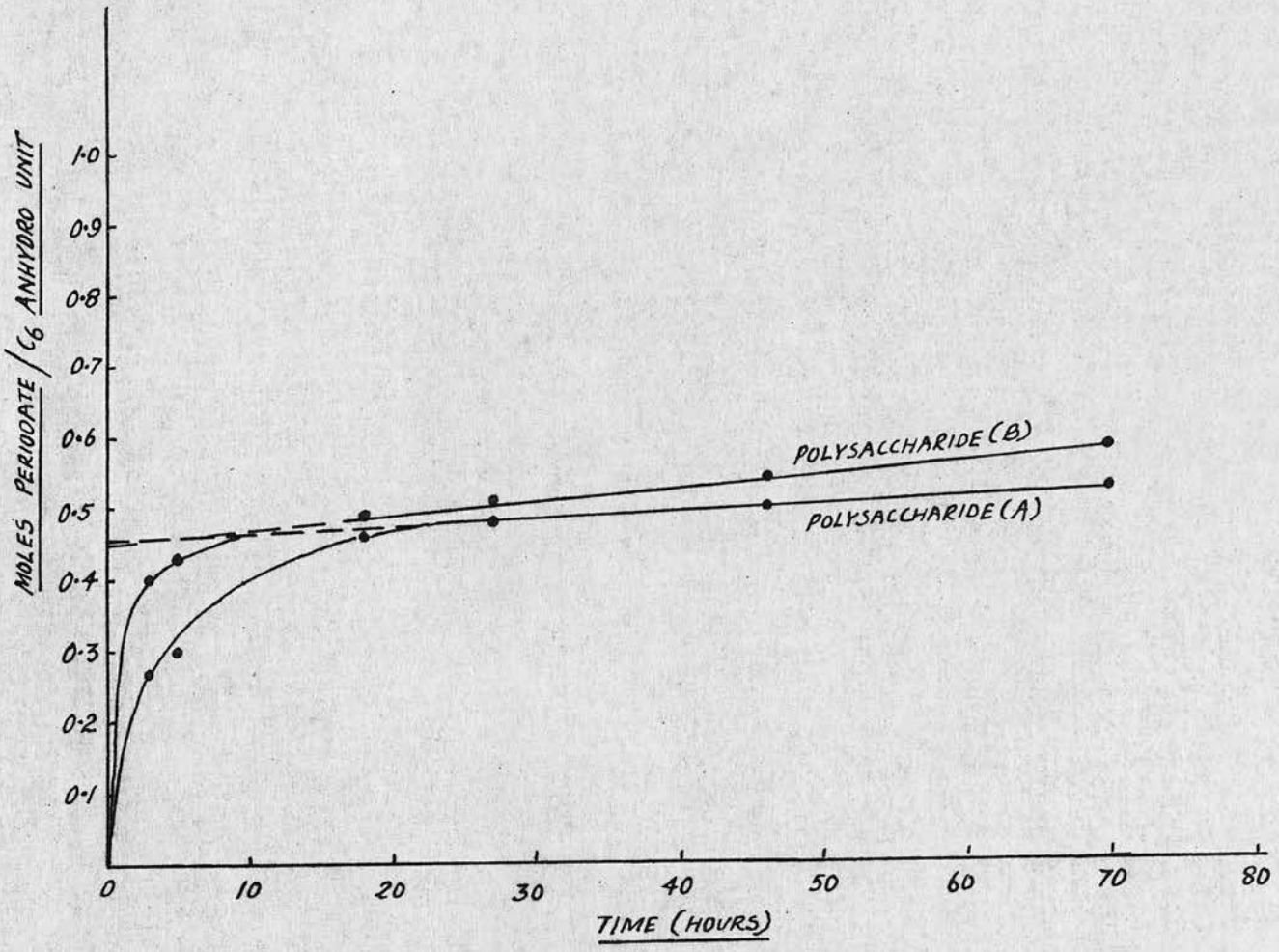
EXPT. 7. PROPORTION OF GLUCURONE TO 4-O-METHYL GLUCURONIC ACID.

The proportions of these sugars were measured in polysaccharides (A) and (B) hydrolysed with 2N sulphuric acid at 100° for 18 hours. The method used was that of Wilson (32) (page 37). Absorbances were measured at 390 mu and the weights of sugars found from calibration graphs (Graph 2). The proportion of glucurone to 4-O-methyl glucuronic acid was found to be the same for both polysaccharides. A typical result is shown below.

Sugar	Absorbance (A)	(B)	Wt. of sugar (µg) (A)	(B)	Molar Proportions (A)	(B)
glucurone	.145	.140	62	60	1.8	1.9
4-O-methyl glucuronic acid	.092	.083	41	37	1	1

GRAPH 3

PERIODATE OXIDATION OF POLYSACCHARIDES (A) AND (B)



EXPT. 8. PERIODATE OXIDATION STUDIES

I. On polysaccharide (A).

a) Measurement of periodate reduced. The polysaccharide (78 mg.) was added to 0.015M sodium metaperiodate (50 ml.). Oxidation was allowed to proceed in the dark at 2° with frequent shaking. At suitable intervals, aliquots (0.2 ml.) were withdrawn and diluted to 50 ml. with distilled water. The amount of periodate in the solution was measured by the method of Aspinall and Ferrier (104) using a Unicam SP 500 spectrophotometer.

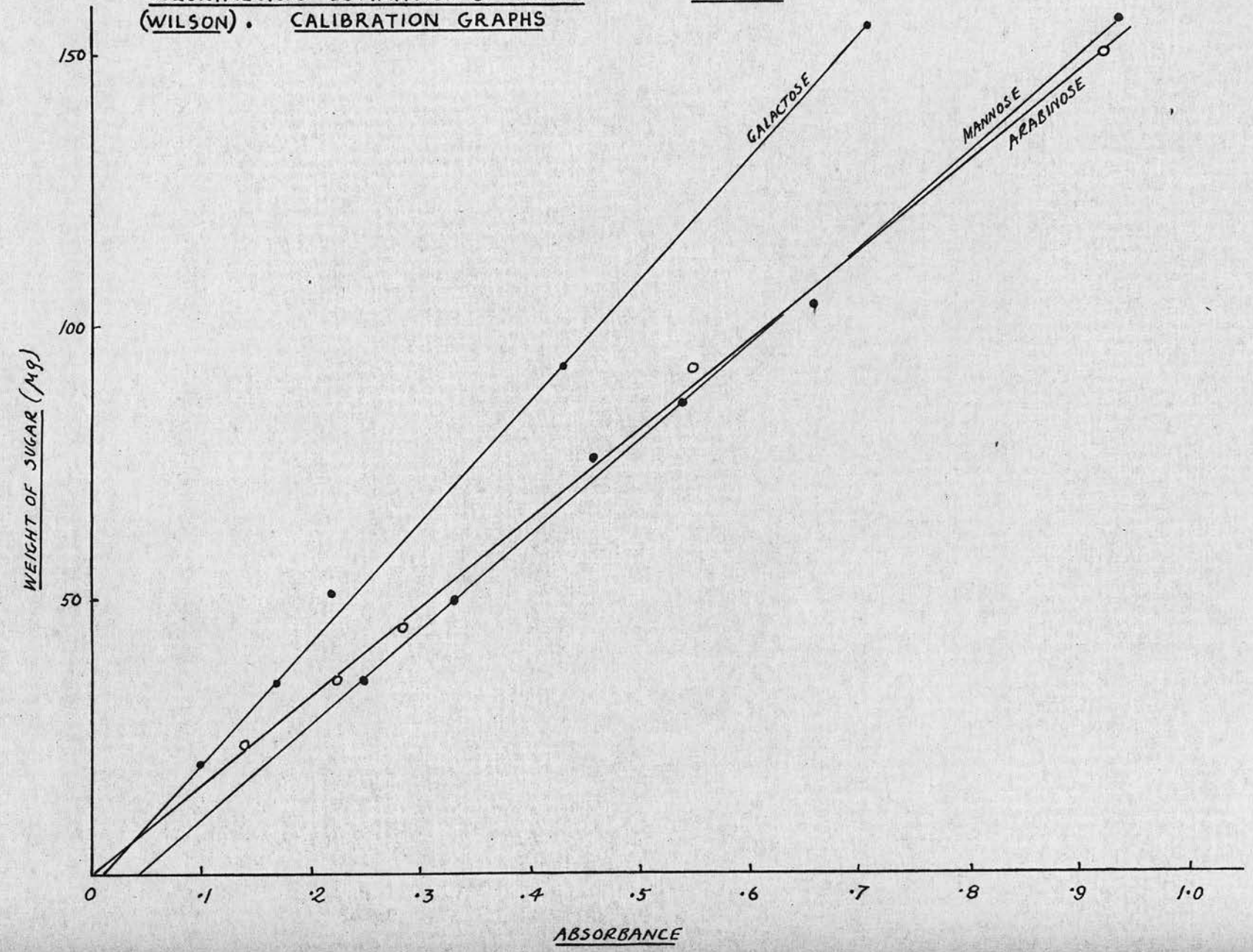
The value of periodate reduced during the primary oxidation was 0.46 moles per anhydro C_6 unit, or 1 mole periodate per 354 g. polysaccharide (Graph 3).

b) Isolation of the oxo-polysaccharide. The oxidation was stopped after 70 hours by addition of excess ethylene glycol to the reaction mixture. The solution was dialysed for 4 days against running water and then freeze-dried to give the oxo-polysaccharide (60 mg.). $[\alpha]_D$ + 3.7° (c, 1.08, H_2O). Chromatographic examination of a hydrolysate of this material in solvents (2) and (3) revealed the presence of arabinose, mannose, galactose and glucurone (trace).

c) Quantitative estimation of the proportions of sugars. The oxo-polysaccharide (10 mg.) was hydrolysed with 2N sulphuric acid (1 ml.) at 100° for 18 hours (i.e. conditions identical with those

COLORIMETRIC ESTIMATION OF SUGARS (WILSON). CALIBRATION GRAPHS

GRAPH 4



used in EXPT. 5 I), and the hydrolysate worked up in the usual way.

Estimation of the sugars was carried out by the colorimetric method of Wilson (32). Aniline phthalate is used as the developing reagent instead of the p-anisidine hydrochloride used by Pridham and the spots are eluted with a solution of 0.7 N hydrochloric acid in 80% aqueous ethanol. As in the Pridham method, the weights of sugars in a hydrolysate are obtained from the corresponding calibration graph of absorbance against weight of sugar (μg) (Graph 4).

The hydrolysate was spotted onto Whatman No.1 paper and eluted with solvent (3) for 36 hours at 25°. After spraying, the spots were developed by heating the chromatogram at 105° for 6 minutes, and then cut out and eluted with the acid eluting reagent (4 ml.). After 1 hour the absorbances were measured in a Unicam SP 500 spectrophotometer at 390 mμ for hexoses and at 360 mμ for pentoses.

Sugar	Absorbance	Wt. of sugar (μg)	Approximate molar proportions
galactose	.515	112	1
mannose	.165	21	.19
arabinose	.235	38	.41
glucurone	.004	-	-

II. On polysaccharide (B).

a) Measurement of periodate reduced. The polysaccharide (218 mg.) was added to 0.015M sodium metaperiodate and oxidation was allowed to proceed under conditions identical with those used for polysaccharide (A).

The value of periodate reduced during the primary oxidation was 0.45 moles per C_6 anhydro unit, or 1 mole periodate per 362 g. polysaccharide (Graph 2).

b) Isolation of the oxo-polysaccharide. Oxidation was stopped after 70 hours by addition of ethylene glycol. A proportion of the polysaccharide remained undissolved throughout the course of the oxidation. This insoluble material (R) was filtered off, dried and weighed (120 mg.). The filtrate was dialysed for 4 days against running water and freeze-dried to give the soluble oxo-polysaccharide (20 mg.) $[\alpha]_D$ +21° (c, 0.82, H_2O). Chromatographic examination of a hydrolysate indicated the presence of arabinose, mannose and galactose. A hydrolysate of the insoluble oxo-polysaccharide (R) showed, in addition, glucurone.

c) Quantitative estimation of the proportions of sugars. As before, the oxo-polysaccharide (10 mg.) was hydrolysed with 2N sulphuric acid (1 ml.) at 100° for 18 hours. Wilson's method was used for determination of the sugars.

i) Soluble oxo-polysaccharide

Sugar	Absorbance	Wt. of sugar (µg)	Approximate molar proportions
galactose	.140	29	1
mannose	.080	7	.24
arabinose	.085	14	.58

ii) Insoluble oxo-polysaccharide (R)

galactose	.515	112	1
mannose	.175	23	.21
arabinose	.190	31	.33
glucurone	.018	7	.06

PARTIAL HYDROLYSIS OF THE POLYSACCHARIDE AND SEPARATION OF THE PRODUCTS

Dried polysaccharide (A) (3.7 g.) was hydrolysed with N sulphuric acid (500 ml.) for 7 hours at 100°. The solution was cooled, neutralised with barium carbonate and then centrifuged. The barium salts were extracted with hot water (2 x 250 ml.) and the extracts added to the main solution. Evaporation to dryness *in vacuo* yielded a brown solid (4.0 g.) consisting of neutral sugars and barium salts of uronic acids.

The hydrolysate (3.5 g.) was made into a slurry with cellulose powder (25 g.) and $^{2}/_{3}$ saturated aq. butan-1-ol and applied to the top of a cellulose column (760 x 37 mm.). The sugars were eluted

with $^{2}/_{3}$ saturated aq. butan-1-ol, which was collected in fractions of 6 ml. Every tenth fraction was evaporated and examined chromatographically in solvent (2). Similar fractions were bulked and evaporated to dryness.

After elution of all neutral sugars, the column was washed with water to elute the barium uronates. Washing was continued until the eluant gave no reaction for barium ions. After evaporation in vacuo at 40°, the free uronic acids were obtained by treatment with IR 120(H) resin. Filtration and evaporation yielded a brown syrup (1.26 g.).

EXPT. 9. CHARACTERISATION OF NEUTRAL SUGARS

Tubes	Fraction	R_{Glu} in solvent (2)	Colour of spot	Wt. (mg.)
100-230	I	2.0	yellow: comparable with rhamnose	79
231-390	II	1.18	pink : " " arabinose	480
		1.16	brown : " " mannose	
391-520	III	1.18	pink : " " arabinose	515
		1.16	brown : " " mannose	
521-605	IV	1.16	diffuse	60
606-1150	V	0.89	brown : comparable with galactose	763
			Total	1897

Each fraction was taken up in the minimum of cold water, filtered and evaporated slowly. Fractions II and V crystallised. The solids were recrystallised from methanol or butan-1-ol.

Fraction I, a colourless syrup, had $[\alpha]_D$ +8.6° (c, 1.16, H_2O) const. Treatment with a saturated ethanolic solution of benzoylhydrazine (66) yielded a crystalline hydrazone, m.p. 188-189° (decomp.). Mixed m.p. 188° with authentic L-rhamnose benzoylhydrazone.

Fraction II, crystalline, had $[\alpha]_D$ +82.3° (c, 1.07, H_2O) const. Recrystallisation from methanol yielded needles, m.p. and mixed m.p. 159° with authentic L-arabinose. The crystals had $[\alpha]_D$ +105° (c, 2.0, H_2O). The derived benzoylhydrazone (105) had m.p. and mixed m.p. 203° with authentic L-arabinose benzoylhydrazone.

Fraction III. Chromatographic examination in solvents (2) and (3) showed the presence of arabinose and mannose (3:1, visual estimation). A sample (135 mg.) was applied to Whatman No.3MM paper and eluted for 56 hours in solvent (3). After spraying side-strips, the portion containing the mannose was extracted with warm water (3 x 60 ml.). Evaporation yielded a colourless syrup (36 mg.), R_{Glu} 1.16 in solvent (2), $[\alpha]_D$ +14.0° (c, 1.23, H_2O) const.

Treatment with phenylhydrazine (106) yielded a crystalline phenylhydrazone, m.p. and mixed m.p. 188° with authentic D-mannose phenylhydrazone.

Fraction IV. Chromatographic examination in solvents (2) and (3) revealed the presence of arabinose, mannose and galactose (3:2:5, visual estimation).

Fraction V. Crystalline, had $[\alpha]_D$ +80.3 (c, 2.32, H_2O) const. Recrystallisation from butan-1-ol yielded white crystals, m.p. and mixed m.p. 164° with authentic D-galactose ($[\alpha]_D$ +142° → +80°). The sugar was further characterised by preparation of the diethylmercaptal derivative (107), m.p. and mixed m.p. 141°.

EXPT. 10. CHARACTERISATION OF THE URONIC ACID FRACTION

The syrup (page 40) was applied to the top of a Grycksbo filter paper column and eluted with solvent (3). Aliquots of every tenth fraction were evaporated and examined chromatographically in solvent (3).

Tubes	Fraction	R_{Glu} in solvent (3)	Colour of Spot	Wt. (mg.)
85-111	A I	3.0	brown : comparable with glucurone	40
112-130	A II	3.0	brown : comparable with glucurone	28
		2.7	red-brown (trace)	
131-174	A III	3.0	brown	42
		2.7	red-brown	
175-390	A IV	3.0	brown	85
		2.7	red-brown	
		1.84	pink : comparable with arabinose	
391-900	A V	1.40	brown : comparable with mannose	140
		1.84	pink	
		0.77	brown	
Water washings		no moving material		840
			Total	1175 mg.

Fraction A I, a syrup, had $[\alpha]_D$ + 17.0° (c, 0.72) and was chromatographically identical with glucurone in solvents (3) and (4). A crystalline p-nitroanilide was prepared (108) and was recrystallised from cold methanol as yellow plates, m.p. and mixed m.p. 130-131° with authentic D-glucuronic acid p-nitroanilide.

Fraction A II had $[\alpha]_D$ + 23.3° (c, 0.56). D-glucurone crystallised from the aqueous solution, and after recrystallisation from water had m.p. and mixed m.p. 177° and $[\alpha]_D$ +18.8° (c, 1.18, H_2O) const., cf. authentic sample $[\alpha]_D$ +19.4 (171).

Fraction A III. Separation on Whatman 3MM paper in solvent (3) gave D-glucurone (10 mg.) and a sugar (27 mg.), $[\alpha]_D$ +42.2° (c, 1.8) which was chromatographically identical with 4-O-methyl-D-glucuronic acid. Methoxyl 14.0% (Calc. for a mono-O-methyl hexuronic acid 14.9%).

The sugar did not crystallise on slow evaporation from aqueous solution. Attempted formation of the amide by the method of Smith (111) was unsuccessful.

Fraction A IV. Separation on 3MM paper in solvent (3) gave the following fractions:

a) 43 mg. D-glucurone, crystallised from aqueous solution.

b) 12 mg. A mixture of D-glucurone and 4-O-methyl-D-glucuronic acid, identified chromatographically.

c) 18 mg. Chromatographically identical with arabinose.

Fraction A V. Separation on 3MM paper in solvent (3) gave the following fractions:

a) 78 mg. Mixture of mannose and arabinose, identified chromatographically.

b) 43 mg. Glucuronic acid, identified chromatographically.

Water washings. The material (840 mg.) showed no movement in basic or acidic solvents in 24 hours.

a) A part of the fraction (540 mg.) was hydrolysed with 90% formic acid (25 ml.) at 100° for 24 hours. Removal of formic acid by

repeated distillation with methanol yielded a dark brown syrup which was then hydrolysed with N sulphuric acid for 3 hours at 100°. After appropriate treatment a pale brown syrup was obtained (123 mg.).

Chromatographic examination in solvents (2), (3) and (4) revealed all the sugars present in the original gum, the arabinose being reduced to trace quantities.

b) The remainder of the fraction (300 mg.) was converted to the methyl ester methyl glycosides and reduced with potassium borohydride. The product, after neutralisation with acetic acid, was hydrolysed with N sulphuric acid at 100° for 5 hours. After working up in the usual way, a syrup (140 mg.) was obtained. Chromatographic examination in solvents (2), (3) and (4) indicated the presence of glucose, 4-O-methyl glucose, arabinose, mannose and galactose. Separation on 3MM paper in solvent (1) yielded the following fractions:

i) D-glucose (24 mg.). The sugar was crystallised from aqueous solution and had m.p. and mixed m.p. 146° with authentic D-glucose.

ii) 4-O-methyl-D-glucose (18 mg.), $[\alpha]_D$ +50.1° (c, 1.36, const.), and R_{Glu} 1.82 in solvent (2), and chromatographically identical with authentic material in solvents (1), (2), (3) and (4). Authentic 4-O-methyl-D-glucose is reported as having $[\alpha]_D$ +53° (114). Electrophoresis in borate buffer (pH 10) at 750 volts for 5 hours gave M_G 0.24, a value identical with that of an authentic sample.

Chromatographic examination of a periodate oxidised sample by the method of Lemieux and Bauer (172) showed an orange-brown spot R_F 0.53 in butan-1-ol : ethanol : water (4 : 1 : 5). Identical treatment of an authentic sample gave the same characteristic spot when sprayed with aniline oxalate.

Attempted osazone formation, by the method of Hough, Jones and Wadman (110) did not yield the expected derivative. Colourless crystals were obtained, m.p. 117°. Infra-red spectroscopic examination suggested that this was a phenylhydrazone.

EXPT. 11. ACETOLYSIS OF THE POLYSACCHARIDE

The polysaccharide (A) (2.0 g.) was added slowly to a rapidly stirred mixture of acetic anhydride (13 ml.), glacial acetic acid (9 ml.) and concentrated sulphuric acid (1.2 ml.) at 0°. The mixture was shaken mechanically at room temperature and aliquots (2 ml.) were withdrawn at intervals. The aliquots were filtered, poured into ice-cold water and neutralised to Congo red with sodium bicarbonate. The precipitated acetylated polysaccharides were removed by centrifugation, washed with water and dried. The centrifugates were extracted with chloroform (4 x 10 ml.) and the combined extracts evaporated to a syrup.

Deacetylation was carried out by dissolution of the solids and syrups in dry methanol (5 ml.) and addition of a 0.5% methanolic solution of sodium methoxide (5 ml.). After allowing to stand

at 3° for 24 hours, water was added until the precipitated sugars just redissolved. After treatment with IR 120(H) resin, the solutions were evaporated to syrups and examined chromatographically in solvents (1), (2), (3) and (5).

Time (hours)	Sugars released
6	Arabinose
15	Arabinose
28	Arabinose
42	Arabinose, galactose, 3 spots R_{Gal} 0.68, 0.38 and 0.26 in solvent (5)

The acetolysis was stopped after 42 hours and the main bulk of the solution worked up as before. A yellow syrup (S) (0.7 g.) was obtained. The aqueous solutions left after extraction of the aliquot filtrates with chloroform were combined, treated with IR 120(H) resin and evaporated to a gel (200 mg.). Dissolution in 0.1N sodium hydroxide, acidification to pH 2 and precipitation with ethanol (5 volumes) yielded a pale brown solid (150 mg.), $[\alpha]_D$ +116.5° (c, 1.0).

A sample (ca. 20 mg.) was hydrolysed (2N sulphuric acid, 18 hours, 100°) and chromatographic examination of the product in solvents (2) and (3) revealed all the sugars present in the original polysaccharide.

Chromatographic examination of the syrup (S) (0.7 g.) in solvent (5) revealed arabinose, galactose and 10 slower spots with R_{Gal} 0.84, 0.77, 0.68, 0.53, 0.49, 0.38, 0.34, 0.26, 0.15 and 0.12, of which 3 were brown, 5 red-brown, 1 pink and 1 orange when sprayed with aniline oxalate. The syrup was separated on Whatman No.17 paper by elution with solvent (5) for 5 days. Side-strips were sprayed with aniline oxalate and the appropriate paper strips cut out and eluted with cold water.

Fraction 1, a syrup (21 mg.), had $[\alpha]_D$ +72° (c, 1.41) and gave a yellow-brown spot R_{Gal} 0.6 in solvent (3) when sprayed with aniline oxalate. The compound was acid. Chromatographic examination after reduction and hydrolysis of the methyl ester methyl glycoside revealed 4-O-methyl glucose and galactose in equal proportions (visual estimation). cf. Fraction A (4) EXPT. 16.

Fraction 2, a syrup (14 mg.) had $[\alpha]_D$ +56.4° (c, 0.93, const.) and R_{Gal} 0.62 and 0.46 in solvents (2) and (3) respectively, identical with authentic 3-O-β-D-galactopyranosyl-L-arabinose run as a control. M_G 0.68 in borate buffer (pH 10).

Hydrolysis of a sample (2 mg.) (N sulphuric acid, 5 hours, 100°) gave galactose and arabinose in equal amounts (paper chromatography, visual estimation). Reduction of a sample (5 mg.) with excess sodium borohydride for 24 hours followed by acid hydrolysis gave galactose only.

Fraction 3, a syrup (286 mg.) had $[\alpha]_D$ +95° (c, 1.0) and showed no chromatographic movement in solvents (2) and (3) during 4 days. Hydrolysis of a sample (5 mg.) (N sulphuric acid, 6 hours, 100°) and chromatographic examination in solvent (3) gave mannose, galactose and arabinose (0.5 : 1 : 1, visual estimation) and unhydrolysed material. A sample (10 mg.), after hydrolysis with 2N sulphuric acid for 18 hours at 100°, showed mannose, galactose, arabinose (equal amounts, visual examination), 4-O-methyl-glucuronic acid, glucurone and unhydrolysed material.

Subsequent fractions proved to be complex mixtures.

EXPT. 12 AUTOHYDROLYSIS OF THE POLYSACCHARIDE (A)

Polysaccharide (A) (1.5 g.), $[\alpha]_D$ +21° was heated with water (1% solution w/v) at 95° for 26 hours. At intervals, aliquots (5 ml.) were withdrawn, cooled and examined polarimetrically in a 0.5 dm. tube. The aliquots were then evaporated at 40° *in vacuo* and the residues examined chromatographically in solvent (2). Further aliquots (5 ml.) were added, after cooling, to a buffer solution of pH 11.4 (12.5 ml.) and 0.1 N iodine solution (2.5 ml.). After allowing to stand in the dark for 2 hours, 3% sulphuric acid (25 ml.) was added and the residual iodine titrated with sodium thiosulphate (112). The iodine number (no. of mls. of 0.1N iodine taken up per gram of polysaccharide) was calculated for each aliquot.

Time (hours)	Rotation (α^{o})	Iodine Number	Sugars released
2	+ 0.08	2.0	arabinose
3	+ 0.10	3.7	"
4	+ 0.11	4.3	"
5	+ 0.11	5.2	"
6	+ 0.12	6.4	"
7	+ 0.12	8.4	"
8	+ 0.13	9.2	"
14	+ 0.14	13.8	arabinose, rhamnose
24	+ 0.18	21.0	" "
26	+ 0.19	21.5	" "

No evidence for the presence of any oligosaccharides could be attained. The residual polysaccharide was precipitated by addition of ethanol and had $[\alpha]_D$ + 45.8 (c, 2.8).

EXPT. 13 PARTIAL ACID HYDROLYSIS

The residual polysaccharide, $[\alpha]_D$ +44° after autohydrolysis of the free acid gum (25 g.) (0.7% solution w/v., 5 hours at 95°), was heated with 0.5 N sulphuric acid (300 ml.) at 95° for 15 hours. Under these conditions maximum yield of aldobiuronic acids from A.karroo gum has been reported (67). Chromatographic examination of an aliquot (5 ml.) showed arabinose, galactose and several slower spots. The entire solution was then cooled, neutralised with barium

carbonate and centrifuged. Addition of the centrifugate to ethanol (6 volumes) precipitated partially degraded barium uronates (10 g.) having $[\alpha]_D$ +56.1° (c, 1.95). The supernatant liquor, after concentration to 100 ml., was poured into ethanol (10 volumes) yielding a further precipitate of barium uronates* (2.6 g.) having $[\alpha]_D$ +61° (c, 1.8). Evaporation of the supernatant solution yielded neutral sugars in the form of a syrup A (8.8 g.). Chromatographic examination of the free acid uronates in solvent (3) indicated that the two fractions* were essentially the same. They were therefore combined.

EXPT. 14 SEPARATION OF THE NEUTRAL FRACTION

Syrup (A) (8.8 g.) was placed on a charcoal column (6 x 35 cm.) and washed with water (6 l.) to remove monosaccharides. These were discarded. The column was then eluted with 5, 10, 12.5, 15 and 17.5% concentrations of aqueous ethanol. As the 5% and 10% ethanol washings gave similar chromatograms they were combined and called fraction (i). Similarly 15% and 17.5% ethanol washings were called fraction (iii).

Solvent	Fraction	Wt. (g)	
Water (6 l.)		5.6 g.	contained arabinose mannose and galactose
5% ethanol (1 l.)	(i)	0.520	oligosaccharides
10% ethanol (1 l.)			+ monosaccharides
12.5% ethanol (2 l.)	(ii)	0.500	
15% ethanol (3 l.)	(iii)	0.980	higher oligosaccharides
17.5% ethanol (4 l.)			
	Total	7.600 g.	

Fraction (i) and (ii) were each separated on Whatman No.17 paper by eluting with solvent (1) for 4 and 5 days respectively. There was a considerable amount of overlapping of spots on both papers. Only those portions of the papers containing clearly separated sugars were cut out and eluted. Similar oligosaccharides from the two fractions were combined and characterised.

Fraction i(1), a syrup (43 mg.) had $[\alpha]_D$ +104° (c, 3.99), R_{Gal} 3.5, R_{Glu} 2.55 in solvent (2) (cf. rhamnose R_{Gal} 2.5, R_{Glu} 1.95) and R_{Gal} 4.8 in solvent (3) (cf. rhamnose R_{Gal} 3.5).
(Found: OMe nil; OEt 23.2%, by infra-red absorption measurement of gases produced by a Zeizel method (179). Calc. for a mono-O-ethylarabinose 25.5%). A sample (5 mg.) was de-ethylated by

treatment with boron trichloride at -80° for 30 minutes (180). Chromatographic examination in solvent (2) gave a single spot identical with arabinose run as a control.

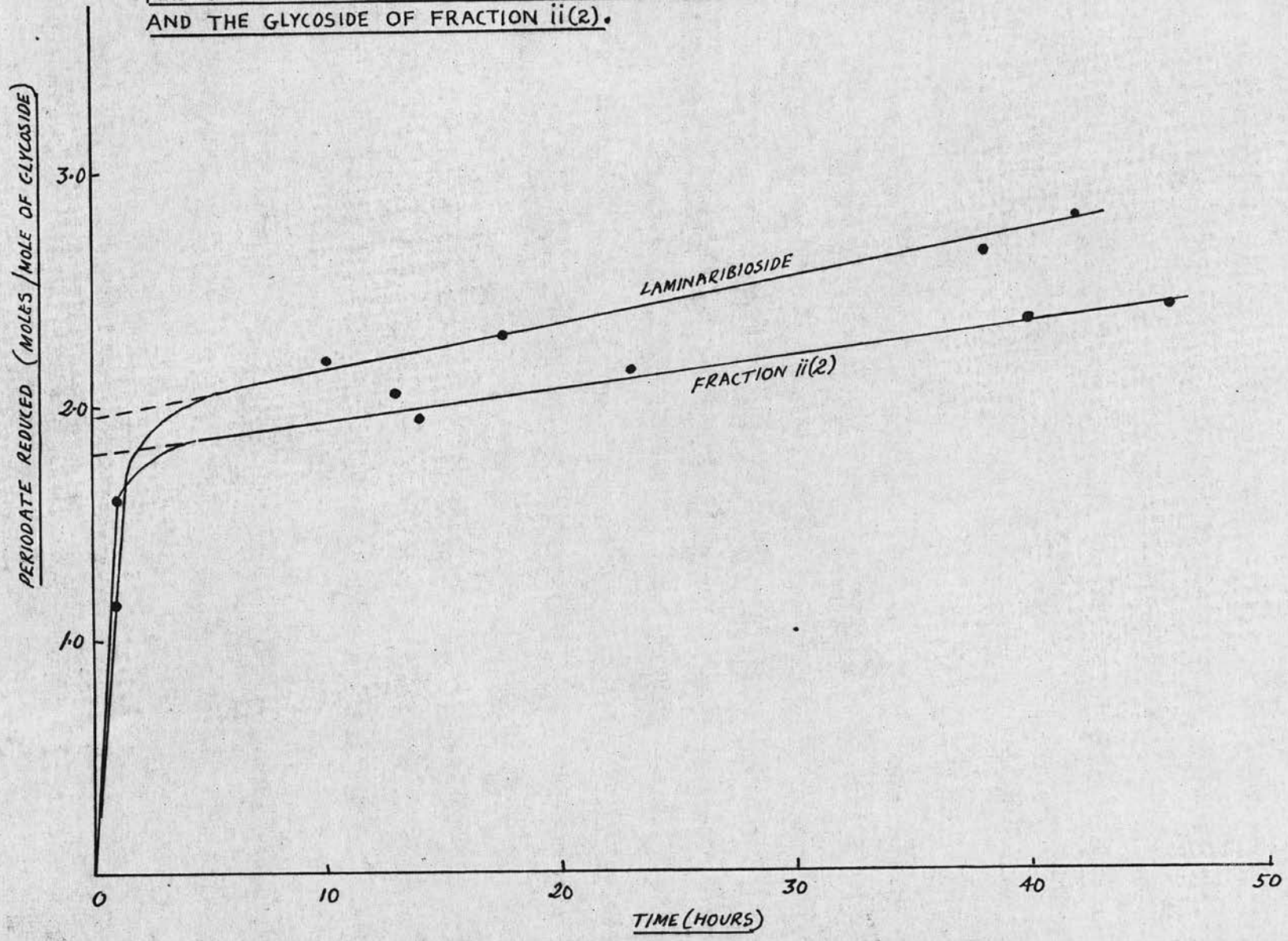
The ethylated arabinose had R_G 0.34 in solvent (6) and M_G 0.30 in borate buffer. 2-O-Methylarabinose had R_{Glu} 1.9 in solvent (2), R_G 0.45 in solvent (6) and M_G 0.35 in borate buffer.

Fraction i(2), identical with fraction 2 (page 48) from the acetolysis experiments (EXPT. 11), was a syrup (20 mg.) which partially crystallised on allowing to stand with aqueous ethanol in the cold. The crystals had m.p. and mixed m.p. with 3-O-β-D-galactopyranosyl-L-arabinose, 200-201°. The syrup had R_{Gal} 0.62 and 0.46 in solvents (2) and (3) respectively and M_G 0.68, identical with an authentic sample run as a control. It had $[\alpha]_D$ +58° (c, 1.85 const.) (cf. 3-O-β-D-galactopyranosyl-L-arabinose $[\alpha]_D$ +62° (70) and gave on acid hydrolysis equal amounts of galactose and arabinose (paper chromatography, visual estimation). Reduction of a sample (6 mg.) with sodium borohydride followed by hydrolysis gave galactose with only trace amounts of arabinose (paper chromatography).

Fraction i(3), a syrup (8 mg.) had R_{Gal} 0.22 in solvent (2) and R_{Gal} 0.19 in solvent (3) identical with 6-O-β-D-galactopyranosyl-D-galactopyranose run as a control. The syrup had $[\alpha]_D$ +26° (c, 0.67 const.) (cf. 6-O-β-D-galactopyranosyl-D-galactose $[\alpha]_D$ +31°) (70) and gave only galactose on acid hydrolysis.

PERIODATE OXIDATION OF METHYL LAMINARIBIOSIDE AND THE GLYCOSIDE OF FRACTION ii(2).

GRAPH 5



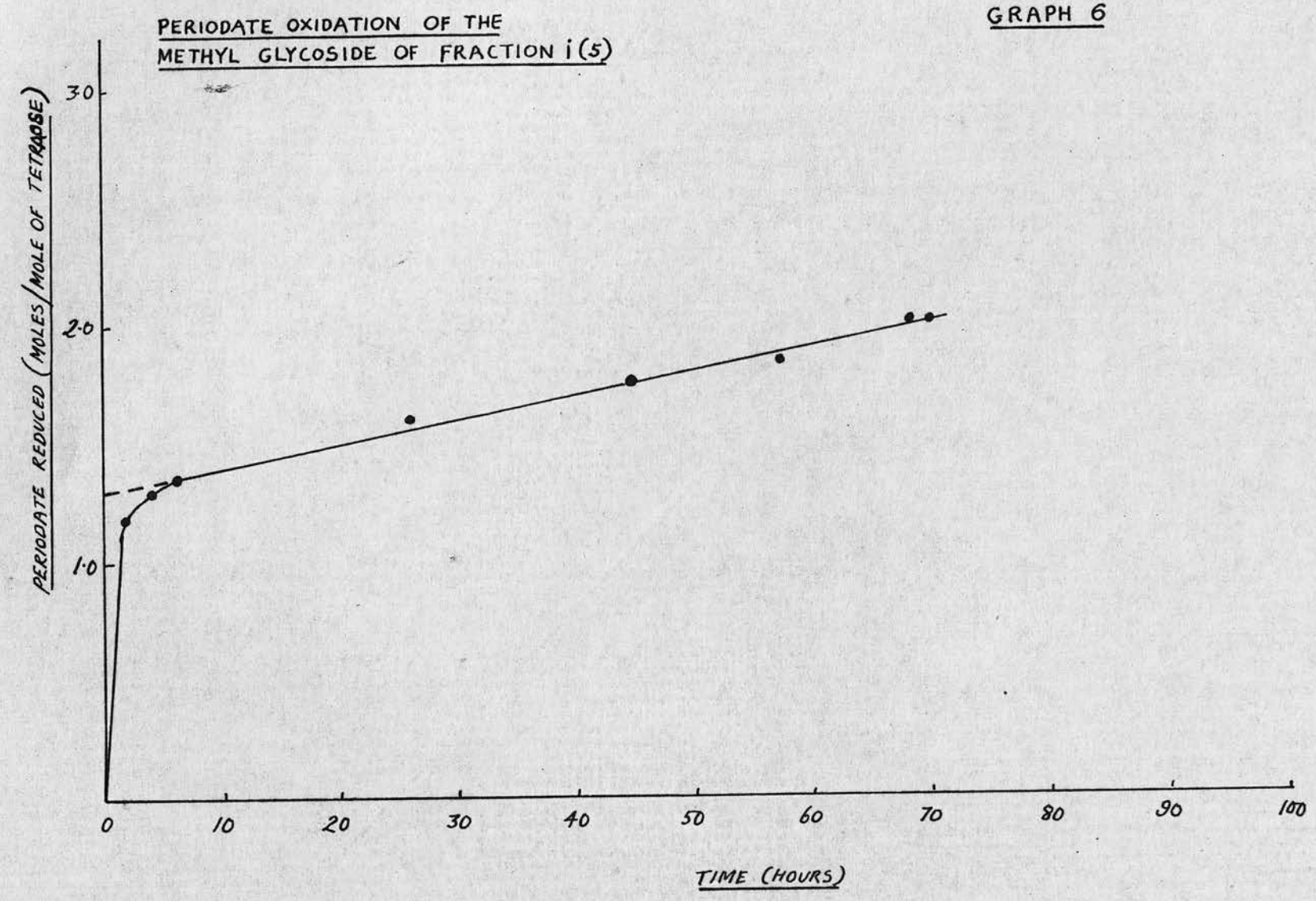
Fractions i(4) and ii(2), a syrup (51 mg.) had R_{Gal} 0.35 in solvent (2) and was identical in solvents (2) and (3) with 3-O-β-D-galactopyranosyl-D-galactopyranose run as a control. Ionophoresis as the N-benzylglycosylammonium ion in formic acid buffer (113) gave a mobility of 0.72. Crystallisation from aqueous ethanol yielded needles of m.p. and mixed m.p. with 3-O-β-D-galactopyranosyl-D-galactopyranose 145-146°. The syrup had $[\alpha]_D$ +58° (c, 3.0 const.) (cf. 3-O-β-D-galactopyranosyl-D-galactopyranose $[\alpha]_D$ +55°) (70) and gave only galactose on acid hydrolysis.

The non-reducing syrup obtained after conversion of a portion (10 mg.) to the methyl glycoside reduced 1.80 moles of periodate per mole of sugar in the dark at 2°. Methyl laminaribioside reduced 1.95 moles per mole in a parallel experiment (Graph 5).

Fraction i(5), a syrup (31 mg.) had $[\alpha]_D$ +71.8° (c, 2.8, const.) and R_{Gal} less than 0.09 in solvent (2). Hydrolysis (2N sulphuric acid, 5 hours, 100°) gave galactose and mannose in the ratio of 3 : 1 (paper chromatography, visual estimation). Partial hydrolysis gave a trace of galactose and unchanged material. Ionophoresis of the N-benzylglycosylammonium ion in formic acid buffer gave a mobility of 0.5.

The non-reducing syrup obtained after conversion to the methyl-glycoside reduced 1.3 moles of periodate per mole of tetraose (Graph 6).

GRAPH 6


PERIODATE OXIDATION OF THE
METHYL GLYCOSIDE OF FRACTION i (5)
PERIODATE REDUCED (MOLES/MOLE OF TETROSE)
3·0
2·0
1·0
0
10
20
30
40
50
60
70
80
90
100
TIME (HOURS)

Fraction ii(1) (34 mg.) proved to be a mixture of monosaccharides.

Fraction ii(2) - see previous page.

It proved to be impossible to separate any of the higher oligosaccharides from fraction (ii).

Fraction (iii) from the charcoal column was separated into 4 fractions on No.17 paper after elution in solvent (1) for 7 days.

Fraction iii(1), an acidic syrup (60 mg.) had $[\alpha]_D$ +72° (c, 3.75) and showed slight chromatographic movement in solvents (2) and (3) during 24 hours. Ionophoresis in borate buffer (pH 10) for 4 hours at 1500 volts yielded 2 spots M_G 0.26 and 0.76. After conversion to the methyl ester methyl glycosides, reduction with sodium borohydride and hydrolysis, the resulting syrup contained equal amounts of 4-O-methylglucose and galactose plus smaller amounts of glucose and mannose (visual estimation, paper chromatography in solvent (2)).

Fraction iii(2), a neutral syrup (35 mg.) had $[\alpha]_D$ +34° (c, 3.2) and gave galactose and arabinose (8 : 1, visual examination) on hydrolysis. The mobility of its N-benzylglycosylammonium ion was less than 0.3. Chromatographic examination of a partial acid hydrolysate in solvent (2) revealed spots with R_{Gal} 1.5, 1.0, 0.62 and 0.35 corresponding to arabinose, galactose, 3-O-galactopyranosyl-L-arabinose and 3-O-galactopyranosyl-D-galactopyranose respectively.

Reduction of a sample (5 mg.) with excess sodium borohydride followed by acid hydrolysis gave galactose and a trace of arabinose (visual estimation, paper chromatography).

Fraction iii(3), an acidic syrup (13 mg.) had $[\alpha]_D$ +9.3 (c, 1.2) and M_G 0.88 in borate buffer. Similar results to fraction iii(1) were obtained after conversion to the methyl ester methyl glycoside, reduction and hydrolysis.

Fraction iii(4), an acidic syrup (50 mg.) had $[\alpha]_D$ +23° (c, 5.1). Ionophoresis in borate buffer gave two spots with M_G 0.80 and 0.90. After reduction and hydrolysis of the methyl ester methyl glycosides the fraction differed from the previous acidic fractions in that it appeared to contain less glucose and only trace amounts of mannose.

EXPT. 15 SEPARATION OF THE ACIDIC FRACTION

The combined barium salts (12 g.) (EXPT. 13) were hydrolysed with 0.5N sulphuric acid (300 ml.) at 100° for 4 hours, the hydrolysate being neutralised in the usual way. The resulting syrup (11 g.) consisting of neutral sugars and barium uronates was placed on a cellulose column (4 x 55 cm.) which was eluted with half-saturated aqueous butan-1-ol until all neutral monosaccharides (3.1 g.) (rhamnose, mannose and galactose) had been removed.

The barium salts were then eluted with butan-1-ol: acetic acid: water (2:1:1) in fractions of 20 ml. An aliquot of every fifth fraction was evaporated at 60°, treated with 1 R 120 (H) resin, filtered and again evaporated. The residues were examined chromatographically in solvent (5). Like fractions were combined, evaporated several times with water to remove acetic acid, treated with 1 R 120 (H) resin and weighed.

Tubes	Fraction	R_{gal} in solvent (5)	Colour of Spot	wt. (mg.)
1 - 47	A	4.5	brown	
		3.7	red-brown	
		1.1	brown	
		0.73	yellow-brown	700
48 - 75	B	1.1	brown	
		0.73	yellow-brown	
		0.40	red-brown	1956
		unhydrolysed material		
76 - 99	C	0.73	yellow-brown	1697
		0.40	brown	
		0.32	yellow	
		0.23	brown	
100 - 119	D	0.06	red-brown	130
120 - 220	E	unhydrolysed material		760
water washings	F	"	"	380
			Total	5.623 g.

Neutral monosaccharides 3.1 g.

% Yield 79.1

EXPT. 16 EXAMINATION OF THE ACIDIC FRACTIONS FROM THE COLUMN

Fractions A and B were separated on Whatman No. 17 paper in solvent (5) for 36 and 72 hours respectively. After drying in air to remove acetic acid, the chromatograms were sprayed with bromocresol green. The appropriate strips were cut out and eluted with cold water (200 ml.). After treatment with charcoal (to remove indicator), filtration and evaporation, the following fractions were obtained:

Separation of Fraction A

Fraction A(1) (186 mg.) was chromatographically identical with glucurone in solvents (3) and (5).

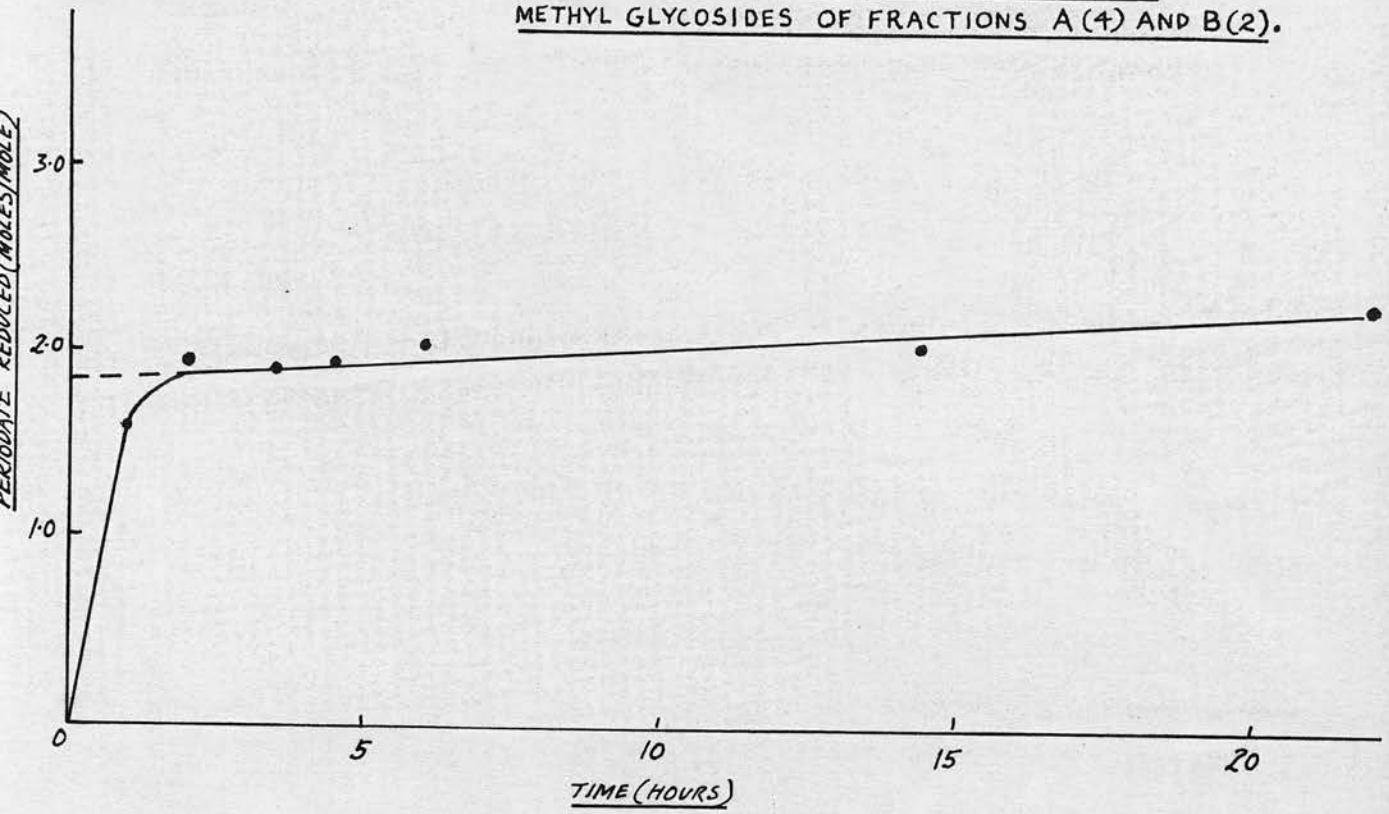
Fraction A(2) (77 mg.) contained 4-O-methylglucuronic acid contaminated with glucurone. Two further separations on 3 MM paper yielded chromatographically pure 4-O-methyl-D-glucuronic acid (38 mg.) having $[\alpha]_D$ + 41° (c, 1.4 const.). Methoxyl 14.0% (Calc. for a mono-O-methyl hexuronic acid 14.9%).

Fraction A(3), a syrup (104 mg.), was found to be a mixture of glucuronic and 4-O-methyl glucuronic acids.

Fraction A(4), a syrup (193 mg.), had $[\alpha]_D$ + 102.8° (c, 2.17) and gave a single yellow-brown spot (spray a) R_{gal} 0.70 in solvent (4). 4-O-α-(4-O-methyl-D-glucopyranuronosyl)-D-galactose has R_{gal} 0.67 in this solvent (77). Ionophoresis gave

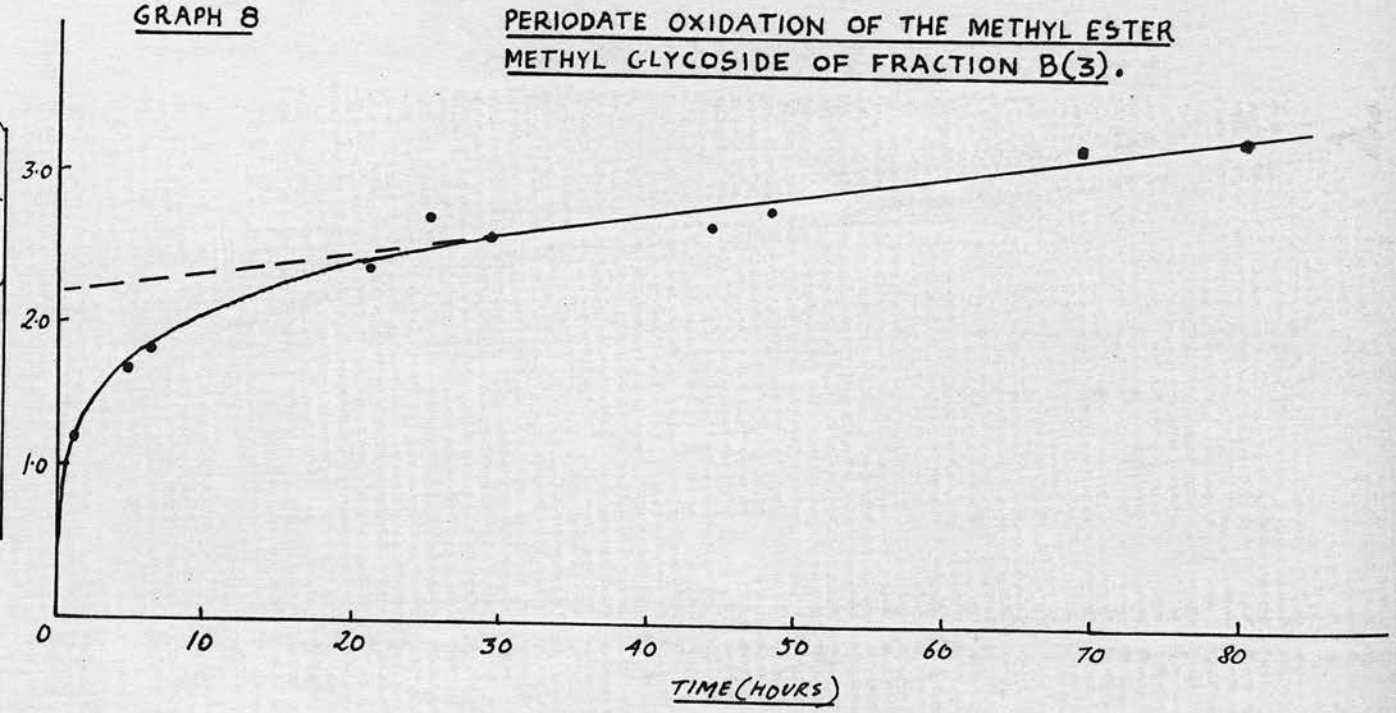
GRAPH 7

PERIODATE OXIDATION OF THE METHYL ESTER METHYL GLYCOSIDES OF FRACTIONS A(4) AND B(2).



GRAPH 8

PERIODATE OXIDATION OF THE METHYL ESTER METHYL GLYCOSIDE OF FRACTION B(3).



a single spot M_G 0.67. The barium salt of the acid had $[\alpha]_D$ + 97° (c, 1.7). The barium salt of 4-O-α-(4-O-methyl-D-glucopyranuronosyl)-D-galactose has $[\alpha]_D$ + 87° ± 4° (77).

The syrup had equivalent weight 360 (Calc. for a monomethyl glucuronosyl galactose 370). A sample (15 mg.) was converted to the methyl ester methyl glycoside and the product reduced with sodium borohydride. The syrup obtained after hydrolysis contained equal amounts of galactose and 4-O-methylglucose (paper chromatography, visual estimation). The methyl ester methyl glycoside reduced 1.8 moles of sodium metaperiodate per mole of glycoside (GRAPH 7).

For further examination of this fraction see B(2).

Separation of Fraction B

Fraction B(1), a syrup (210 mg.), was mainly glucuronic acid.

Fraction B(2), a syrup (338 mg.), was a mixture which after a further separation on Whatman No. 3 MM paper yielded 4-O-α-(4-O-methyl-D-glucopyranuronosyl)-D-galactose (102 mg.). This material was added to the remainder of fraction A(4) and the mixed fractions were converted to the methyl ester methyl glycoside.

Methylation. The glycoside (92 mg.) was dissolved in water (30 ml.) and cooled to less than 10°. Methylation was carried out by the Haworth procedure. Dimethyl sulphate (6 ml.) and 30% sodium hydroxide (15 ml.) were added slowly to the cooled,

rapidly-stirred solution. After addition, the mixture was allowed to reach room temperature and stirred rapidly for 24 hours. This procedure was repeated twice. The solution was then heated to boiling for 30 minutes, acidified with dilute sulphuric acid, and sodium sulphate precipitated by addition of methanol. The filtrate was made slightly alkaline, concentrated to 50 ml., acidified again and extracted with chloroform (5 x 50 ml.). The combined chloroform extracts were evaporated to dryness. The resulting methylated ester glycoside was reduced in dry tetrahydrofuran solution (5 ml.) with lithium aluminium hydride (5 g.). After refluxing for 1 hour, excess lithium aluminium hydride was destroyed by addition of water (0.1 ml.). The solution was diluted with ethyl acetate, filtered and evaporated. After twice extracting the residual material with dry acetone, the residue obtained on removal of the acetone was heated with N hydrochloric acid (1 ml.) at 100° for 4 hours. The product was neutralised with silver carbonate, filtered, evaporated and the residue extracted with acetone. Chromatographic examination of the acetone solution in solvent (6) showed 2,3,4-tri-O-methylglucose and 2,3,6-tri-O-methylgalactose with R_G 0.87 and 0.74 respectively, identical with authentic specimens run as controls. (cf. 2,4,6- and 2,3,4-tri-O-methylgalactoses R_G 0.67 and 0.64 respectively.)

Fraction B(3), a syrup (739 mg.), was a mixture of the previous aldobiuronic acid, a slower spot with R_{gal} 0.33 and 0.46 in solvents (3) and (4) respectively, and higher oligosaccharides.

Two further separations on No. 17 paper in solvent (4) yielded a syrup (90 mg.). This gave a single red-brown spot (spray a) R_{gal} 0.46 in solvent (4) and had $[\alpha]_D$ - 32.4° (c, 0.83). The free acid had $R_{Glucurone}$ 0.14 in solvent (4) identical with that reported for 2-O-glucopyranuronosyl-mannose (174), and the syrup had equivalent weight 365 (Calc. for a glucuronosyl mannose 356). Hydrolysis of a sample (5 mg.) (2 N sulphuric acid, 5 hours, 100°) and chromatographic examination in solvent (4) showed mannose, glucurone and a faint spot for glucuronic acid. A sample (6 mg.) was converted to the methyl ester methyl glycoside, and after borohydride reduction and hydrolysis yielded glucose and mannose in equal amounts (paper chromatography, visual estimation). The methyl ester methyl glycoside reduced 2.2 moles per mole of .015 M sodium metaperiodate at 2° in the dark (GRAPH 8).

Methylation. The methyl ester methyl glycoside (35 mg.) was reduced with sodium borohydride. After neutralisation and removal of borate by repeated distillation with methanol, the product was subjected to Haworth methylation in the same way as for the previous aldobiuronic acid. After extraction with chloroform, the methylated disaccharide was hydrolysed by heating with N hydrochloric acid (1 ml.) at 100° for 5 hours. Chromatographic examination of the product showed the presence of two spots R_G 1.00 and 0.77 in solvent (6), identical with 2,3,4,6-tetra-O-methyl glucose and 3,4,6-tri-O-methyl-mannose respectively. (cf. 2,3,6-tri-O-methyl-mannose R_G 0.81.)

DISCUSSION

Albizzia zygia (Macbride), also known as A. brownei (Walp.) or West African walnut, is a tree of widespread distribution throughout West and Central Africa. It is particularly abundant in Ghana and Southern Nigeria, where it grows usually of thirty to forty feet in height in the deciduous and secondary forests (162). The leaves, bark and roots of the tree are used medicinally in S. Nigeria (163) and the wood provides a good timber suitable for building purposes (163, 164). The tree is, in fact, grown in plantations for this purpose (165).

The genus Albizzia, comprising some twenty-six species, is a member of the Mimosaceae, a family which also includes such gum-bearing genera as Acacia and Prosopis. Only two species of Albizzia, however, are known to produce gum. These are A. sassa and A. zygia. Samples of A. zygia gum from Uganda have been described as irregular dark brown tears, varying in weight from 8 oz. to 0.25 oz. or less and in most cases containing woody impurities (166). A purer form, of a pale yellow colour (167) is of some commercial value, but the gum is not collected on a large scale as is gum arabic. The gum is apparently exuded from insect borings in the bark of the tree (167).

The crude gum.

The present sample of gum, collected from the Gold Coast (Ghana), West Africa, consisted of brittle red-brown nodules, of varying size and having an odour of acetic acid. In contact with

cold water, the nodules swelled, forming a pale-brown gel, and leaving an insoluble, horn-like residue. The gel was slightly acid to litmus. Analysis of individual nodules showed the presence of moisture (17%), ash (5.8%), probably due, at least in part, to metallic salts of uronic acids, and acetyl (3.6%). The presence of acetyl groups is a common feature of unpurified gum nodules, particularly in Sterculia spp. (93, 94, 66), and has been reported in the exudates of Cochlospermum gossypium (168) and Fagara Xanthoxyloides (169).

The crude gum did not reduce Fehling's solution and did not form an insoluble copper complex. Acid hydrolysis and chromatographic examination of the products gave evidence for the presence of galactose, mannose, arabinose, glucuronic acid, 4-O-methyl-glucuronic acid and trace quantities of rhamnose.

Purification and properties of the polysaccharide (EXPTS. 2 and 4)

The gum was treated with aqueous sodium hydroxide solution at room temperature and the mixture acidified with hydrochloric acid. Addition of ethanol precipitated polysaccharide (A). After washing to remove chloride ions, the dried, de-acetylated polysaccharide amounted to 58% by weight of the crude dried gum. A brown residue left after the alkali treatment was extracted with water at 100°. Acidification and precipitation with ethanol gave de-acetylated polysaccharide (B) (16% by weight of crude dried gum).

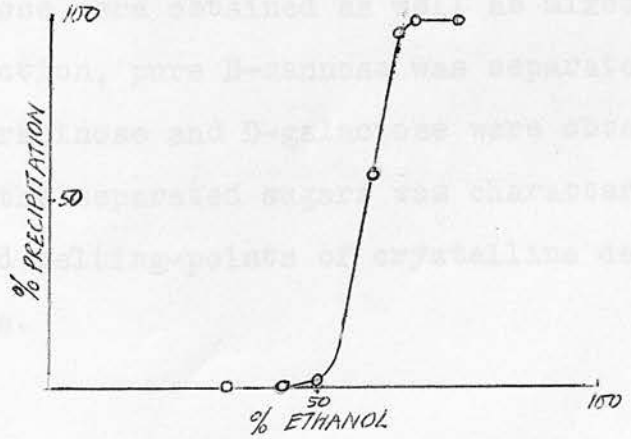
Polysaccharide (A), a white powder, had $[\alpha]_D$ + 21°, ash 0.25%, methoxyl 1.3% and ethoxyl 1.3%. The proportion of uronic acid

present was determined by two methods. Direct titration with alkali gave an equivalent weight of 723, a value which requires a uronic anhydride content of 24.3%. Thermal decarboxylation gave a value of 23.5% for the uronic anhydride content.

Polysaccharide (B) was insoluble in water and formed a gel, but a solution in 0.1 N sodium hydroxide solution had $[\alpha]_D$ + 38.7°. The polysaccharide had ash 0.22% and acetyl nil. Uronic anhydride content, as determined by thermal decarboxylation, was 32.6%. This proportion of uronic acid, although considerably more than is reported in any other species of the Mimosaceae, is not so high as in Sterculia gums.

Attempted fractionation of polysaccharide (A)

Graded precipitation by addition of ethanol did not result in any fractionation (EXPT. 3). The major precipitates when hydrolysed under identical conditions and examined chromatographically, showed identical constituents. Furthermore, a plot of the fraction of polysaccharide precipitated against the concentration of ethanol in the solution gave a curve typical for a homogeneous polysaccharide (170). Polysaccharide (B) gave a similar result.



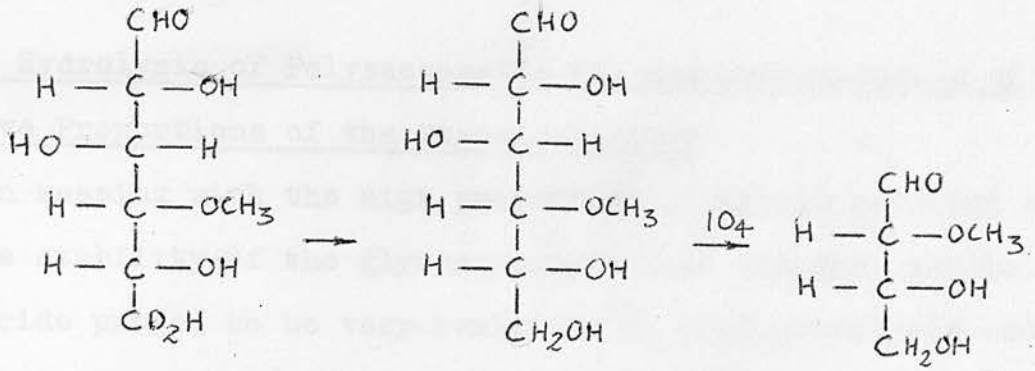
Cetyltrimethylammonium bromide, "Cetavlon," has been used with success for the separation of acidic from neutral polysaccharides (24) and for fractionation of neutral polysaccharides (26). Under certain conditions, polysaccharides may be precipitated with this reagent in the form of a complex from which the free polysaccharide may be obtained by treatment with acetic acid. Polysaccharide (A) was found to complex with "Cetavlon" in unbuffered alkaline solution and in buffered solutions of pH 4, 7, and 9 (EXPT. 3). Addition of ethanol to the supernatant liquor did not precipitate any material and polysaccharide (A) was recovered unchanged from the precipitated complexes. It was concluded, therefore, that no fractionation had occurred.

Isolation and characterisation of the neutral sugars (EXPT. 9)

A syrup, consisting of neutral sugars and barium salts of uronic acids was obtained by hydrolysing polysaccharide (A) with N sulphuric acid for seven hours at 100°. This was separated into its constituent sugars by partition chromatography on a cellulose column which was eluted with two-thirds saturated aqueous butan-1-ol. Pure fractions of L-rhamnose, L-arabinose and D-galactose were obtained as well as mixed fractions. From one such fraction, pure D-mannose was separated by paper chromatography. Both L-arabinose and D-galactose were obtained crystalline and each of the separated sugars was characterised by melting-points and mixed melting-points of crystalline derivatives and by optical rotations.

Separation and characterisation of the barium uronate fraction (EXPT. 10)

The barium uronates were eluted from the cellulose column with water. After deionisation with 1 R 120 (H) resin and evaporation, the mixture of free acids was separated on a Grycksbo filter-paper column. D-glucurone was obtained and was characterised by optical rotation, melting-point and mixed melting-point, and the formation of the crystalline p-nitroanilide which was identical with an authentic sample. Most of the other fractions were contaminated with arabinose and mannose, but after separation on paper, chromatographically pure syrupy 4-O-methyl-D-glucuronic acid was obtained having $[\alpha]_D$ + 42.2°, and ome 14.0%. / The specific rotation of this sugar (syrupy) has been reported as + 48° and + 45° (slightly impure) (109, 110). Attempted formation of the crystalline amide by the method of Smith (111) was unsuccessful. Reduction and hydrolysis of the methyl ester methyl glycoside yielded material chromatographically and ionophoretically identical with 4-O-methylglucose. This sugar, on oxidation with sodium meta-periodate, yields 2-O-methylerythrose, characterised as an orange spot R_F 0.53 on a paper chromatogram (172). The above syrup gave this spot on chromatographic examination after periodate oxidation, confirming that the syrup was indeed 4-O-methylglucose, and that the polyuronide from Albizzia zygia contains 4-O-methyl-D-glucuronic acid in addition to D-glucuronic acid.



4-O-methylglucuronic acid 2-O-methylerythrose

Attempted formation of the osazone from the syrupy 4-O-methyl-D-glucose was unsuccessful. Whereas the melting-point of the osazone has been reported as 158-159° (110), colourless plates of m.p. 117° were obtained. Infra-red spectroscopic data gave evidence for the presence of a phenylhydrazone grouping but in the absence of any authentic material, the identity of this derivative must remain in doubt.

The water-washing from the column, consisting of high-molecular weight material was submitted to hydrolysis with 90% formic acid (page 44). Chromatographic examination of the product revealed the presence of all the sugars in the original polysaccharide, the arabinose being present in trace quantities. A portion of the water washings, after glycosidation, was reduced with sodium borohydride. Separation of the products after hydrolysis yielded pure fractions of D-glucose and 4-O-methyl-D-glucose. The former was obtained crystalline and characterised by melting-point and mixed melting-point. The latter, a syrup, had optical rotation, chromatographic and ionophoretic constants identical with authentic material.

Acidic Hydrolysis of Polysaccharide (A) and Determination of the Relative Proportions of the Monosaccharides

In keeping with the high proportion of uronic acid and the extreme stability of the glycosiduronic acid linkage, the polysaccharide proved to be very resistant to acid hydrolysis and a completely quantitative hydrolysis was impossible. The conditions required for complete hydrolysis caused degradation of fragments already cleaved by the acid. Accordingly the relative proportions of neutral sugars in the gum were determined on hydrolysates obtained under different conditions (EXPT. 5). The sugars, after separation, were estimated by two methods; firstly by periodate oxidation and titration of the liberated formic acid (101) and secondly by a colorimetric reaction with p-anisidine hydrochloride (Pridham, 102), the intensity of colour being measured spectrophotometrically.

When the gum polysaccharide (A) was heated at 100° with 2 N sulphuric acid for eighteen hours, the molar proportions of neutral sugars in the hydrolysate were:

galactose	mannose	arabinose	
1	0.36	0.86	by periodate oxidation
1	0.36	0.90	by Pridham's method

On hydrolysis with N sulphuric acid for seven hours at 100°, the following molar ratios were obtained:

galactose	mannose	arabinose	
1	trace	1.4	by Pridham's method

The proportion of rhamnose on the hydrolysates was too small to be accurately measured. This sugar could not always be detected on chromatograms and when faint spots were obtained in quantitative estimations, titres and absorbances were not significantly different from those of blanks. An attempt was made to measure the amount of rhamnose in unhydrolysed polysaccharide (A). Under appropriate conditions, the colour produced by the reaction of methylpentoses with cysteine hydrochloride may be measured spectrophotometrically. In this case, (EXPT. 6), the distinctive yellow colour was not obtained, and the solutions showed pink tints due to very large excess of hexose (103). It was therefore concluded that the rhamnose content is very small, possibly less than 3%.

It appears from the molar proportions obtained, that under the drastic conditions necessary to cleave the mannose, some of the arabinose is degraded. This indicates that the mannose is tightly bound within the molecule possibly by glycosiduronic linkages (although mannans themselves are hydrolysed with difficulty) while the arabinose is considerably more acid-labile. This conclusion is substantiated by autohydrolysis experiments (EXPT. 12). The polysaccharide was dissolved in water (1% solution) and heated at 95°. Two hours' heating or less was sufficient to release some arabinose, while after twenty-four hours this sugar was present in the hydrolysate in considerable quantities together with trace amounts of rhamnose. This indicates that some of the arabinose is present as end units, possibly in the furanose form. During twenty-six hours' heating, the optical rotation of a

measured sample of solution rose from + 0.08° to + 0.19° and an iodine number of 21.5 was obtained. The residual unhydrolysed polysaccharide had $[\alpha]_D$ + 45.8°.

The failure to detect any arabinose oligosaccharides in the autohydrolysis solution may be due to the fact that the commonly occurring disaccharide (3-O-β-L-arabinosyl-arabinose) of this sugar has R_{Gal} values of 2.0 and 1.4 in solvents (3) and (2) respectively, identical with those of arabinose itself.

The relative proportions of glucuronic and 4-O-methyl-glucuronic acids were determined on hydrolysates of polysaccharides (A) and (B) (EXPT. 7). Both polysaccharides were hydrolysed under the same conditions as used for complete acid hydrolysis (EXPT. 5) and using the method of Wilson for colorimetric analysis, the following results were obtained:

	Molar Proportions in (A)	Molar Proportions in (B)
glucurone	1.8	1.9
4-O-methyl-glucuronic acid	1.0	1.0

The methoxyl content of polysaccharide (A) is in agreement with this result. A polyuronide containing about 24% uronic anhydride with a methoxyl value of 1.3% requires a ratio of glucuronic acid to 4-O-methylglucuronic acid of 2:1. The slightly lower ratio attained in the above experiments may be explained by the fact that the small proportion of glucurone which runs in the acid form on the paper chromatogram is not included in the determination.

When the results from the various quantitative estimations are correlated, the molar ratios of the monosaccharides in poly-

saccharide (A) are as follows:

Sugar	Molar Ratios	Approx. Molar Ratios
D-galactose	1	4
D-mannose	0.36	1.5
L-arabinose	1.4	6
L-rhamnose	trace	-
D-glucuronic acid	0.53	2
4-O-methyl-D-glucuronic acid	0.25	1

Periodate oxidation studies (EXPT. 8)

Polysaccharides (A) and (B) were treated with sodium meta-periodate, the course of the reaction being followed spectrophotometrically. Polysaccharide (A) consumed 0.46 moles of periodate per anhydro hexose unit, or, alternatively, 1 mole of periodate for every 354 g. of polysaccharide. Polysaccharide (B), oxidised under identical conditions, reduced 0.45 moles per anhydro hexose unit or 1 mole of periodate for every 362 g. of polysaccharide. While polysaccharide (A) was completely soluble in the reaction mixture, approximately half (residue R, page 38) of polysaccharide (B) remained undissolved. The relatively low periodate uptake of both polysaccharides indicates that a considerable proportion of the monosaccharide units do not contain adjacent hydroxyl groups and are either linked 1,3' or are triply linked.

Isolation of the oxopolysaccharides was effected, after dialysis against running water, by freeze-drying the resulting ion-free solutions. Oxopolysaccharides (A) and (B) had specific

rotations of + 3.7° and + 21° respectively. Acid hydrolysis of a sample of each and chromatographic examination showed the presence of galactose, mannose and arabinose in both, but in different proportions. Residue (R) showed, in addition, glucurone. 4-O-Methylglucuronic acid was not detected in any of the oxopolysaccharides. A recent improved colorimetric method, that of Wilson (32) was used to determine quantitatively the molar ratios of the sugars. The following results were obtained:

	galactose	mannose	arabinose	glucurone
Oxopolysaccharide (A)	1	0.19	0.41	-
Oxopolysaccharide (B)	1	0.24	0.58	-
Residue (R)	1	0.21	0.33	0.06

It will be seen, on comparison with the unoxidised polysaccharide (page 34), hydrolysed under identical conditions, that the arabinose and mannose residues have been cleaved by the periodate in considerably higher proportion than those of galactose. This would seem to indicate that the galactose comprises the backbone of the molecule and is, to a large extent, linked 1,3' or is triply linked, while a large proportion of the arabinose and mannose units are involved in linkages which permit adjacent hydroxyl groups or are terminal units. Except for the presence of mannose and of two uronic acids, polysaccharides of this type are general in other members of the Mimosaceae (page 85). As the glucuronic acid was reduced to trace quantities and 4-O-methylglucuronic acid was not detected at all, these residues are probably present either

as end groups or are linked 1,2' or 1,4', thus permitting α-glycol cleavage. The small proportion of uronic acid remaining in residue (R) may result from under-oxidation due to steric hindrance and is not definite evidence for other types of linkage. The further differences between polysaccharides (A) and (B) revealed by periodate oxidation increases the possibility that although these two fractions may have the same basic constitution, they differ in the fine details of structure.

Partial acid hydrolysis and isolation of oligosaccharides

After autohydrolysis of polysaccharide (A) (25 g.), the residual material was heated with 0.5 N sulphuric acid at 95° for 15 hours (EXPT. 13). The neutralised solution was separated into an amorphous degraded barium salt of the polysaccharide and a neutral syrup containing mono- and oligosaccharides. Elution of this syrup on a charcoal column with successively greater concentrations of ethanol yielded a series of mixed fractions, each of which was separated on thick paper (EXPT. 14). In all cases, the mixtures were complex and a quantitative separation was impossible. However pure samples of the following neutral disaccharides were obtained and characterised.

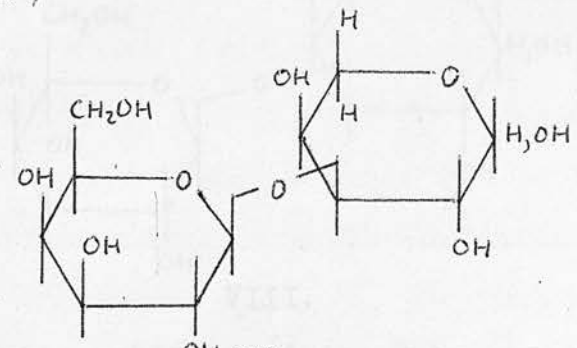
3-O-β-D-galactopyranosyl-L-arabinose

3-O-β-D-galactopyranosyl-D-galactose

6-O-β-D-galactopyranosyl-D-galactose

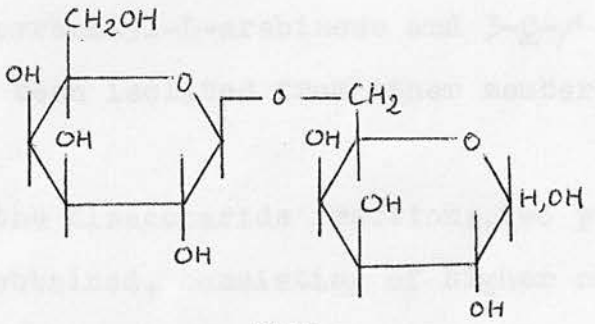
3-O-β-D-Galactopyranosyl-L-arabinose fraction i(2) had specific rotation (70) and chromatographic and ionophoretic mobilities

identical with those of authentic material. Hydrolysis yielded equal quantities of galactose and arabinose while sodium borohydride reduction eliminated the pentose residue, showing that this unit carried the reducing group. Crystallisation from aqueous ethanol afforded crystals the melting point of which was not depressed on admixture with 3-O-β-D-galactopyranosyl-L-arabinose (VI).



VI.

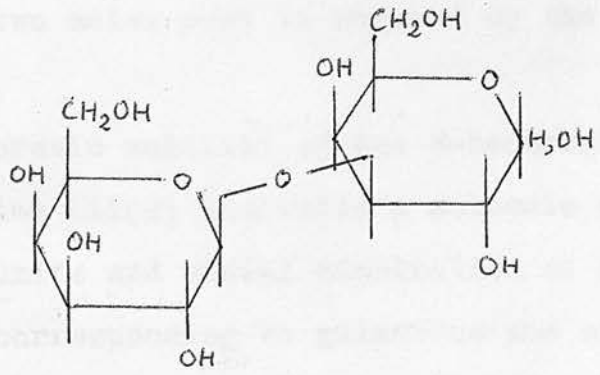
Although the rotation of the syrupy 6-O-β-D-galactopyranosyl-D-galactose fraction i(3) (VII) was slightly low it had chromatographic mobilities identical with an authentic sample of this disaccharide in several solvents and gave only galactose on hydrolysis. The small amount of material available precluded further characterisation.



VII.

3-O-β-D-Galactopyranosyl-D-galactose, fractions i(4) and ii(2), (VIII) was characterised by its melting-point, rotation and

chromatographic mobilities and the fact that only galactose was derived on acid hydrolysis. Furthermore, the amount of periodate reduced by the methyl glycoside of this fraction was the same as that consumed by methyl laminaribioside, a further proof of the 1,3'-linkage.



VIII.

The ionophoretic mobility of its N-benzylglycosylammonium ion provided additional evidence of its disaccharide nature. This technique gives an indication of molecular size regardless of the type of linkage present in the molecule. Both fraction ii(2) and laminaribiose had mobilities of 0.72, corresponding to disaccharides (113).

The above three disaccharides are common in plant gums and 3-O-β-D-galactopyranosyl-L-arabinose and 3-O-β-D-galactopyranosyl-D-galactose have been isolated from other members of the Mimosaceae (page 20).

As well as the disaccharide fractions, two pure fractions i(5) and iii(2) were obtained, consisting of higher neutral oligosaccharides. Fraction i(5) was a syrupy oligosaccharide containing galactose and mannose. The ionophoretic mobility of its N-benzylglycosylammonium ion indicated that it was probably a tetraose and

visual examination of a paper chromatogram of an acid hydrolysate revealed the ratio of galactose to mannose as about 3:1. The small amount of periodate reduced by the methyl glycoside of this fraction can be explained only by the presence of 1,3' linkages, since at least two moles must be reduced by the non-reducing end unit alone.

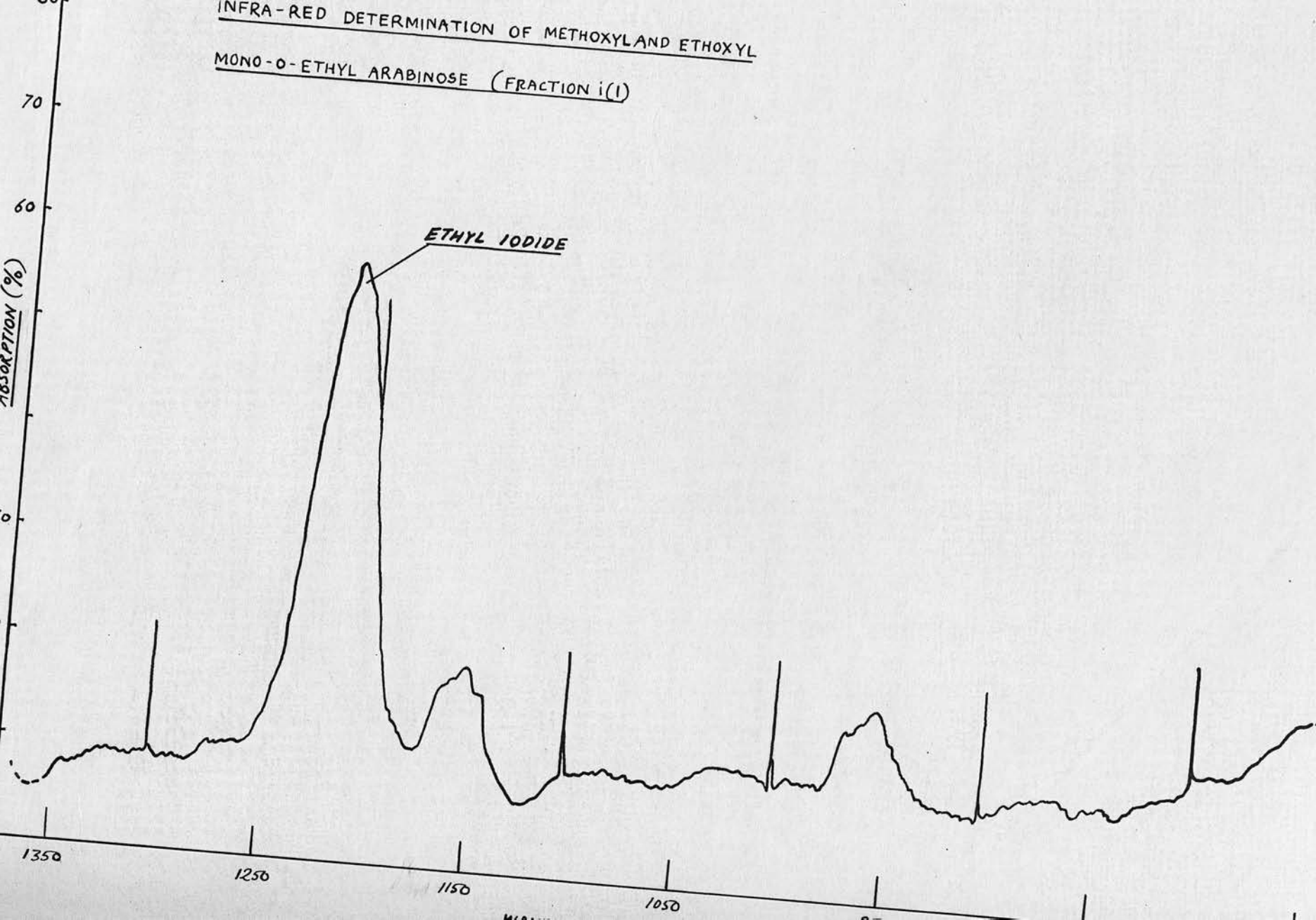
The ionophoretic mobility of the N-benzylglycosylammonium ion of syrupy fraction iii(2) indicated a molecule of more than six monosaccharide units and visual examination of a total hydrolysate revealed spots corresponding to galactose and arabinose in the ratio of about 8:1. Chromatographic examination of a partial acid hydrolysate showed, in addition to galactose and arabinose, spots with the mobilities of 3-O-galactosyl-galactose and 3-O-galactosylarabinose. Borohydride reduction of this fraction removed the greater part of the arabinose, indicating that this sugar was present as the reducing end unit.

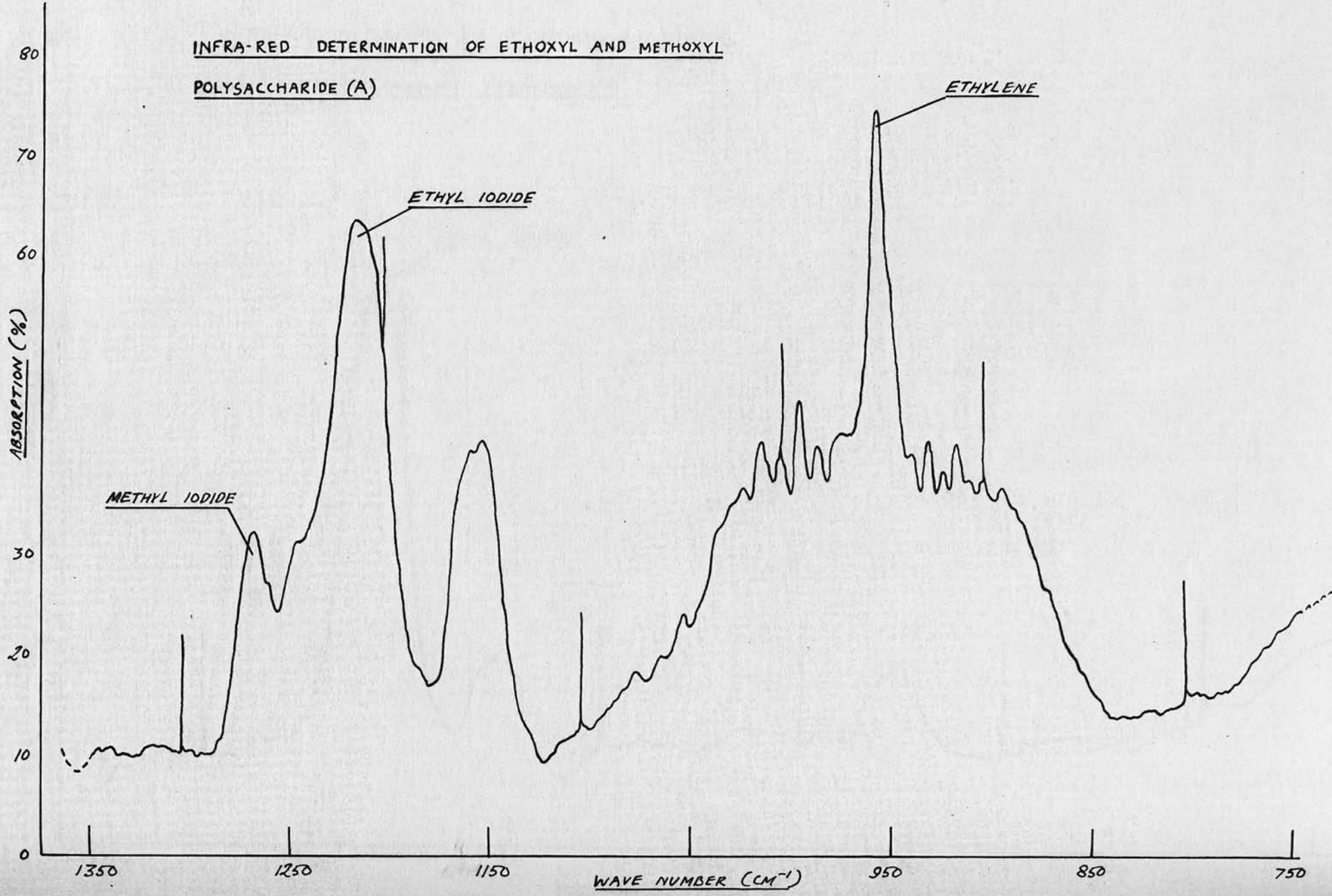
Although no acidity was detected in the original syrup, three acidic fractions iii(1), iii(3) and iii(4) were obtained, consisting of complex oligosaccharides comprising different proportions of galactose, mannose, glucuronic acid and 4-O-methyl-glucuronic acid.

Partial hydrolysis also afforded an unusual sugar which was found to be a mono-O-ethyl arabinose. Examination of its chromatographic mobilities proved it to be the unidentified sugar present in trace quantities in preliminary hydrolysates of polysaccharide (A) (page 30). The sugar, fraction i(1) , isolated as a syrup,

INFRA-RED DETERMINATION OF METHOXYL AND ETHOXYL

MONO-O-ETHYL ARABINOSE (FRACTION i(1)




INFRA-RED DETERMINATION OF ETHOXYL AND METHOXYL
POLYSACCHARIDE (A)
ETHYLENE
ETHYL IODIDE
METHYL IODIDE
ABSORPTION (%)
80
70
60
30
20
10
0
1350
1250
1150
WAVE NUMBER (CM⁻¹)
950
850
750

had a chromatographic mobility similar to a mono-O-methylpentose. It proved, however, to be devoid of methoxyl, and infra-red spectroscopic examination, which distinguishes ethoxyl from methoxyl and from ester groupings, indicated the presence of ethoxyl residues. Quantitative determination by infra-red absorption gave an ethoxyl content equivalent to that of a mono-O-ethylpentose. Chromatographic examination of a de-ethylated sample revealed a single spot identical with arabinose run as a control. This mono-O-ethylarabinose must be present as a minor constituent of the gum, since no treatment which could have caused ethylation of the polysaccharide was utilised either during extraction or manipulation.

Polysaccharide (A) was treated with hydriodic acid in the same way as the mono-O-ethylarabinose and the evolved gases examined in the infra-red spectrometer. The spectrograph contained a peak corresponding to ethyl iodide, which, by itself, would show that ethoxyl was present in the polysaccharide. Surprisingly, the peak indicated a comparatively high proportion (1.3%) of ethoxyl, corresponding to approximately 8% of mono-O-ethylarabinose in the polysaccharide. This large proportion was not confirmed, however, in hydrolytic experiments, in which only trace quantities of the sugar were detected and isolated.

However, the spectrograph also revealed the presence of ethylene, which was completely absent from the spectrograph of the mono-O-ethylarabinose (see opposite). The presence of ethylene in conjunction with ethyl iodide could only mean that a

glycol grouping such as (IX) exists in the polysaccharide. The reactions of this group and of ethoxyl with hydriodic acid are as follows:

$$-O-CH_2-CH_2-O- \xrightarrow{2HI} ICH_2-CH_2I \longrightarrow CH_2=CH_2 \xrightarrow{HI} CH_3CH_2I + I_2$$

IX.

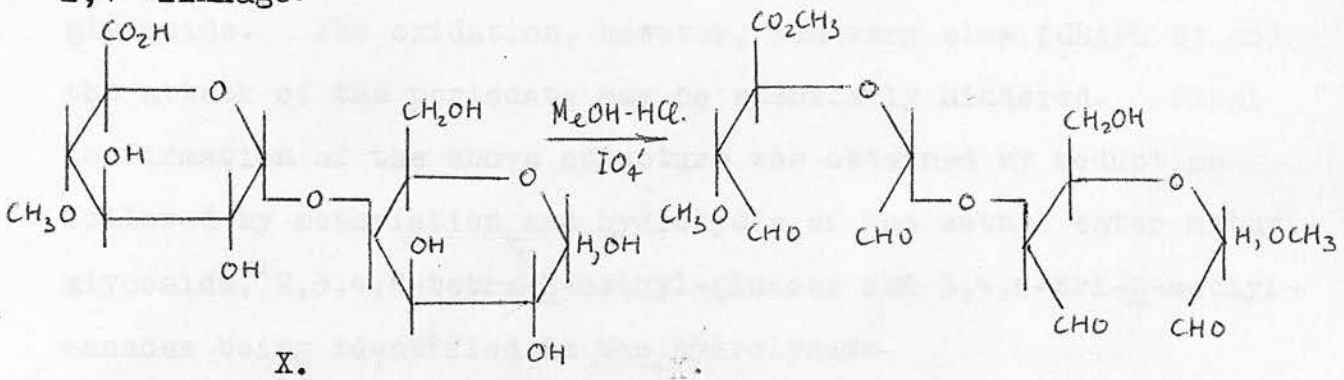
$$-O-CH_2CH_3 \xrightarrow{HI} CH_3CH_2I$$

As it is impossible to produce an ethoxyl group from the glycol group (IX) merely by dilute acid hydrolysis, it must be assumed, on the present facts, that the polysaccharide contains both the glycol group and free ethoxyl groups.

Examination of the degraded polysaccharide - Isolation of Aldobiuronic Acids

The barium salts (EXPT. 15), after hydrolysis with sulphuric acid for four hours, were placed on a cellulose column. Elution with half-saturated aqueous butan-1-ol separated galactose, mannose and rhamnose from the acidic material. No arabinose or neutral disaccharides were detected. The acidic fractions, after elution with the acidic eluant, were deionised and separated on thick paper. D-Glucurone and 4-O-methyl-D-glucuronic acid were obtained in the ratio of 2.5:1, the other major sugars being two aldobiuronic acids, fractions A(4) and B(3). The higher uronic acid fractions C, D, E and F (page 57) were not examined further.

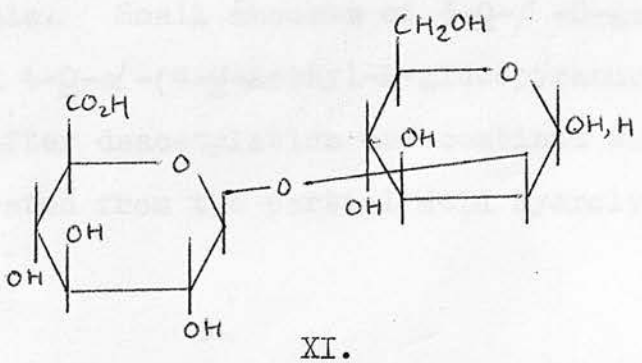
The first aldobiuronic acid, fractions A(4) and B(2) was characterised as 4-O-α-(4-O-methyl-D-glucopyranuronosyl)-D-galactose (X) by its equivalent weight, chromatographic mobilities, and rotation of the barium salt. Confirmation of this structure was obtained by examination of the derived disaccharide glycoside and of the methylated aldobiuronic acid. Hydrolysis of the former gave equal quantities of 4-O-methylglucose and galactose (visual examination, paper chromatography). The reduction of periodate by this disaccharide glycoside was in agreement with a 1,4'-linkage.



X.

Confirmation that the linkage was indeed 1,4' was obtained by the chromatographic identification of 2,3,4-tri-O-methylglucose and 2,3,6-tri-O-methylgalactose in the hydrolysate of the methylated, reduced, ester glycoside of the original aldobiuronic acid.

The second aldobiuronic acid, fraction B(3) proved to be 2-O-β-D-glucopyranuronosyl-D-mannopyranose (XI).



XI.

The chromatographic mobilities were identical with those reported by Aspinall, Hirst and Wickstrom (174) for this acid which was isolated from Gum Ghatti. The rotation, when calculated for the barium salt, was identical with the value reported by Jones (76) for this aldobiuronic acid separated from cherry gum. It had the correct equivalent weight for such a molecule and gave on hydrolysis mannose and glucurone. Reduction and hydrolysis of the derived ester glycoside yielded equal amounts of glucose and mannose. The amount of periodate reduced by the ester glycoside did not reach the theoretical figure of three moles per mole of glycoside. The oxidation, however, was very slow (GRAPH 8) and the attack of the periodate may be sterically hindered. Final confirmation of the above structure was obtained by reduction followed by methylation and hydrolysis of the methyl ester methyl glycoside, 2,3,4,6-tetra-O-methyl-glucose and 3,4,6-tri-O-methyl-mannose being identified in the hydrolysate.

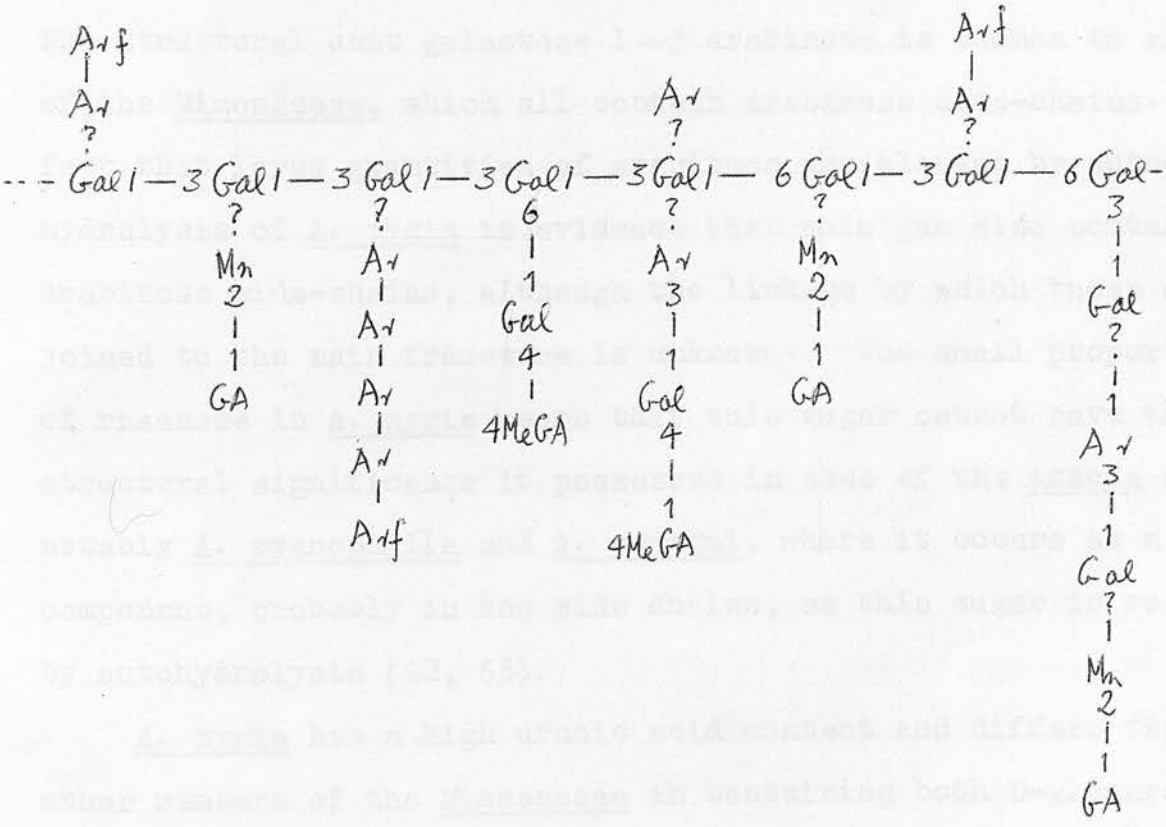
Acetolysis experiments (EXPT. 11)

From these experiments, it was hoped to obtain oligosaccharides differing from those obtained by partial acid hydrolysis. However, the products obtained after hydrolysis of polysaccharide (A) were extremely complex and complete separation proved impossible. Small amounts of 3-O-β-D-galactopyranosyl-L-arabinose and 4-O-α-(4-O-methyl-D-glucopyranuronosyl)-D-galactose were isolated after deacetylation and combined with these respective materials separated from the partial acid hydrolysate.

The isolation of six different monosaccharides from the gum shows that the polysaccharide of Albizzia zygia is extremely complex and possibly heterogeneous. The separation of two distinct fractions designated polysaccharides (A) and (B), which differ at least in their fine structure, supports this contention. The large proportion of galactose and the isolation of 1,3'- and 1,6'-linked galactose disaccharides and of an oligosaccharide containing at least eight contiguous galactose units provides evidence that the framework of the polysaccharide is composed of 1,3'- and 1,6'-linked galactose residues. The occurrence of 3-O-β-D-galactopyranosyl-L-arabinose as one of the three disaccharides isolated indicates that galactose linked to the C_3 position of arabinose is one of the major structural units in the molecule. From the evidence so far available, however, it cannot be decided whether this unit is present in the main chain or only in the side-chains attached to the galactan framework. The large proportion of arabinose in the polysaccharide is evidence in favour of side-chains consisting of arabinose residues, in spite of the failure to isolate any arabinose disaccharides. Autohydrolysis experiments indicate that some of the arabinose residues are in the furanose form and a proportion of the residues are end units. Periodate oxidation studies confirm the presence of end-unit arabinose.

The separation of 4-O-α-(4-O-methyl-D-glucopyranuronosyl)-D-galactose and 2-O-β-D-glucopyranuronosyl-D-mannose provides evidence for direct union of 4-O-methyl-glucuronic acid to C_4 of galactose and of glucuronic acid to C_2 of mannose in the macro-

molecule. As both the uronic acid residues are almost entirely eliminated on oxidation with periodate, these acidic residues most probably occur as end-units. Periodate oxidation also reduced the proportion of mannose, supporting the presence of C_2'-linked mannose units. It seems likely, therefore, that arabinose, mannose and galactose are present in side-chains and to these uronic acid units may be linked as end-groups. One of a number of possible diagrammatic formulae which incorporates all the linkages so far established for this polysaccharide is given below; all the evidence is in favour of a highly-branched molecule.



The position of rhamnose in the polysaccharide is still in doubt as no rhamnose-containing oligosaccharides have been isolated. This sugar must, in any case, be of minor structural significance

in view of the small proportion present in the polysaccharide.

In general structure, the polysaccharides of Albizzia zygia are similar to those of other gums of the Mimosaceae in that the galactose residues constitute the framework of the molecule. In view of the proportions of galactose disaccharides isolated, the galactose residues must be linked predominately 1,3' with some 1,6'-linkages. This is similar to the Acacia gums in which the main framework of the molecule consists of 1,3'-linked galactose residues, with 1,6'-linkages occurring to a much smaller extent. The structural unit galactose 1→3 arabinose is common to all species of the Mimosaceae, which all contain arabinose side-chains. The fact that large quantities of arabinose are cleaved by autohydrolysis of A. zygia is evidence that this gum also contains arabinose side-chains, although the linkage by which these are joined to the main framework is unknown.. The small proportion of rhamnose in A. zygia means that this sugar cannot have the structural significance it possesses in some of the Acacia gums, notably A. cyanophylla and A. senegal, where it occurs as a major component, probably in the side chains, as this sugar is released by autohydrolysis (62, 63).

A. zygia has a high uronic acid content and differs from the other members of the Mimosaceae in containing both D-glucuronic acid and its 4-O-methyl ether. A few other gum exudates, such as Khaya grandifolia and K. senegalensis, contain two uronic acids but in these species the acids are D-glucuronic acid and D-galact-

uronic acid. The Khaya gums, however, are more related to the Sterculia type in their molecular structure (page 19).

A. zygia also differs from the other members of the Mimosaceae in containing D-mannose (TABLE 4). The isolation of 2-O-β-D-glucopyranuronosyl-D-mannose shows that at least some of the mannose content is linked directly to D-glucuronic acid. This aldobiuronic acid, more characteristic of gums of the Prunus spp., has been isolated from P. insitia and P. cerasus. The present hydrolytic studies also reveal the direct union of mannose to galactose. Although a mannose-galactose oligosaccharide was separated, no mannose-containing neutral disaccharide has been completely characterised in the present experiments. 6-O-α-D-Galactosyl-D-mannose has, however, been isolated from Guar gum (181). 4-O-α-(4-O-Methyl-D-glucopyranuronosyl)-D-galactose, the other aldobiuronic acid isolated from A. zygia, has also been obtained from Prosopis Juliflora (182), Khaya grandifolia (7) and Citrus limona (183). The 4-O-methyl-D-glucuronic acid occurs as end group in K. grandifolia, and the evidence is in favour of this in A. zygia.

Gums containing 4-O-methyl-D-glucuronic acid, although not numerous, are widely distributed among different botanical families. They are all similar to A. zygia in containing a galactan framework with arabinose side-chains, but A. zygia alone of these species contains D-mannose (TABLE 5). Moreover, A. zygia, with the possible exception of Citrus limona (Lemon gum) is the only gum polysaccharide known to contain both D-glucuronic acid and its 4-O-methyl ether.

TABLE 4 STRUCTURAL UNITS IN MIMOSACEAE

Species	Uronic acid	Neutral Mono-saccharides Gal Ar Rh Mn	Aldobiuronic acids	Neutral Disaccharides
Acacia catechu	GA	+ + +	GA 1 - 6 Gal	
A. cyanophylla	GA	+ + +	GA 1 - 6 Gal	Gal 1 - 3 Ar
A. karroo	GA	+ + +	GA 1 - 6 Gal GA 1 - 4 Gal	Ar 1 - 3 Ar
A. mollissima	GA	+ + +	GA 1 - 6 Gal	Ar 1 - 3 Ar
A. pycnantha	GA	+ + +	GA 1 - 6 Gal	Ar 1 - 3 Ar Gal 1 - 3 Gal
A. senegal	GA	+ + +	GA 1 - 6 Gal	Ar 1 - 3 Ar Gal 1 - 3 Ar Gal 1 - 3 Gal
A. sundra	GA	+ + +	GA 1 - 6 Gal	
Prosopis juliflora	4 Me GA	+ + +	4 Me GA 1 - 6 Gal 4 Me GA 1 - 4 Gal	
Albizzia zygia	4 Me GA GA	+ + + +	4 Me GA 1 - 4 Gal GA 1 - 2 Mn	Gal 1 - 3 Ar Gal 1 - 3 Gal Gal 1 - 6 Gal

TABLE 5 GUMS CONTAINING 4-O-METHYL-D-GLUCURONIC ACID

Species	Uronic acids			Neutral Monosaccharides				Aldobiuronic acids	Ref.
	Gal A	GA	4 Me GA	Gal	Mn	Ar	Rh		
Prosopis juliflora			+	+		+		4 Me GA 1 - 6 Gal 4 Me GA 1 - 4 Gal	182
Fagara Xanthoxyloides			+	+		+			169
Citrus limona		+(?)	+	+		+		4 Me GA 1 - 4 Gal	183, 184
Commiphora myrrha			+	+		+			110
Khaya grandifolia	+		+	+		+	+	4 Me GA 1 - 4 Gal Gal A 1 - 2 Rh	7
K. senegalensis	+		+	+		+	+		185
Albizzia zygia		+	+	+	+	+	+	4 Me GA 1 - 4 Gal GA 1 - 2 Mn	

PART TWO

THE CONSTITUTION OF ALGINIC ACID FROM LAMINARIA DIGITATA

INTRODUCTION

Classification of the algae is based partly on their photosynthetic pigmentation (115), the major groups being the green, blue-green, red and brown algae. While the green and blue-green types are often fresh-water forms, species of all types are found in marine habitats and are commonly referred to as seaweeds. The larger species of brown algae are known collectively as kelp.

In 1883, Stanford (116, 117) noticed that after extraction of mineral salts from species of Laminaria, a "peculiar principle" remained in the residue. Fresh seaweed also yielded this material in the form of a colourless jelly, and Stanford realised that this was the sodium salt of a new substance which he called alginic acid.

Alginic acid is now known to occur in all the Phaeophyceae, or brown algae, where it is the main structural constituent of the cell-walls (118). The seaweeds used by Stanford were Fucus vesticulosis and Laminaria stenophylla, but the principal commercial source today is Macrocystis pyrifera, a giant kelp growing in the Californian coastal waters. Other important sources are Ascophyllum nodosum, a British seaweed, and species of Laminaria, which are of world-wide occurrence, but found particularly in France, Norway and Japan (119).

The alginic acid content of brown algae varies from species to species (TABLE 6). There is also a marked seasonal variation in the content of the fronds, where most of the photosynthesis occurs. In the spring, when the new frond is forming, the carbohydrate

constituents are similar to those of the stipe, i.e. a high proportion of alginic acid, mineral salts and water, but a low content of mannitol and laminarin. As photosynthesis proceeds, however, mannitol and laminarin accumulate, while the alginic acid content is decreased, reaching a minimum in late summer (120). Depth of immersion also affects the proportion of alginic acid, and in the case of L. digitata, maximum yield is obtained from plants growing at a depth of twelve metres (121). An apparent decrease has been reported in the viscosity of sodium alginate solutions extracted from seaweed at successively greater depths (121).

TABLE 6

Species	Alginic acid Content (%) Min.	Max.	Ref.
Laminaria cloustoni	12	24	120
L. saccharina	10 - 16	18 - 20	120, 122
L. digitata (frond)	16 - 18	25 - 27	120
" (stipe)	27	33	120
L. stenophylla (frond)	24.1		119
" (stipe)	25.7		119
Ascophyllum nodosum	24	28	123
Pelvetiaa canaliculata	13.3	18.6	124
Fucus spiralis	13.0	16.6	124
F. vesiculosis	13.8	17.2	124
F. serratus	17.0	22.1	124
Macrocystis pyrifera (frond)	15		125
Durvillaea antartica	33.1		126

Extraction of alginic acid

In the usual manner of extraction, first used by Stanford (117), the dried powdered seaweed is washed with dilute acid to remove acid-soluble material. Digestion with warm aqueous sodium carbonate yields a viscous solution of sodium alginate from which the free acid or one of its insoluble salts may be precipitated by addition of a suitable reagent. The concentration of sodium carbonate employed has varied from 0.2% to 3.0%, the higher concentrations apparently yielding a product of higher viscosity (127). Digestion has been carried out at temperatures ranging from 16° to 80°. Increase in temperature results in a higher yield of alginic acid from the Laminariaceae (128), but yields from Sargassum horneri and S. microcanthum are apparently independent of temperature (127). Other extracting agents which have been utilised include ammonium, sodium and potassium oxalates (129) and aqueous ammonia (130).

Precipitation from sodium alginate solution is usually effected by addition of calcium chloride, the calcium alginate being isolated as gelatinous sheets. However, as alginic acid forms insoluble salts with most of the heavy metals, many other compounds may be employed, although the alginates of potassium, magnesium, lithium and ammonium are soluble in aqueous solution (131). Acidification of an alginate solution with mineral acids gives a precipitate of free alginic acid, usually as a gel, which can be dehydrated by extraction with ethanol (125). Alginic acid is also precipitated by oxalic, phosphoric and tartaric acids, but not by acetic, formic or benzoic acids (117).

Bleaching the sodium alginate solution with sodium hypochlorite or hydrogen peroxide produces a white or near-white alginic acid. This process does not noticeably affect the viscous qualities of the final product, but Black (128) has found that bleaching decreases the yield of acid from the Fucaceae.

Uses of alginates

Alginic acid, in the form of its highly-hydrated sodium salt finds extensive use in the food, cosmetic and pharmaceutical industries. As sodium alginate forms stable gels and high-viscosity solutions at low concentrations (ca. 1 - 2%), its use is indicated wherever thickening, emulsifying, suspending and gel-forming applications are involved, as for example in creams, ointments and jellies. By extrusion of alginate solutions into baths containing a calcium salt solution, films and filaments of insoluble calcium alginate may be produced. As this substance is soluble in soap and dilute alkali, the fibres cannot be used for weaving into permanent fabrics but are used as temporary textile yarns and as absorbable medical gauze (132).

Chemical constitution of alginic acid

Stanford established alginic acid as a weak organic acid which readily reacts with bases to form salts. He prepared the alginates of some twenty-seven different metals and also obtained compounds of alginic acid with alkaloids and other organic bases (131). Analysis of alginates led to the conclusion that the free acid

contained some 3% of nitrogen and that the molecular formula was $C_{76}H_{80}N_2O_{22}$. This was expressed by Stanford as a diamide (XII).

$$NH_2$$
$$NH_2$$
$$C_{76}H_{76}O_{22}$$

XII.

On the basis of molecular formula determinations, it was decided that alginic acid was very similar to albumin, but did not resemble pectic acid. Later researches, however, revealed that the pure acid contained no nitrogen (125, 133).

The first indication that alginic acid might be polysaccharide in nature was given by Kylin in 1915 (118) who reported the presence of pentoses in the product obtained on acid hydrolysis. The specific rotation of calcium alginate was found to be -136°. About the same time, Hoagland and Lieb (133) isolated alginic acid from Macrocystis pyrifera and found the neutralisation equivalent to be 325. Analytical data indicated a compound of formula $C_{21}H_{27}O_{20}$ and having two replaceable hydrogen atoms. Treatment of an acid hydrolysate with phenylhydrazine yielded an osazone whose melting-point, optical rotation and solubilities closely resembled those of D-xylosazone. On the basis of these observations, it seemed possible that alginic acid was composed of an acidic nucleus in combination with pentose sugars.

Evidence for the presence of uronic acid was furnished by Atsuki and Tomada (122) in 1926. These workers found that on

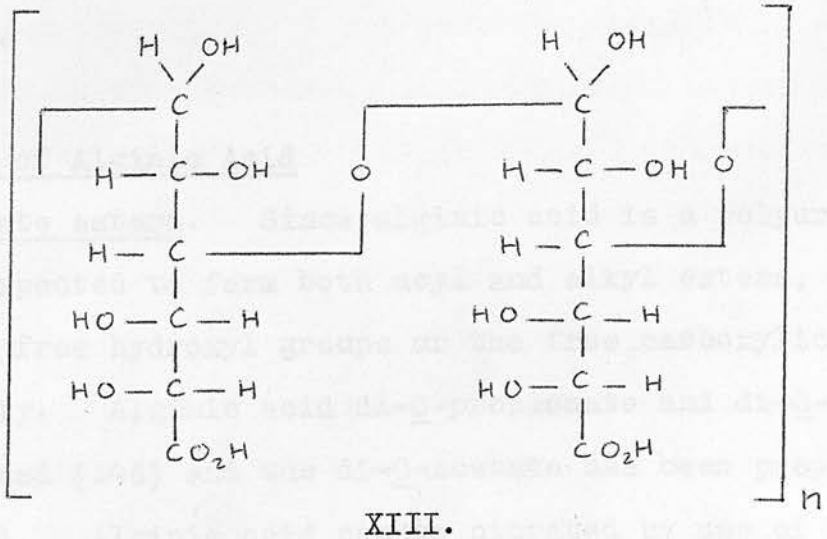
boiling alginic acid with hydrochloric acid (d, 1.06), carbon dioxide (19.9%) and furfuraldehyde (14.2%) was produced. The neutralisation equivalent was found to be 262 and the specific rotation -130°. Nelson and Cretcher, in 1929 (125), using alginic acid isolated from F. serratus, L. digitata and M. pyrifera, obtained a value of 176 - 184 for the neutralisation equivalent, and a value of -133° for the specific rotation of sodium alginate. Decarboxylation with 18% hydrochloric acid yielded carbon dioxide (24.2%), equivalent to a uronic acid content of 100%. Carbon and hydrogen determinations indicated the formula $(C_6H_8O_6)_n$. As pure alginic acid gave a strong naphtho-resorcin test and did not reduce Fehling's solution, it was concluded that the polysaccharide is a polyuronic acid in which all the carboxyl groups are free and all the aldehydic groups involved in linkage.

Identification of the Uronic acid: Hydrolytic studies

The hexuronic acid yielded by hydrolysis of alginic acid was first thought to be D-glucuronic acid. This was reported by Schmidt and Vocke (134) who subjected alginic acid from F. serratus to hydrolysis by 80% sulphuric acid at room temperature. Identification of the acid, however, rested solely on the melting-point of the cinchonine salt (204°) and was therefore inconclusive. D-galacturonic acid was suggested by Atsuki and Tomada (122) on the basis of the similarity between alginic and pectic acids.

Further hydrolytic experiments were carried out by Nelson and Cretcher (125). By submission of alginic acid from F. serratus to

more drastic conditions than had been used hitherto, an acid was obtained which yielded a cinchonine salt of melting-point 152°. Alginic acid from M. pyrifera gave a uronic acid which was apparently identical. Oxidation of this compound produced D-mannosaccharic acid, which was characterised as its diamide and its osazone. Confirmation of this result came in 1930 when D-mannuronic acid itself, in the form of its lactone, was isolated from hydrolysates of M. pyrifera (135). This lactone, hitherto unknown, was reported as having a melting-point of 140 - 141° and specific rotation + 89.8°. No muta-rotation was observed. Two years later, D-mannuronic acid, characterised as cinchonine and brucine salts, was obtained by Miwa from eight species of brown algae (136). Dillon and McGuiness (137), by analysis of barium alginate, came to the conclusion that the free acid could be formulated as $(C_6H_{10}O_7)_n$ and, on drying, formed the lactone of formula $(C_6H_8O_6)_n$. A structural formula for the free acid was suggested (XIII).



XIII.

Isolation of D-mannuronic acid lactone from alginic acid hydrolysates has been achieved by use of mineral acids under a variety of conditions (138, 139, 140), the yields ranging from 4% to 27%. In an effort to avoid some of the degradation which accompanies strong mineral acid hydrolysis, 90% formic acid has been employed (141, 142). The resulting lactone was isolated in 45% yield and was reported as having melting-point 145° and specific rotation + 92° (141).

Enzymic hydrolysis, although useful for the structural investigation of many polysaccharides, has not been generally employed in the analysis of alginic acid, as alginase activity is not present to any great extent in bacteria. However, some decomposition of alginic acid by certain species of bacteria has been reported (143, 144). More recently, chromatographic evidence has indicated that enzyme preparations from soil bacteria are capable of degrading alginic acid, to some extent, to D-mannuronic acid (145).

Properties of Alginic Acid

Alginate esters. Since alginic acid is a polyuronide, it would be expected to form both acyl and alkyl esters, involving either the free hydroxyl groups or the free carboxylic acid groups respectively. Alginic acid di-O-propionate and di-O-butyrate have been obtained (146) and the di-O-acetate has been prepared in 97% yield (147). Alginic acid can be nitrated by use of a nitric-sulphuric acid mixture, the degree of nitration depending upon the

particular conditions employed (148). Esters of the second type, i.e. with the carboxyl group esterified, have also been obtained. The methyl ester has been prepared by the action of anhydrous methanolic hydrogen chloride on alginic acid (35, 149), but the glycol esters are more easily obtainable. Alkylene oxides react with alginic acid under mild conditions to give water-soluble glycol esters (150). Ethylene alginate, propylene alginate and similar esters have been prepared.

Alginate ethers. The potassium hydroxide-dimethyl sulphate procedure has been used with success to methylate a degraded alginic acid (151) and a fully methylated polymannuronide has been obtained by use of thallous hydroxide and methyl iodide (35). Treatment with diazomethane in ethereal solution has also afforded some degree of methylation (148).

Physical properties. The specific rotation of sodium alginate has been variously reported as having values between -120° and -150° (128). The value is apparently dependent upon the species of weed and the method of extraction employed. The more vigorous conditions will cause a greater degree of degradation of the molecular chains.

The soluble alginates have properties typical of hydrophilic colloids. Solutions of sodium alginate, for example, are considerably more viscous than those of simple molecules of the same concentration, and can be separated, by dialysis, from compounds of low molecular weight. The viscosity of sodium alginate

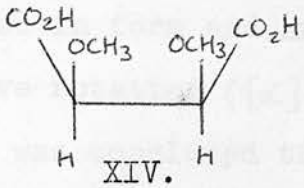
solution at any given concentration and temperature is dependent upon the method of extraction used, although it seems to be immaterial from which particular species of seaweed the alginate is obtained (152). Solutions of highly-polymerised alginates undergo a decrease in viscosity if stored at room temperature for any length of time.

By viscometric measurements, Heen (153) found the molecular weight of sodium alginate to be 15,000. Later researches, however, revealed that the degree of polymerisation is considerably higher. Osmotic pressure methods have given values ranging from 48,000 to 186,000 for the molecular weight (154) and ultra-centrifuge measurements indicate a value of 30,000 to 210,000 (155). It seems likely, therefore, that the chain length of undegraded alginic acid is considerably greater than 100 units.

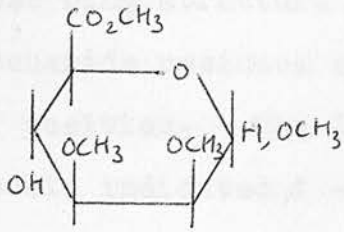
Structural Studies

Methylation. Hirst (35) found that treatment of alginic acid with boiling anhydrous methanolic hydrogen chloride (10%) yielded a degraded polymer plus a high proportion of the methyl ester of methyl-D-mannuronoside. The latter, after methylation and oxidation, gave 2,3,4-tri-O-methyl-D-mannosaccharic acid. The degraded material, of considerably lower viscosity than the original alginic acid, was methylated using thallous hydroxide and methyl iodide. The methylated product, although exceptionally stable towards the usual hydrolytic agents, was simultaneously hydrolysed and oxidised by treatment with boiling 50% nitric acid. This

process yielded meso-dimethoxysuccinic acid (XIV).

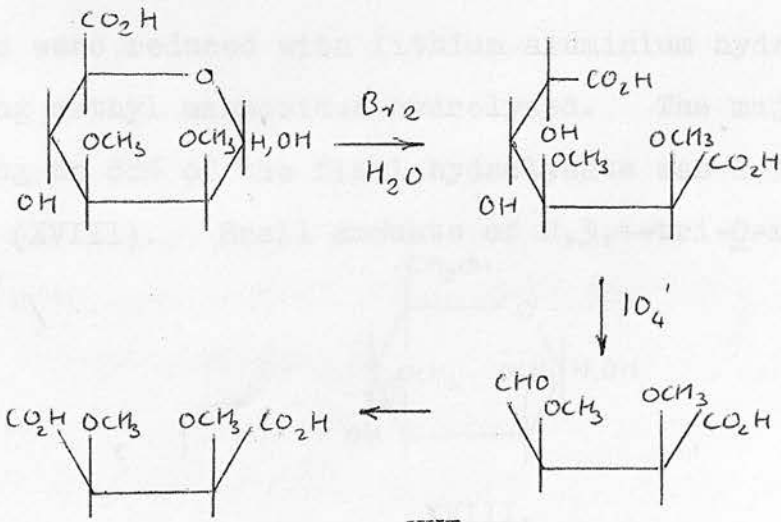


XIV.



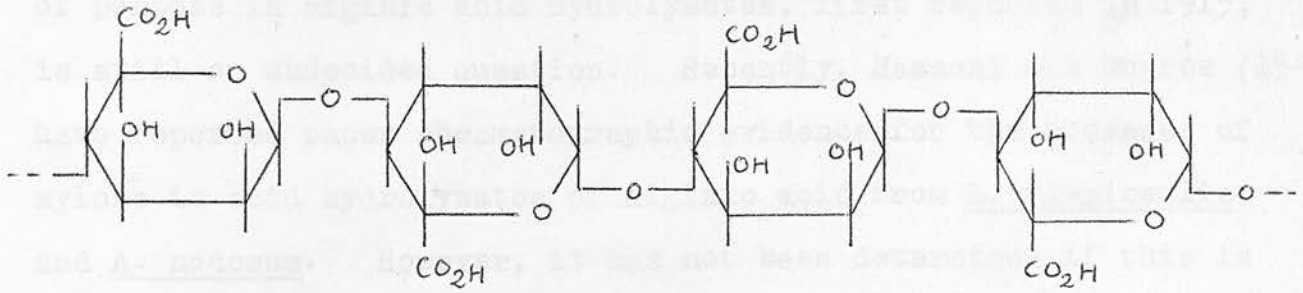
XV.

This indicated that in each of the methylated D-mannuronic acid residues, the methyl groups were attached either to C_2 and C_3 or to C_4 and C_5. Proof that they were, in fact, attached to C_2 and C_3 was obtained by heating methylated alginic acid under pressure with 4% anhydrous methanolic hydrogen chloride at 150° to form the methyl ester of 2,3-di-O-methyl-methyl-D-mannuronoside (XV). As this compound underwent a change in specific rotation on hydrolysis, it was evident that an oxide ring is present and that substitution on C_4 and C_5 is precluded. Furthermore, the compound was characterised as 2,3-di-O-methyl-D-mannuronic acid by oxidation to meso-dimethoxy-succinic acid via 2,3-di-O-methyl-D-mannosaccharic acid (XVI).



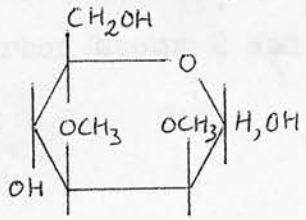
XVI.

In view of these results and the fact that the extreme stability of alginic acid rendered a furanose ring structure most unlikely, it was concluded that the monosaccharide residues are pryanose in form and are linked in the 1,4' position. The large negative rotation ($[\alpha]_D$ -139°) of the molecule indicated β -links and it was concluded that at least 50% of the molecule could be represented as D-mannuronic acid residues joined in this manner (XVII).



XVII.

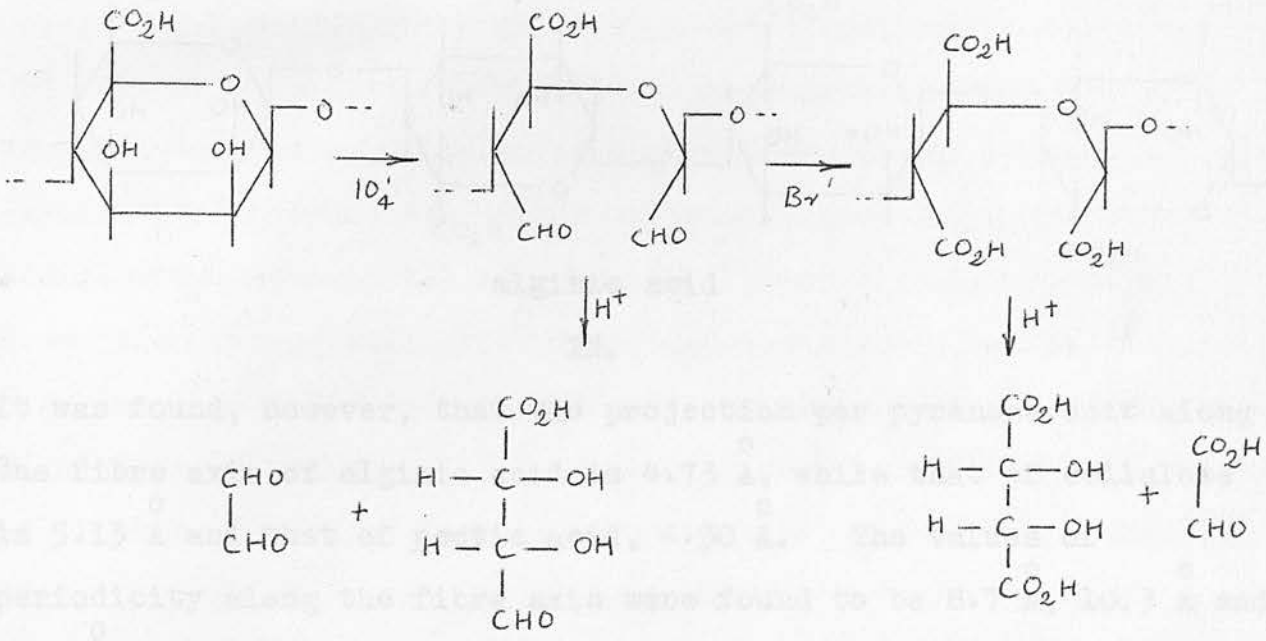
These results were later extended using a less degraded alginic acid with chain length ca. 100 units (151). The chain length was deduced from viscosity measurements on the methylated acid. Methylation was, in this case, effected by means of dimethyl sulphate and sodium hydroxide. After hydrolysis with 98% formic acid, the products were reduced with lithium aluminium hydride, and the resulting methyl mannosides hydrolysed. The major component, amounting to 88% of the final hydrolysate was 2,3-di-O-methyl-D-mannose (XVIII). Small amounts of 2,3,4-tri-O-methyl-D-mannose,



XVIII.

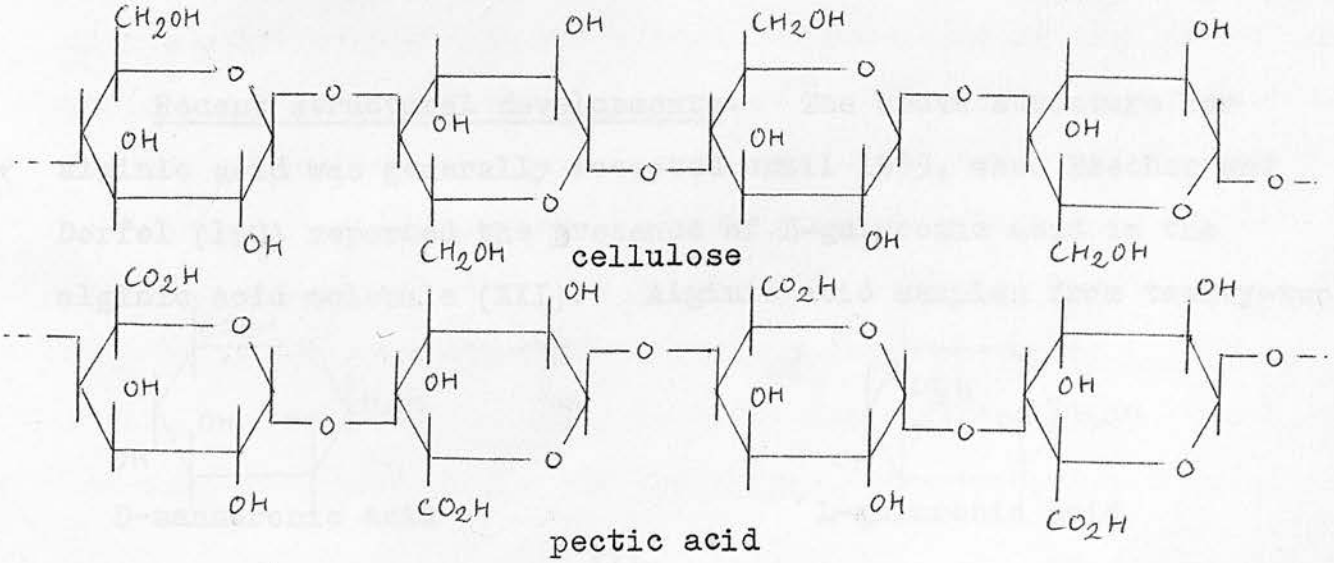
mono-O-methyl-D-mannose and di-O-methyl-D-glucose were obtained. The proportion of the main constituent, together with the large negative rotation, again indicated that the greater part of the molecule, or perhaps the entire molecule, was composed of 1 , 4'-linked D-mannuronic acid units. The "glucose" was not definitely characterised and in view of recent investigations (160), the presence of another hexose is of some significance. The presence of pentose in alginic acid hydrolysates, first reported in 1915, is still an undecided question. Recently, Massoni and Dupres (156) have reported paper chromatographic evidence for the presence of xylose in acid hydrolysates of alginic acid from L. flexicaulis and A. nodosum. However, it has not been determined if this is an integral part of the molecule, a degradation product, or an artifact.

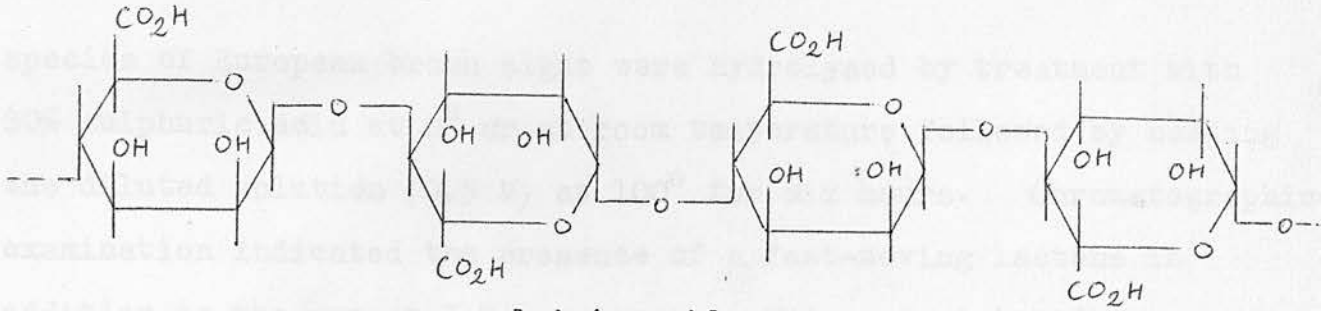
Oxidation by Periodate. Work by Lucas and Stewart (157), using periodic acid to oxidise the adjacent hydroxyl groups, confirmed the results of Hirst's methylation studies. Macrocystis pyrifera was employed as the source of alginic acid. These workers were able to isolate glyoxal in 42% yield from hydrolysis products of the dialdehyde. Further oxidation with bromine and subsequent hydrolysis yielded meso-tartaric acid in 25% yield (XIX). These results confirmed that carbon atoms 2 and 3 are not involved in ring or bridge formation.



XIX.

Alginate Fibres. X-ray diffraction investigations on stretched fibres of alginic acid and sodium alginate have revealed well-defined diffraction patterns, which indicate a high degree of orientation. In this respect, alginic acid resembles cellulose and pectic acid, with which there is a close formal similarity (XX).



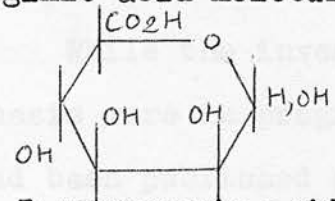


alginic acid

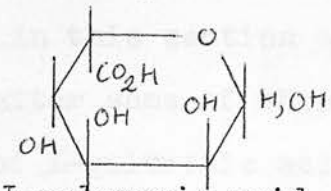
XX.

It was found, however, that the projection per pyranose unit along the fibre axis of alginic acid is 4.73 Å, while that of cellulose is 5.13 Å and that of pectic acid, 4.30 Å. The values of periodicity along the fibre axis were found to be 8.7 Å, 10.3 Å and 13.0 Å respectively (158, 159), and it is considered that the pyranose rings of alginic acid and pectic acid exist in a different chain conformation from that in cellulose. The angle between the rings is approximately 90° instead of 20° and the molecule is therefore more buckled. As sodium alginate is found to have a periodicity of 15.0 Å and a projection per unit of 5.0, it seems probable that the conformation of the pyranose rings in the sodium salt differs from that in the free acid.

Recent structural developments. The above structure for alginic acid was generally accepted until 1955, when Fischer and Dorfel (160) reported the presence of L-guluronic acid in the alginic acid molecule (XXI). Alginic acid samples from twenty-two



D-mannuronic acid



L-guluronic acid

XXI.

species of European brown algae were hydrolysed by treatment with 80% sulphuric acid at 0° or at room temperature followed by heating the diluted solution (0.5 M) at 100° for six hours. Chromatographic examination indicated the presence of a fast-moving lactone in addition to the expected D-mannurone. This material, after separation and crystallation was found to be identical with synthetic L-gulurone and had m.p. 141-142° and specific rotation + 81° (cf. D-mannurone m.p. 191°, $[\alpha]_D$ + 92.5°) (160). Infra-red investigation indicated a 3,6' lactone ring and the compound was considered to be α -L-gulopyranurono-3,6'-lactone.

The ratio of D-mannurone to L-gulurone was found to vary between 2 to 1 and 1 to 2 according to the starting material and the hydrolytic conditions employed. Prolonged heating with 0.5 M sulphuric acid caused more decomposition of L-gulurone than of D-mannurone. The possibility that the L-gulurone could have arisen from D-mannurone by C_5 epimerisation was excluded on the grounds that the proportions of the lactones resulting from any one set of hydrolytic conditions were reproducible. Pre-extraction of the alginic acid with hot alkali had no effect on the proportions, nor was D-mannurone found to epimerise in hot dilute acid solution. Fischer and Dorfel also found traces of D-glucuronic acid in extracts of the Phaeophyceae, but none was detected in hydrolysates of alginic acid.

While the investigations described in this section of the thesis were in progress, and, in fact, after some of these results had been published (178), the presence of L-guluronic acid was

confirmed by Whistler and Kirby (161). These workers, using Fischer and Dorfel's methods on alginic acid from M. pyrifera, estimated the ratio of D-mannuronic to L-guluronic acid to be 4.5 to 1. In contrast with the present results (page 114), L-gulurone was not detected, however, when the alginic acid was hydrolysed with hot formic acid in the manner of Spoehr (141). This failure was attributed to the sensitivity of L-guluronic acid to hot formic acid. The L-gulurone isolated from sulphuric acid hydrolysates had m.p. 142° and $[\alpha]_D$ + 79°.

In view of these investigations, the di-O-methyl-D-glucose reported by Hirst (151) in his experiments on reduced and methylated alginic acid hydrolysates is significant. This sugar was not characterised completely and may have been a di-O-methyl-L-gulose.

More recently, Vincent (177) has isolated oligosaccharides from partial hydrolysates of alginic acid. Alginic acid from commercial sodium alginate was subjected to the action of 80% sulphuric acid at 20° for 15 minutes. Two disaccharides and a trisaccharide were isolated by paper chromatography. Reduction and hydrolysis of the methyl ester methyl glycosides of each yielded mannose and gulose (identified chromatographically) in varying ratios, indicating that mannuronic acid and guluronic acid are chemically combined in alginic acid. The fractionation of alginic acid by Haug (176) does not conflict with these results. Although two distinct fractions of alginic acid were obtained, both contained mannuronic and guluronic acids.

The present investigations describe further experiments which

not only confirm the presence of L-guluronic acid residues in the alginic acid molecule but also show that they are involved in 1,4' linkage. Extensive periodate oxidation studies on alginic acid have also indicated that the molecule is more complex than straight chains of 1,4'-linked mannuronic and guluronic acid residues.

EXPERIMENTAL

GENERAL METHODS OF INVESTIGATION

Unless otherwise stated, the general experimental methods described in PART ONE of this thesis were used.

Chromatographic solvents

The following additional solvents were employed:

(7) ½-Saturated aqueous methyl ethyl ketone + 1% .880 ammonia.

(8) Pyridine: ethyl acetate: acetic acid: water (5:5:1:3).

(9) Benzene: ethanol: water (169:47:15).

(10) Phenol: water (4:1).

(11) Butan-1-ol: acetic acid: water (4:5:1).

(12) Pyridine: ethyl acetate: water (11:40:6).

(13) Butan-1-ol: acetic acid: water: conc. hydrochloric acid (20:5:25:1).

(14) Ethyl acetate: acetic acid: water (6:3:2).

(15) Methyl ethyl ketone: water (1:3) + 1% .880 ammonia.

(16) Ethyl acetate: acetic acid: water (2:1:1).

(17) Amyl alcohol: acetic acid: water (4:1:5).

(18) Methyl ethyl ketone: acetic acid: water (9:1:1) saturated with boric acid.

Source of Alginic acid

Free alginic acid was extracted from *Laminaria digitata* frond.

The weed, collected at Thorntonloch, had been dried for 2 hours at 45° and then 20 hours at 25°.

Commercial alginic acid (Hopkins & Williams Ltd.) and commercial calcium alginate (Alginate Industries Ltd.) were also used.

EXPT. 1. EXTRACTION OF ALGINIC ACID FROM L. DIGITATA

The method used was based on that of Black, Cornhill and Dewar (128). In a typical experiment, the dried, milled and powdered seaweed (100 g.) was stirred at 60° for 30 minutes with water (2 l.) containing calcium hydroxide (5 g.). After centrifugation, the seaweed was washed with water (2 x 1 l.) and then stirred at room temperature with 0.2 N sulphuric acid (2 l.) for 1 hour. After centrifugation and washing with water (1 l.), the residue was digested at 50-70° with 3% sodium carbonate solution (1 l.) for 3 hours with occasional stirring. The resulting brown-green jelly was diluted with water (5 l.) and stirred rapidly overnight at room temperature. The solution was then diluted further with water (5 l.) and centrifuged. The centrifugate was bleached by allowing to stand overnight with sodium hypochlorite solution (3 g. sodium hydroxide in 300 ml. chlorine water). The solution of sodium alginate was then poured, with stirring, into a solution of calcium chloride (200 g.) in water (1 l.) to precipitate calcium alginate as gelatinous sheets. The product was squeezed through muslin and washed with a dilute sulphur dioxide solution (1 l.). Washing was

continued with water until the calcium alginate was free from sulphur dioxide.

Free alginic acid was obtained by stirring the calcium salt for 8 hours with 2 N hydrochloric acid (1 l.) at room temperature. After filtration, the product was washed with ethanol containing 10% of N hydrochloric acid until free from calcium ions and then with ethanol until free from chloride ions. The alginic acid was dried in air at room temperature. Yield, 25.8 g.

PROPERTIES OF ALGINIC ACID

The acid was obtained as a white, fibrous solid, having $[\alpha]_D$ -136° to -139° in 0.015 N sodium hydroxide, (cf. Schoeffel and Link (140) $[\alpha]_D$ -136° in NaOH) ash 0.96% and sulphated ash 2.4%. Equivalent weight (by direct titration) was 144 to 170 (Calc. for a polymer of hexurone residues 176). On storing for some time, the acid was found to absorb moisture to the extent of 15%.

EXPT. 2. CHROMATOGRAPHIC IDENTIFICATION OF GULURONE AND GULOSE

a) Gulurone. As no authentic sample of gulurone was available, the chromatographic constants of alginic acid hydrolysates (EXPTS. 3 and 4) were compared with those reported by Fischer and Dorfel (160) using identical conditions. The R_F values of mannurone and gulurone (in solvent 11) reported by these workers were confirmed. In addition, these two lactones were found to be easily separable from each other and from glucurone in a variety of other solvents

(TABLE 7). It was not possible, however, to distinguish between mannuronic and guluronic acids chromatographically. Mannurone from alginic acid hydrolysates was found to have chromatographic constants identical with an authentic sample.

TABLE 7

Solvent	(3)	(4)	(5)	(12)	(14)
Mannurone R_F				0.53	0.40
" R_{glu}	2.8	2.3	2.7	1.63	1.8
Glucurone R_F				0.65	0.46
" R_{glu}	3.9	2.5	3.6	2.0	1.9
Gulurone R_F				0.77	0.54
" R_{glu}	4.8	2.8	4.4	2.4	2.5

b) Gulose. Authentic gulose for preliminary chromatographic work was obtained by treatment of a commercial gulose-calcium chloride complex with 1R 4B OH and 1R 12 O(H) ion-exchange resins. Solvents (8), (10), (9) and 13) did not separate gulose from mannose. In solvents (7) and (15) the R_{glu} values of mannose and gulose were 1.00 and 1.04 - 1.07 respectively, but separation of a co-spot of these two sugars proved impossible. In solvent (18), however, mannose had R_{glu} 1.36 and gulose had R_{glu} 1.88, identical with

authentic samples. Ionophoresis in borate buffer also gave a separation, mannose and gulose having M_G 0.46 and 0.59 respectively.

EXPT. 3. SULPHURIC ACID HYDROLYSIS OF ALGINIC ACID

Alginic acid from the different sources were hydrolysed under various conditions (TABLE 8), but based on the method of Fischer and Dorfel (160). In all cases, considerable degradation of the material occurred and yields were poor. In general, the experimental procedure was as follows:

Alginic acid was added gradually to a rapidly-stirred mixture of concentrated sulphuric acid (4 volumes) and water (1 volume) (100 ml.) at the required temperature. After the specified time, the brown pasty mass was poured into the calculated quantity of water to give a 0.5 N sulphuric acid solution, which was heated under reflux at 100°. After cooling, any undissolved material was removed by filtration, washed with water and ethanol, dried in vacuo, and weighed. The filtrate was neutralised with barium carbonate, filtered and evaporated to small bulk. It was found necessary to remove colour by addition of charcoal. Treatment with 1 R 120 H resin gave an acid solution from which partially degraded alginic acid was precipitated by addition of ethanol or acetone. Evaporation of the residual solution yielded a syrup which was examined chromatographically, any crystals being removed by filtration. The results of the hydrolyses are summarised in TABLE 8.

Hydrolysis 1. (TABLE 8). The hydrolysis was accompanied by an extremely large degree of degradation and no identifiable products were isolated.

Hydrolysis 2. (TABLE 8). After hydrolysis with 0.5 N sulphuric acid the hydrolysate was neutralised with barium hydroxide and barium carbonate. Concentration of the filtrate yielded barium mannuronate (0.7 g.), $[\alpha]_D$ -1° (c, 2.0) and a syrup (2 c.) (3.6 g.) having $[\alpha]_D$ +40° (c, 0.32). This syrup was further hydrolysed by heating with 0.2 N hydrochloric acid (300 ml.) at 100° for 3 hours. However, chromatographic examination of the product (a dark brown syrup) revealed no identifiable components.

Hydrolysis 3. (TABLE 8). The partially degraded material (3b) had $[\alpha]_D$ +10.8° (c, 1.1). The mannurone crystals (3c) had m.p. and mixed m.p. 187° and $[\alpha]_D$ +91° (c, 0.47). Chromatographic examination of the residual syrup (a) in solvents (12) and (14) revealed mannurone together with a faint spot corresponding to gulurone.

EXPT. 4. FORMIC ACID HYDROLYSIS OF ALGINIC ACID

The general experimental method, based on that of Spoehr (141), is as follows:

Alginic acid was added gradually, with stirring, to formic acid (500 ml.) and the mixture heated under reflux with occasional

shaking. In some experiments, hydrolyses were carried out under an atmosphere of nitrogen or carbon dioxide. In all hydrolyses, the solution turned dark brown or black after about 9 hours. After cooling, undissolved material was removed by filtration, washed with water and ethanol, dried in vacuo and weighed. The filtrate was distilled in vacuo at 50° to remove most of the formic acid, the last traces of which were removed by repeated distillation with water. The residue was dissolved in water, treated with charcoal, filtered and evaporated. Any crystals were removed by filtration. The residual syrup was hydrolysed with N sulphuric acid at 100° for 3 hours to destroy any stable formyl esters. The hydrolysate was neutralised with barium carbonate. In some experiments, ethanol was added to the final hydrolysate to precipitate high polymers. Results are summarised in TABLE 9.

Hydrolysis 1. (TABLE 9). D-Mannurone (0.8 g.) (3%) crystallised from the solution after removal of formic acid, and had $[\alpha]_D$ +87° (c, 1.2). Chromatographic examination in solvent (11) revealed a trace of gulurone. In another experiment under identical conditions, chromatographically pure D-mannurone was obtained (0.3 g.) (1.5%), having $[\alpha]_D$ +88° (c, 1.0) and m.p. and mixed m.p. 188°.

Hydrolysis 2. During removal of formic acid, a waxy solid (1.9 g.) separated from the solution. This material had $[\alpha]_D$ ca. 0° (c, 0.9) and chromatographic examination showed that it consisted

TABLE 9

No.	Source of Alginic acid	wt.(g.)	Experimental Conditions	Products	Total %
1	Commercial alginic acid (Hopkins & Williams)	25	90% formic acid; 100°; 10 hours	a) Undissolved residue (7 g.) b) Mixture of mannurone and higher polymers (2.8 g.) c) Mannurone crystals (contaminated with gulurone) (0.8 g.)	42
2	Alginic acid (Hopkins & Williams)	20	90% formic; 100°; 10 Hours (under nitrogen)	a) Undissolved material (5 g.) b) Impure mannurone (1.4 g.)	32
3	Calcium alginate (Alginate Industries)	17	75% formic; 100°; 12 hours	a) Undissolved residue (9.2 g.) b) Mannurone crystals (1.0g.) c) Mixture of mannurone, gulurone and unhydrolysed material (1.3 g.)	68
4	L. digitata	20	90% formic; 105°; 10 hours (under carbon dioxide)	a) Undissolved residue (5 g.) b) Mannurone and partially degraded acid (1.3 g.)	32
5	L. digitata	17	60% formic; 100°; 30 hours	a) Undissolved residue (4.3 g.) b) Mixture of mannurone, gulurone and partially degraded acid (2 g.)	37

of unhydrolysed material and a trace of mannurone. Hydrolysis with N sulphuric acid at 100° for 3 hours gave a solid (1.4 g.) consisting mainly of mannurone. No gulurone was detected.

Hydrolysis 3. D-mannurone (1.0 g.) having $[\alpha]_D$ +90° (c, 0.72) crystallised from the residual syrup after removal of formic acid. The crystals had m.p. and mixed m.p. 188°. The residual syrup (3c) (1.3 g.), on chromatographic examination in solvents (12) and (14) was found to contain mannurone, gulurone and partially hydrolysed material.

Separation of syrup (3c)

The syrup (1.0 g.) was placed on a cellulose column (70 x 3 cm.) and eluted in solvent (14), fractions of 20 ml. being collected. Every fifth fraction was concentrated and examined chromatographically in solvents (12) and (14). The following fractions were obtained.

Fraction i, a syrup (23 mg.), had $[\alpha]_D$ +76° and R_{glu} 2.3 and 4.5 in solvents (14) and (3) respectively. Reduction and hydrolysis of the methyl ester methyl glycoside yielded a sugar having M_G 0.59 in borate buffer, identical with authentic gulose.

Fraction ii, a syrup (430 mg.). Chromatographic examination in solvents (14) and (3) showed spots corresponding to mannurone, gulurone and a diffuse spot of a speed comparable with mannuronic or guluronic acids. A sample (50 mg.) was converted to the methyl ester methyl glycosides, reduced with borohydride and hydrolysed.

Ionophoresis in borate buffer showed mannose (M_G 0.46) and a trace of gulose (M_G 0.59).

Fraction iii (370 mg.) was found to be unhydrolysed material, showing no chromatographic movement in solvent (14) during 18 hours.

A part of syrup (3 c) (300 mg.), separated on 3 MM paper in solvent (3), yielded mannose and gulose in the ratio 6:1.

Hydrolysis 4. After evaporation of the reaction mixture to 100 ml., formic acid was removed by repeated extraction with ether. No gulurone was detected.

Hydrolysis 5. After removal of formic acid, the residual syrup was heated with N sulphuric acid at 100° for 3 hours. After working up in the usual manner, the resulting syrup (2.0 g.), $[\alpha]_D$ +61° (c, 1.04) was heated at 100° for 1 hour to lactonise the uronic acids. Chromatographic examination in solvent (14) showed mannurone, partially hydrolysed material and a trace of gulurone.

EXPT. 5. ACTION OF HOT FORMIC ACID ON MANNURONE

Chromatographically pure mannurone crystals (64 mg.) were heated at 100° with 98% formic acid (5 ml.) for 1 hour. The solution was diluted to 10 ml. with distilled water and evaporated to dryness. More formic acid (5 ml.) was added and the process repeated. After treatment with charcoal, filtration and evaporation, the residue weighed 50 mg. Recrystallisation from water yielded

D-mannurone, m.p. and mixed m.p. 188°.

Chromatographic examination of the residual syrup in solvents (12) and (3) revealed mannurone, and a trace of material on the starting line. Gulurone was not detected.

EXPT. 6. FRACTIONATION OF ALGINIC ACID

Following the method of Haug (176), alginic acid was fractionated to give a fraction rich in gulurone. Alginic acid (10 g.), freshly extracted from L. digitata, and sodium bicarbonate (5 g.) were dissolved in water (1200 ml.) to give a 1% solution of sodium alginate. An equal volume of saturated aqueous potassium chloride was added with stirring and the mixture allowed to stand overnight. The precipitated gel (mannurone-rich fraction) was removed by centrifugation and the remaining material precipitated from the centrifugate by addition of concentrated hydrochloric acid. This precipitate was washed with water and acetone until free from chloride ions and dried in vacuo. Refractionation of this material was repeated twice, yielding a final gulurone-rich fraction (5.5 g.).

Hydrolysis. This fraction (5 g.) was heated with 90% formic acid (300 ml.) at 100° for 10 hours, and the product worked up in the usual way, giving an insoluble residue (1.6 g.) and a pale brown syrup (1.8 g.). Chromatographic examination in solvent (3) showed spots corresponding to mannurone, gulurone (5:1 visual estimation) and a diffuse mannuronic acid/guluronic acid spot.

Attempted Separation. The syrup (1.8 g.) was placed on a cellulose column (70 x 4 cms.) and eluted with solvent (3). Every fifth fraction was examined chromatographically and like fractions were combined. The following fractions were obtained.

Fraction (1), a syrup (14 mg.), had $[\alpha]_D$ ca 0. Chromatographic examination in solvent (3) showed that it was non-reducing but gave a bright yellow fluorescent spot R_{Gal} 6.6 under U.V. light when sprayed with aniline oxalate. The material, presumably a degradation product, was not examined further.

Fraction (2), a syrup (128 mg.), had $[\alpha]_D$ +46.4° (c, 0.54). Chromatographic examination in solvent (3) showed gulurone and guluronic acid. Attempted crystallisation from ethanol was unsuccessful, both before and after lactonisation by heating at 100° for 4 hours. Separation of the lactone spot on 3 MM paper yielded a syrup (30 mg.) having $[\alpha]_D$ +71° +56° (c, 0.33) 2 hours. A portion (10 mg.) was reduced by borohydride giving gulose (paper chromatography, solvent 18).

Fraction (3), a syrup (438 mg.), consisting of gulurone and mannurone (1:6) (paper chromatography, visual estimation) and a diffuse acid spot.

Fraction (4) (600 mg.) was crystalline, chromatographically identical with mannurone, and had m.p. and mixed m.p. 188° with authentic D-mannurone.

Fraction (5) (water washings) (500 mg.) had $[\alpha]_D$ +81.1° (c, 1.0). Chromatographic examination in solvent (3) showed mannuronic and guluronic acids, plus unhydrolysed material on the

starting line. Heating with 90% formic acid for 12 hours at 100° caused lactonisation of the mannuronic acid but did not degrade the unhydrolysed material.

EXPT. 7. CHARACTERISATION OF L(+)-TARTARIC ACID

L(+)-tartaric acid was found to be separable from meso-tartaric acid in a variety of solvents (TABLE 10). Chromatograms were dried in air at room temperature for 24 hours and sprayed with bromocresol green (spray c). Reducing sugars, subsequently found in oxidation products, were detected on the chromatograms by re-spraying with

TABLE 10

Solvent	3	4	8	16	17
L(+)-tartaric acid R_{Glu}	4.0	2.5	0.63	1.30	10.0
meso-tartaric acid R_{Glu}	3.3	2.1	0.72	1.23	7.1

aniline oxalate (spray a). It was possible to separate glyoxylic acid from L(+)-tartaric acid only in solvent (4). However, glyoxylic acid gave a characteristic bright yellow spot with aniline oxalate.

Action of ammonium molybdate on L(+)-tartaric acid

It has been reported (173) that addition of ammonium molybdate (1 mole) to L(+)-tartaric acid (3 moles) in aqueous solution causes a 60-fold increase in the observed rotation. The rotations of solutions containing L(+)-tartaric acid were, in fact, found to increase at least 20-fold, and on standing, the rotation solutions

turned green. Ammonium molybdate had no effect on the rotations of meso-tartaric acid or of D-mannurone.

EXPT. 8. OXIDATION OF ALGINIC ACID AND ISOLATION OF L(+)-TARTARIC ACID

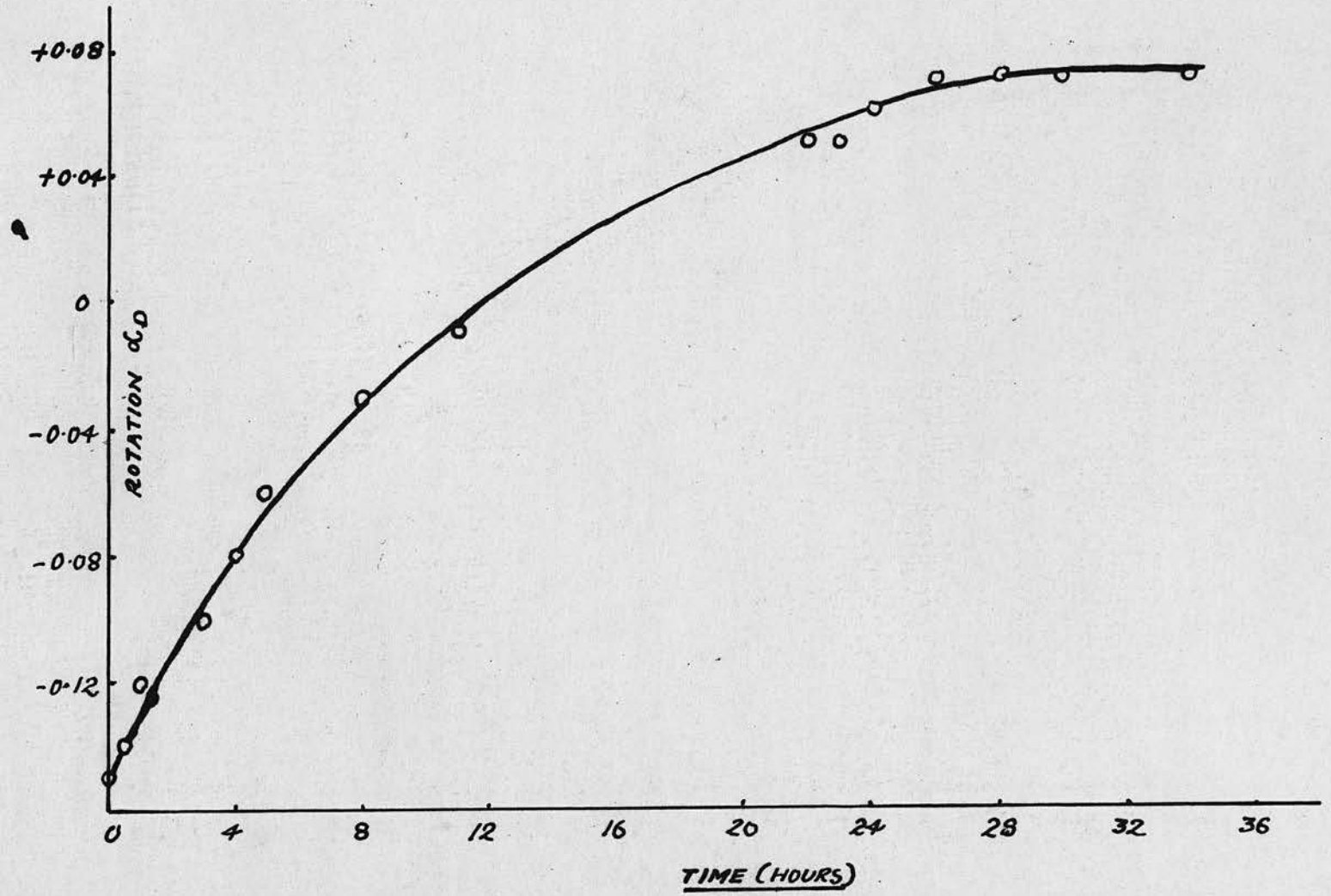
a) Periodate oxidation. In a typical experiment, alginic acid (10 g.) from L. digitata was stirred with a saturated solution of sodium metaperiodate (200 ml.) for 26 hours at room temperature (157). A small quantity of inorganic material was filtered off and the filtrate poured into tert. butanol (1300 ml.) with stirring. Oxopolysaccharide was precipitated as a white fibrous solid. After washing with tert. butanol : water (3:1) (4 x 200 ml.), the product was dried over concentrated sulphuric acid in vacuo. Yield, 8.2 g.

b) Bromine oxidation. The oxopolysaccharide (8.2 g.) was dispersed in water (350 ml.) and stirred rapidly at room temperature for 24 hours with bromine (16 g.) and barium carbonate (35 g.). Excess bromine was then removed by aeration and the calculated quantity of sulphuric acid added to precipitate barium ions as sulphate. After dialysis against running water for 3 days the solution was freeze-dried, yielding a white solid (2.4 g.) having $[\alpha]_D$ -32.5° (c, 1.18). The material was extremely soluble in water.

c) Hydrolysis of bromine-oxidised oxopolysaccharide. The product

HYDROLYSIS OF BROMINE-OXIDATION PRODUCT

GRAPH 7A



(0.74 g.) was dissolved in 0.05 N sulphuric acid (100 ml.) and heated at 100° under reflux for 36 hours. The observed $[\alpha]_D$ of a cooled aliquot (10 ml.) in a 1 dm. tube rose from -0.15° to +0.07° (const.) (GRAPH 7A). Addition of ammonium molybdate (100 mg.) to the final aliquot immediately raised the rotation to +0.39°, the solution turning green after a few hours. After cooling, the main solution was extracted with a 5% solution of di-n-octylmethylamine in chloroform (3 x 50 ml.) to remove sulphuric acid (197). In a trial experiment, this reagent did not extract tartaric acids from aqueous solution. The aqueous extract was treated with 1 R 120(H) resin, filtered and concentrated to a syrup (108 mg.). Chromatographic examination in solvent (3) showed spots corresponding to meso- and L(+)-tartaric acids.

d) Isolation of L(+)-tartaric acid. The syrup (108 mg.) was placed on Whatman No. 3 paper and eluted with solvent (3). After irrigation for 36 hours, the papers were dried in air for 36 hours at room temperature. After spraying guide strips with spray (c), the appropriate paper sections were cut out and eluted with ethanol: water (1:1). After evaporation to dryness, the following fractions were obtained.

Fraction (1), a syrup (27 mg.), had $[\alpha]_D = 0$ (c, 2.0) and was chromatographically identical with meso-tartaric acid in solvents (3), (4), (8), (16) and (17).

Fraction (2), a syrup (23 mg.), had $[\alpha]_D$ +8.8° (c, 2.3). Extraction with cold water and treatment with 1 R 120 (H) resin yielded

a colourless glass (14 mg.) having $[\alpha]_D$ +10.8° (c, 1.4). Cf. L(+)-tartaric acid $[\alpha]_D$ +12.0° (186). The material was chromatographically identical with L(+)-tartaric acid in solvents (3), (4), (8), (16) and (17). Attempted crystallisation from aqueous solution was unsuccessful. A partially crystalline mass was obtained by drying the syrup at 61° over phosphorus pentoxide for 3 days. An X-ray powder photograph of the material was essentially identical with one of authentic L(+)-tartaric acid, and only certain very faint bands could not be identified with certainty. Comparison of the photograph with those of meso-tartaric acid and D-mannurone revealed no similarity with these compounds. Addition of the calculated quantity of potassium hydroxide to a solution of the syrup and evaporation to dryness yielded the crystalline potassium hydrogen salt. Comparison of the X-ray powder photograph with one of the corresponding salt of meso-tartaric acid revealed major differences, while all the major bands in the photograph were identical with those in a powder photograph of potassium hydrogen L(+)-tartrate.

Fraction (3), a syrup (54 mg.). Deionisation with 1 R 120(H) resin and treatment with charcoal yielded a syrup (46 mg.) which on chromatographic examination in solvent (3) showed an acid streak with most of the material on the starting line. Measurement of specific rotations was impossible owing to charring of the syrup on concentration. Hydrolysis with 90% formic acid at 100° for 20 hours, and chromatographic examination of the product in solvent (3) showed meso-tartaric acid, mannuronic acid and mannurone.

EXPT. 9. INCOMPLETE OXIDATION OF ALGINIC ACID

a) Periodate oxidation. In further experiments of the same type (detailed below), it was found that oxidation of the alginic acid was incomplete as unattached mannurone and gulurone were detected in hydrolysates of the poly-acid obtained after bromine oxidation. Alginic acid (20 g.) from commercial calcium alginate was stirred with saturated sodium metaperiodate (500 ml.) for 24 hours at 18°. The resulting viscous solution was centrifuged to remove inorganic material. Addition of tert. butanol (2 l.) with rapid stirring precipitated oxopolysaccharide as before but, in addition, a sticky solid (M). This was removed, and, after drying, weighed 4.3 g. The remainder of the solid was dried over concentrated sulphuric acid in vacuo. Yield, 25 g. of impure oxopolysaccharide. A sample (1 g.) was dispersed in water (150 ml.), dialysed and freeze-dried yielding an off-white fluffy solid (0.54 g.) having $[\alpha]_D$ +108.2° (c, 0.57).

The sticky material (M) (4.3g), after hydrolysis with 90% formic acid for 20 hours at 100° and chromatographic examination in solvent (3), showed mannurone and mannuronic acid.

b) Bromine oxidation. The crude oxopolysaccharide (15 g.) in water (700 ml.) was stirred with bromine (16 g.) and barium carbonate (45 g.) for 40 hours at 18°. After removal of excess bromine and precipitation of barium ions as before, the solution was dialysed and freeze-dried. The resulting solid was only slightly soluble

in water and had $[\alpha]_D$ +36.8° (c, 0.33). An aqueous solution was reducing to ammoniacal silver nitrate and to Fehling's solution. The material was hydrolysed with .05 N sulphuric acid at 100° for 30 hours, yielding a syrup (1.2 g.) which on chromatographic examination in solvent (3) showed mannurone, gulurone, meso-tartaric acid and unhydrolysed material.

c) Reduction of oxidation products. Both the oxopolysaccharide (157 mg.) and the bromine-oxidised oxopolysaccharide (65 mg.) were converted to the methyl ester methyl glycosides and reduced with sodium borohydride. Hydrolysis of the products with N sulphuric acid at 100° for 6 hours gave mannose in both products on chromatographic examination in solvents (2) and (3).

EXPT. 10.

In a further experiment, the reduction of periodate was measured. Alginic acid from L. digitata (8.5 g.) was stirred with 0.43 M sodium meta-periodate solution (500 ml.) at room temperature. The course of the oxidation was followed by titration of aliquots (2 ml.) with standard sodium arsenite and iodine (175), with the following result.

Time (hours)	0.5	1.0	2.0	4.0	5	6	8.5	26
Periodate reduced (Moles/anhydro C_6 unit)	0.64	0.67	0.70	0.72	0.75	0.78	0.85	0.96

After 26 hours, when the consumption of periodate per anhydro C_6 unit

was 0.96 moles, the oxidation was stopped by blowing sulphur dioxide through the solution. Excess sulphur dioxide was removed by aeration.

The solution, after oxidation with bromine in the usual way until it was non-reducing, was dialysed for 4 days against running water. An aliquot (10 ml.) in a 1 dm. tube had $[\alpha]_D$ +0.96°.

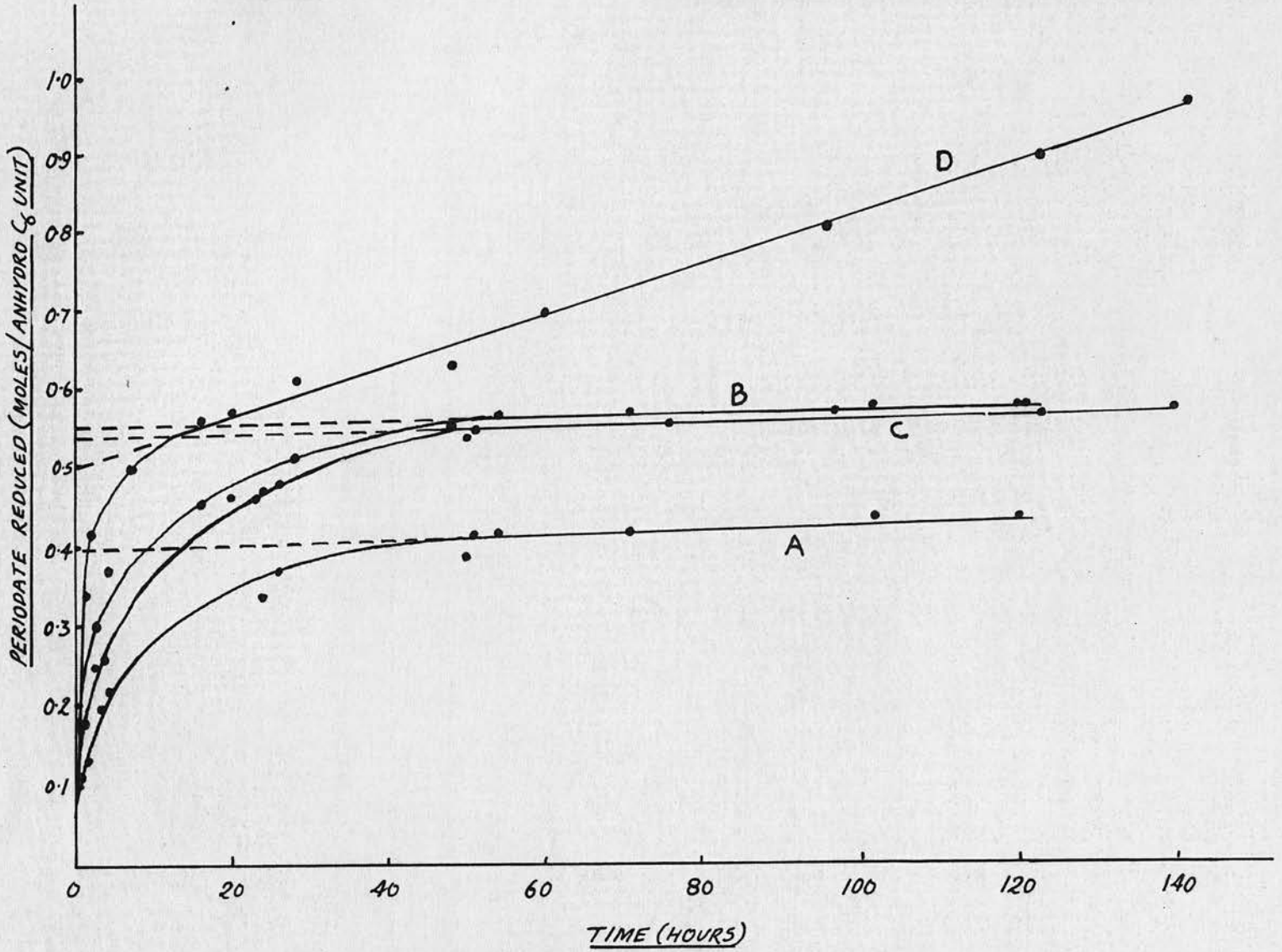
After hydrolysis (0.5 N sulphuric acid, 100°, 28 hours) an aliquot (10 ml.) had $[\alpha]_D$ +0.66° (const.). Neutralisation with barium carbonate, filtration, evaporation and treatment with 1 R 120(H) resin yielded a brown solid (4.1 g.). A chromatogram in solvent (3) was diffuse showing mannurone and possibly tartaric acids.

Reduction with borohydride. A part of the product (2 g.) was reduced with sodium borohydride in the usual way, after conversion to ester glycosides. An ionophoretogram of a hydrolysate in borate buffer revealed mannose and gulose as the predominant components.

In further experiments, under conditions identical with those used in EXPT. 8, mannurone, L(+)-tartaric acid, meso-tartaric acid and glyoxylic acid were detected chromatographically in hydrolysates of the products of bromine oxidation.

GRAPH 9.

PERIODATE OXIDATION OF ALGINIC ACID (EXPT. II).



EXPT. 11. OXIDATION WITH DILUTE PERIODATE SOLUTIONS

As the course of periodate oxidation is considerably affected by experimental conditions (43, 45), further experiments were carried out using dilute sodium meta-periodate and controlled conditions. Alginic acid, extracted from L. digitata by the usual method, was dried at 61°/10 mm. to constant weight. Samples (1 g.) were oxidised by 0.015 M sodium meta-periodate (800 ml.) under various conditions of temperature and pH. Buffered solutions were made by dispersing the alginic acid in the appropriate buffer (400 ml.) and adding 0.03 M periodate (400 ml.). Each solution contained 2.11 moles of periodate for every anhydro hexuronic acid unit. At intervals, aliquots (1 ml.) were withdrawn and periodate uptake measured spectrophotometrically by the method of Aspinall and Ferrier (50). At the end of the reaction, sulphur dioxide was blown into the solutions to destroy excess periodate. The solutions, after dialysis, were treated with 1 R 120 (H) resin and the oxopolysaccharides precipitated by addition of tert.-butanol. The precipitates were washed with acetone and ether, dried in vacuo and weighed. The results are summarised in TABLE 10, while GRAPH 9 depicts the course of the oxidations.

TABLE 10

Sample (1 g.)	Temp.	pH	Buffer	Periodate uptake (moles)	Wt. of oxopoly-saccharide (g.)
A	2°	1.65	0.1 N H_2SO_4	0.40	0.41
B	2°	3.72	acetate	0.55	0.83
C	2°	3.2 after 48 hours	-	0.54	1.08
D	25°	3.2 after 48 hours	-	0.50	0.99

EXPT. 12. REDUCTION OF OXO-ALGINIC ACIDS

1. Preliminary Reductions on (C).

Oxo-alginic acid (C) (530 mg.) was added to 0.05 M boric acid (50 ml.) and cooled to 0°. Sodium borohydride (1 g.) in water (40 ml.) was added dropwise with stirring at 0°. The solution (pH 9) was then allowed to stand overnight at 2°. After acidification with acetic acid and dialysis, the solution was treated with 1 R 120 (H) resin and the reduced oxo-polysaccharide precipitated by addition of ethanol (6 volumes). Centrifugation and drying yielded a white powder (436 mg.).

Esterification. A portion of the product (275 mg.) was refluxed with 5% anhydrous methanolic hydrogen chloride (6 ml.) for 6 hours. The resulting solution was neutralised with silver carbonate, filtered and concentrated to a syrup (200 mg.) (i).

The remainder of the reduction product (160 mg.) was shaken with 5% anhydrous methanolic hydrogen chloride (5 ml.) at room temperature for 3 days (187). Working up in the usual way yielded a syrup (91 mg.) (ii).

Reduction of the esters. Syrup (i) was dissolved in 0.4 M boric acid, cooled to 0° and sodium borohydride (0.4 g.) in water (10 ml.) added with stirring, at 0°. The resulting solution, after standing overnight at 2°, was acidified with acetic acid, dialysed and freeze-dried yielding a solid (3 mg.). Hydrolysis with N sulphuric acid at 100° for 4 hours and chromatographic examination in solvent (18) revealed mannose and gulose (4:1).

Syrup (ii) was treated in exactly the same way using proportional quantities of borohydride. A final yield of 1 mg. was obtained, which on hydrolysis as before gave mannose and gulose (4:1).

2. Reductions and diazomethane esterification

a) Reduction of poly-dialdehyde. Oxo-alginic acid (A) (410 mg.) was dissolved in 0.4 M boric acid (30 ml.) and cooled to 0°. Sodium borohydride (0.8 g.) in water (10 ml.) was added with stirring at 0° and the solution was allowed to stand at 2° overnight. The solution was then acidified with acetic acid, dialysed, treated with 1 R 120 (H) resin and freeze-dried, yielding a fluffy white powder (250 mg.) which was acid in aqueous solution.

Oxo-alginic acids (B), (C) and (D) (820 mg.) (540 mg.) and

(980 mg.) respectively were treated in the same way using proportional amounts of borohydride.

Yields: (B) 450 mg.; (C) 330 mg.; (D) 580 mg.

b) Esterification. The reduced oxo-alginic acids were dissolved in absolute methanol (50 ml.) in 100 ml. flasks. The solutions were titrated with an ethereal solution of diazomethane at room temperature, until a persistent yellow colour indicated that excess diazomethane was present. Distillation at 60° removed the excess diazomethane and ether. An equal volume of water was added to the solutions, which were neutral.

c) Reduction of the esters. The aqueous methanolic solutions were reduced by allowing to stand overnight at 2° with excess sodium borohydride. The solutions were then acidified with dilute acetic acid. Conc. sulphuric acid was then added to bring the solutions to N and after hydrolysing for 6 hours at 100°, the products were cooled, neutralised with barium carbonate and filtered. Repeated evaporation with methanol yielded solids still containing inorganic material. Treatment of samples with 1 R 120 (H) resin and chromatographic examination in solvents (2), (3) and (18) showed that the products from each of the oxoalginic acids were identical and showed no trace of organic acids (spray C). The bulk of the solids were therefore combined, treated both with 1 R 120 (H) and 1 R 4 B (OH) resins to remove all trace of inorganic material and evaporated to a syrup (780 mg.). Chromatographic examination in

solvents (2), (3) and (18) gave spots corresponding to mannose, gulose, erythritol, threitol and glycerol (run against controls). The proportion of hexose to tetritol was ca. 2:2.9.

Separation of Products.

The syrup,(780 mg.), was placed on 3 MM paper and eluted with solvent (3) overnight. Elutes of the appropriate paper strips with water yielded glycerol (26 mg.), mannose (+ gulose) (200 mg.) and erythritol (+ threitol) (290 mg.).

Glycerol was characterised as the tri-O-tosyl derivative (188). To an ice-cold solution of glycerol (26 mg.), in anhydrous pyridine (0.5 ml.) was added p-toluene-sulphonyl chloride (182 mg.). The mixture was kept overnight at room temperature and poured into cold water (10 ml.). The gum-like product was washed with cold water, dissolved in ethanol and cooled to 0°. ~~The resulting oil was crystallised from acetone-ethanol and had m.p. and mixed m.p. 103-105°.~~

Mannose was characterised as the phenylhydrazone. Mannose (50 mg.) was dissolved in 75% acetic acid (0.1 ml.). Phenylhydrazine (0.1 ml.) was added and the mixture kept at 0° overnight. The product was washed with cold water and ethanol and had m.p. and mixed m.p. 188° (decomp.).

DISCUSSION

Prior to 1955, the accepted structure for alginic acid from the available evidence was that proposed by Hirst and his co-workers, namely, a straight chain of 1,4'-β- linked mannuronic acid residues (page 79). However, the detection, by Fischer and Dorfel (160), by the use of improved chromatographic techniques, of L-guluronic acid in hydrolysates of alginic acid from various species of European brown algae, indicated that the molecule was more complex than had been supposed. The experiments in part two of this thesis were undertaken with a view to confirming that acid hydrolysates of alginic acid from Laminaria digitata contain L-guluronic acid. It was also intended, by chemical modification of the polysaccharide and isolation of end-products, to prove indirectly that L-guluronic acid residues form an integral part of the alginic acid molecule.

Source of Alginic acid. The primary source of material was from a sample (1 kg.) of Laminaria digitata fronds. This seaweed, collected at Thorntonloch in December (and having, therefore, a near-maximum alginic acid content), had been dried at 45° for 2 hours and then 25° for 20 hours. The alginic acid was extracted from the powdered seaweed by dilute sodium carbonate and precipitated in the form of its insoluble calcium salt. Treatment of this salt with hydrochloric acid yielded the free-acid polysaccharide. For hydrolytic experiments, commercial calcium alginate and alginic acid afforded further sources of starting material.

The acid from L. digitata was obtained as a pure white, fibrous solid, practically insoluble in water, and having $[\alpha]_D$ -134° - 139° in dilute sodium hydroxide, a value in agreement with those reported by other workers (128). A sample dried to constant weight had ash 0.96% and equivalent weight 144-170. This slightly low result could possibly be due to the presence of traces of hydrochloric acid. The commercial alginic acid were pale brown in colour and had probably been degraded slightly by excessive heating.

Hydrolysis of Alginic acid. Fischer and Dorfel employed sulphuric acid for the hydrolysis, while Spochr, hoping to reduce the degradation caused by mineral acids, used formic acid. Both these hydrolytic media were used in the present work and the results are summarised in TABLES 8 and 9. Hydrolysis with formic acid was carried out at 100-105° for several hours, after which formic acid was removed by repeated distillation with water. Following the method of Fischer and Dorfel, sulphuric acid hydrolysis was carried out at low temperatures with concentrated acid followed by heating the diluted solution at 100°.

In all cases, it was found that very considerable degradation of the material occurred and that the yields of identifiable products from the hydrolysates were extremely small. Formic acid hydrolysis was found to give the better yields of monosaccharides. Although the greater part of the hydrolysates consisted of charred or unhydrolysed material, mannurone was detected chromatographically as its lactone in all the hydrolysates and, in some cases, was

isolated in crystalline form. The crystalline lactone had $[\alpha]_D$ + 90° and melting-point 188°. This value for the melting-point, also reported by Fischer and Dorfel (160) and, more recently, by Whistler and Kirby (161), is different from that obtained by Spoehr (141) and Nelson and Cretcher (135) who reported 145° and 142° respectively. As this lactone was the main hydrolysis product isolated by these workers, it seems unlikely that it was L-gulurone, and the possibility exists, therefore, that D-mannurone has been isolated in more than one crystalline form.

Both sulphuric and formic acid hydrolysates afforded chromatographic evidence for the presence of L-gulurone. This sugar, however, was apparently present in much smaller proportion than D-mannurone and, in some experiments, was not detected at all (TABLES 8 and 9). This lactone, moving considerably faster than mannurone or gulurone in various solvents (TABLE 7), had chromatographic mobilities identical with those reported by Fischer and Dorfel.

Separation of the hydrolysates on cellulose columns was not entirely satisfactory, but the products of formic acid hydrolysis 3, when separated in this way, yielded a syrup having specific rotation and chromatographic mobilities identical with those reported by Fischer and Dorfel for L-gulurone. The material, however, could not be induced to crystallise. Reduction and hydrolysis of the methyl ester methyl glycoside yielded a syrup with M_G 0.59 in borate buffer, identical with authentic gulose. This syrup was also chromatographically identical with gulose in solvent 18, which was

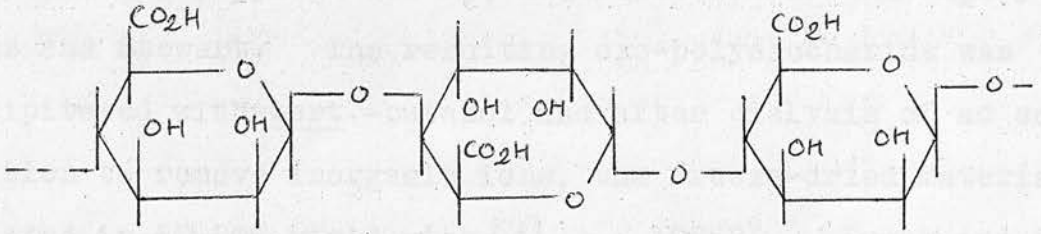
found to give a satisfactory separation of gulose and mannose.

L-Guluronic acid has not been previously reported in formic acid hydrolysates of alginic acid. Whistler and Kirby, although able to isolate L-gulurone from sulphuric acid hydrolysates of Macrocystis pyrifera, failed to detect this lactone when formic acid was employed as the hydrolytic agent. This result they attributed to the instability of L-guluronic acid to hot formic acid (161).

In the present experiments, it was found that the alginic acid was never completely disintegrated under the hydrolytic conditions employed. If the concentration of formic acid was less than 90%, a considerably longer time was required to achieve the same degree of hydrolysis (TABLE 9). No significant difference was found between the behaviour of commercial alginic acids and that of freshly-extracted material. The extent of degradation was not apparently decreased by carrying out the hydrolysis in inert atmospheres such as nitrogen and carbon dioxide. In the sulphuric acid hydrolyses, the amount of alginic acid disintegrated depended to a considerable extent on the temperature of the reaction with concentrated sulphuric acid. At 18°, no insoluble residue remained, whereas at -5°, almost half the alginic acid remained undissolved (TABLE 8). L-gulurone was obtained both from commercial alginic acids and from the freshly-extracted polysaccharide from L. digitata.

Fractionation of alginic acid by the method of Haug (176) did not give a homopolysaccharide, each fraction containing different proportions of the two hexuronic acids. Recently, Vincent (177) has separated oligosaccharides from sulphuric acid hydrolysates of

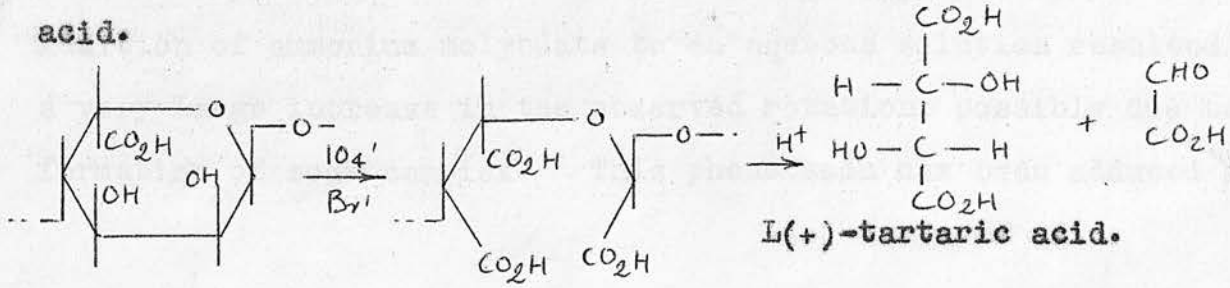
commercial alginic acid. Paper chromatographic evidence indicated that these oligosaccharides contained both mannuronic and guluronic acids. However, until a more clear-cut fractionation is achieved, and oligosaccharides are fully characterised, it cannot be definitely decided whether D-mannuronic and L-guluronic acids occur in separate polymers or whether alginic acid consists of chains comprising varying proportions of both uronic acids (XXII).



XXII.

Both the fractionation experiments of Haug and the hydrolytic data of Fischer and Dorfel suggest that the constitution of alginic acids from different species of seaweed may differ.

Oxidation of Alginic acid. Lucas and Stewart, in 1940, (157) subjected alginic acid from M. pyrifera to oxidation with 0.38 M periodic acid at room temperature. After oxidation of the product with bromine, the solution was hydrolysed until the observed rotation had fallen to 0°. Meso-tartaric acid was isolated from the hydrolysate (page 101). Oxidation of a 1,4'-linked L-guluronic acid residue under the same conditions should yield L(+)-tartaric acid.



L(+)-tartaric acid.

As L(+)-tartaric acid is optically active, the final hydrolysed solution should have a positive optical rotation. Experiments were therefore undertaken with a view ot isolation of L(+)-tartaric acid and the provision of indirect proof for the presence of 1,4'-linked L-guluronic acid units in alginic acid. Alginic acid from L. digitata was oxidised at room temperature with concentrated sodium metaperiodate (approx. 0.4 M), under the conditions employed by Lucas and Stewart. The resulting oxo-polysaccharide was precipitated with tert.-butanol and after dialysis of an aqueous solution to remove inorganic ions, the freeze-dried material was isolated in 60-80% yield with $[\alpha]_D$ + 108.2°. Lucas and Stewart report $[\alpha]_D$ + 35° for this product but as these authors did not dialyse and freeze-dry the material, it was probably contaminated with inorganic ions. Oxidation of the oxopolysaccharide with bromine in the presence of barium carbonate to reduce the acidity yielded a poly-tricarboxylic acid with $[\alpha]_D$ - 32.5°. When this material was heated at 100° with 0.05 N sulphuric acid the observed optical rotation of a measured aliquot rose from -0.15° to +0.07° (const.). Chromatographic examination of the syrup obtained after removal of mineral acid and concentration revealed spots identical with meso- and L(+)-tartaric acids. Separation on thick paper gave the optically-active component as a syrup with specific rotation identical with that reported for L(+)-tartaric acid. Addition of ammonium molybdate to an aqueous solution resulted in a very large increase in the observed rotation, possibly due to formation of some complex. This phenomenon has been adduced as

a means of detection and analysis of L(+)-tartaric acid (173). Comparison of the X-ray powder photographs of the partially crystalline acid and of an authentic sample, and also of the acid potassium salts, showed that the material was, in fact, L(+)-tartaric acid.

It is considered that the positive rotation observed in the hydrolysis solution (page 121) could be due only to L(+)-tartaric acid and thus to 1,4'-linked L-guluronic acid in the alginic acid molecule. A 1,3'-linked L-guluronic acid residue would be unattacked by the periodate, while this uronic acid, if linked 1,2'-, would give rise to optically inactive products on oxidation. D-Galacturonic acid residues would also give rise to L(+)-tartaric acid, but this possibility is ruled out on the grounds that no D-galacturonic acid has ever been detected in hydrolysates of alginic acid. The failure of Lucas and Stewart to detect L(+)-tartaric acid is probably due to the fact that when the hydrolysis solution of the bromine-oxidised products became optically inactive (16 hours), they stopped the reaction. The expected meso-tartaric acid was isolated and characterised, and in the absence of chromatographic techniques, and L(+)-tartaric acid in the mixture would necessarily be missed.

In the present work, it became evident that although the uptake of periodate reached the theoretical value of 1 mole per anhydro acid unit, (EXPT. 10), α -glycol cleavage was incomplete. This suggested that considerable over-oxidation of the cleaved residues was taking place. In EXPT. 10, using 0.43 M sodium

metaperiodate at room temperature, the periodate reduced attained a value of 0.96 moles per anhydro hexuronic acid unit. The solution was bromine-oxidised and hydrolysed. Subsequent analysis revealed that D-mannurone was present in the hydrolysate. Reduction of the ester glycosides and ionophoretic examination revealed mannose and gulose, thus indicating that a proportion of both types of uronic acid residues had been unattacked by the periodate. From an examination of the oxidation curve published by Lucas and Stewart, in which 1.1 moles per anhydro uronic acid unit were reduced, it appears that over-oxidation had taken place and that the primary oxidation was complete after consumption of 0.7 moles of periodate.

Oxidation with dilute sodium meta-periodate. In view of the above anomalous results, further oxidation experiments were carried out under carefully controlled conditions. The work of Hough and others (45, 46) has shown that unless dilute periodate is used at controlled temperatures and acidity, (pH), anomalous results may be expected due to hydrolysis of formyl esters and consequent degradation of the polysaccharide from the reducing end. Formyl esters are most stable at pH 3.6 (47, 48).

Therefore, samples of alginic acid were oxidised with dilute periodate under various conditions of acidity and temperature. Four samples (1 g.) of alginic acid from L. digitata, dried to constant weight, were oxidised in the dark in solutions containing 2.11 moles of periodate per anhydro uronic acid unit (TABLE 10). The method of estimating the periodate reduced has the advantage

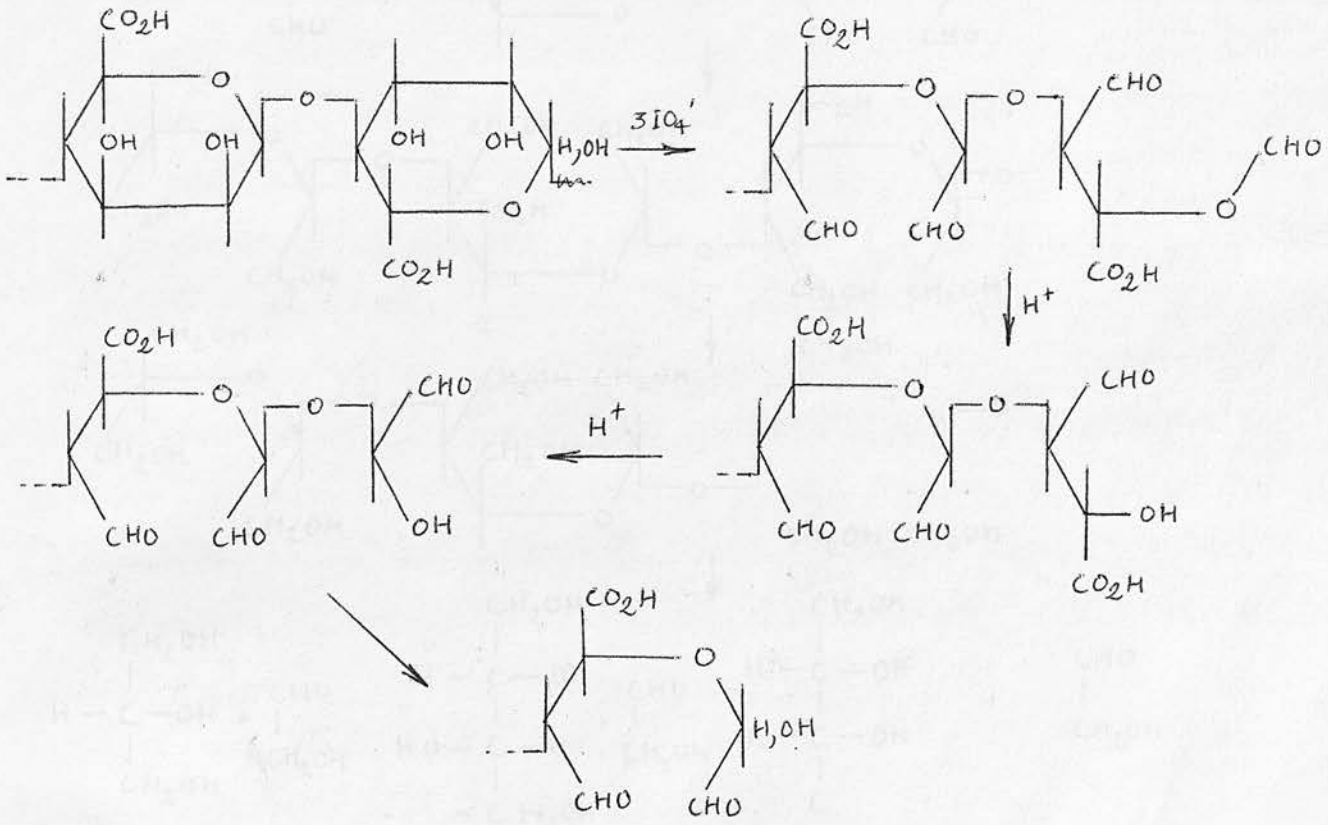
of not changing the pH of the solution and thus obviates the risk of errors due to hydrolysis of formyl esters (46) during the determination.

The results are shown in GRAPH 9. The samples were completely dissolved in the periodate solution during 1 to 2 hours. After about 60 hours, when the primary oxidation had ceased, the excess periodate was destroyed by saturation with sulphur dioxide. Dialysis and precipitation with tert-butanol gave the oxo-polysaccharides. The poor yield of (A) is probably due to the fact that a poly-dialdehyde is sufficiently acid-labile to be degraded at pH 1.65.

As expected, the curves for oxidation at 2° (A, B, C) flattened out completely, showing that hydrolysis of formyl esters was at a minimum and therefore over-oxidation arrested. It is surprising that the periodate reduced by A was not higher as formyl esters should be hydrolysed to some extent at pH 1.65. The curve for oxidation at 25° (D) showed a considerable gradient after completion of the primary oxidation, indicating that over-oxidation was taking place.

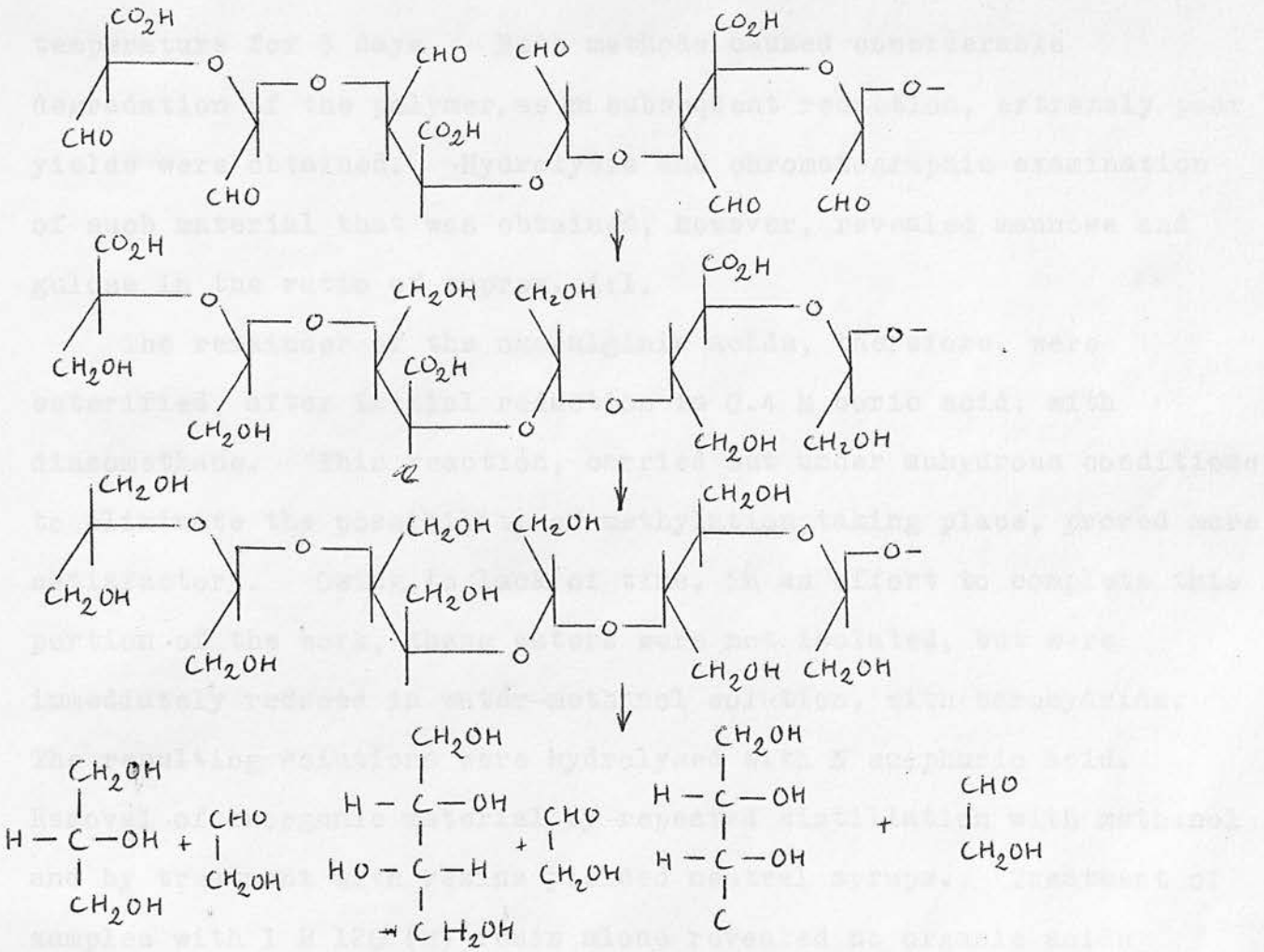
A surprising feature of the oxidations is that a periodate uptake of only 0.40 to 0.55 moles per anhydro acid unit was obtained in all cases. For a 1,4'-linked straight chain poly-saccharide an uptake of 1 mole would be expected. The low figure actually obtained indicates that approximately one half of the hexuronic acid residues were unattacked by the oxidant. This result is in agreement with earlier work (EXPT. 9), in which

mannuronic and guluronic acids were found after oxidation and hydrolysis. The periodate uptake of 1 mole per anhydro uronic acid unit obtained in other experiments (EXPT. 10) must therefore be partly due to degradation from the reducing end. This would arise from hydrolysis of formyl esters and other groupings, followed by further oxidation. A possible scheme for this degradation mechanism is as follows:



Reduction of oxo-alginic acids. The periodate-oxidised alginic acids were subjected to reduction procedures similar to those used by Smith and co-workers on cellulose and amylopectin (189, 190).

A fully reduced oxo-alginic acid would give on hydrolysis, erythritol from residues which had been derived from D-mannuronic acid and threitol from residues derived from L-guluronic acid. From the non-reducing end of the polymer, one mole of glycerol would be produced (XXIII).



XXIII

A sample of oxo-alginic acid (C) was first reduced with sodium borohydride to convert the aldehydic groups to primary alcoholic groupings. This reaction was carried out in 0.05 M boric acid

solution to reduce the alkalinity in the initial stage of the reaction and thus ensure that the polymer remained intact (191). The reduced product, obtained by precipitation with ethanol (page 127) was esterified both by refluxing with 5% anhydrous methanolic hydrogen chloride for 6 hours, and by shaking with this reagent at room temperature for 3 days. Both methods caused considerable degradation of the polymer, as on subsequent reduction, extremely poor yields were obtained. Hydrolysis and chromatographic examination of such material that was obtained, however, revealed mannose and gulose in the ratio of approx. 4:1.

The remainder of the oxo-alginic acids, therefore, were esterified, after initial reduction in 0.4 M boric acid, with diazomethane. This reaction, carried out under anhydrous conditions to eliminate the possibility of methylation taking place, proved more satisfactory. Owing to lack of time, in an effort to complete this portion of the work, these esters were not isolated, but were immediately reduced in water-methanol solution, with borohydride. The resulting solutions were hydrolysed with N sulphuric acid. Removal of inorganic material by repeated distillation with methanol and by treatment with resins yielded neutral syrups. Treatment of samples with 1 R 120 (H) resin alone revealed no organic acids showing that no unreduced fragments remained in the solutions. The syrups were combined, as chromatographic examination showed that their composition was identical.

Chromatographic examination in three different solvents gave spots identical with authentic mannose, gulose, erythritol, theitol

and glycerol. Glycollic aldehyde was not detected. This product was probably lost by volatilisation during evaporation of the hydrolysis solution. The hydrolysate was separated on thick paper into three fractions: glycerol, erythritol + threitol and mannose + gluose. When these experiments were initiated, it was hoped that quantitative assay and characterisation of the above hydrolysis products would be carried out. Unfortunately, many experimental difficulties were encountered not only in isolating quantitative yields of oxo- and reduced polysaccharides, but also in the purification of the hydrolysate. Consequently it has been possible only to separate the material into glycerol, tetritols and hexoses and to characterise the mannose and glycerol as crystalline derivatives.

The weights of isolated tetritols to hexoses were approximately in the ratio of 2:9:2. This provides evidence that about 40% of the uronic acid residues were immune to periodate attack, and is in agreement with the observed reduction of periodate of approximately 0.55 moles per anhydro uronic acid unit. It should be stressed that this figure is not appreciably altered by changes in temperature and pH (1.65 - 3.72).

The total yield of hydrolysate from the oxo-alginic acid was about 34% of the theoretical (allowing for the loss of glycollic aldehyde), and a surprisingly large proportion of glycerol (26 mg. from 780 mg.) was separated from this. It can only have arisen from the non-reducing end of the oxo-alginic acid and corresponds to a chain length of 100 units. Since molecular weight determinations on alginic

acid have given values of the order of 15,000, a chain-length of less than 100 units could only be accommodated in a branched molecule. However the similarity in the X-ray patterns of alginic acid and cellulose makes this very unlikely. It should be mentioned in this connection that Smith and co9workers (190) also found unexplained glycerol in the products from a similar treatment of cellulose.

Molecular Structure of Alginic Acid

The low reduction of periodate by alginic acid is very surprising since all the evidence from this and other studies on alginic acid indicate that the residues are 1,4'-linked.

It has been found, however, (192, 193) that failure to undergo periodate oxidation does not necessarily imply the absence of adjacent hydroxyl groups. 1,6'-Anhydro-β-D-glucofuranose, for example, does not undergo oxidation because the vicinal hydroxyl groups are locked in a trans position. In polysaccharides, this failure to oxidise could arise if pyranose rings were constrained into a conformation giving an angle of 180° between trans hydroxyl groups (193). This would make the formation of a complex with the periodate ion extremely difficult, if not impossible. Alginic acid, however, contains no trans hydroxyl groups if the hexuronic acid residues are linked 1,4'-. If the residues were linked 1,2'-, trans hydroxyl groups would occur, but no evidence for such a linkage has been found.

This form of steric hindrance, therefore, would seem to be precluded in alginic acid. Moreover, construction of models of 1,4'-linked chains comprising both D-mannuronic and L-guluronic acid

units shows no evidence of steric hindrance and both types of hexuronic acid residues should be equally vulnerable to periodate attack.

Cellulose fibres are thought to be bundles of polymer chains held together by hydrogen bonds (194, 195). The similarity in physical properties between cellulose and alginic acid suggests that hydrogen bonding may occur also in the latter. It is unlikely, however, that hydrogen bonds could persist in dilute aqueous solution, and thus reduce the number of hydroxyl groups available for periodate attack. Nevertheless, it is to be noted that cellulose requires oxidation for 9 to 37 days with 0.5 M periodate at 20° before the theoretical quantity of periodate is reduced. Subsequent hydrolysis of the oxo-cellulose gave the products expected from 1,4'-linked glucose units.

A possible explanation of the low periodate oxidation in alginic acid could be that, under the conditions employed, a large proportion of the mannuronic and guluronic acid residues are present as the 3,6-lactone in the macro-molecule. Such residues would not be oxidised by periodate. (cf Kaye and Kent, 198)

Another explanation of the apparent under-oxidation of alginic acid is the presence of 1,3'-linkages in the molecule. No evidence for this linkage was found in earlier work, but the straight-chain formula of 1,4'-β-linked D-mannuronic acid residues has never been claimed to account for the whole of the molecule. It should be pointed out that the 88% yield of 2,3'-di-O-methylmannose isolated by Hirst and his colleagues from a methylated, hydrolysed, reduced

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THE PRESENCE OF L-GULURONIC ACID RESIDUES IN ALGINIC ACID

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The presence of L-guluronic acid residues in alginic acid has been reported by Fischer and Dörfel.[1] This has been confirmed by recent work in this Department. Paper chromatography of the syrup obtained after hydrolysis of alginic acid showed four spots corresponding to gulurone, mannurone, guluronic acid and/or mannuronic acid and partially hydrolyzed material. Separation of this syrup on a cellulose column, followed by glycosidation and reduction of the two fractions containing respectively the lactones and the mono-uronic acids gave syrups, each of which on hydrolysis contained only mannose and gulose (paper chromatography and ionophoresis). Although it is difficult to distinguish between mannose and gulose by paper chromatography, ionophoresis in borate buffer (pH 10) for 5 hours at 750 volts gives two well-defined spots for these two sugars with M_G 0·46 and 0·59 respectively. Although small variations in M_G were obtained on changing the conditions of ionophoresis of the hydrolysates the spots obtained were always identical with mannose and gulose run as controls. It is considered that the gulose can only have arisen by reduction of guluronic acid residues present in the parent molecule.

If alginic acid consisted entirely of 1:4-linked mannuronic acid residues then periodate oxidation followed by bromine oxidation and hydrolysis would result in a mixture of glyoxylic and *meso*tartaric acids with zero rotation. 1:4-Linked L-guluronic acid residues, if present, would, however, give rise to L(+)tartaric acid and not the *meso* acid and the final mixture would have a positive rotation. We have found that the polymeric material isolated after periodate and bromine oxidation of alginic acid had $[\alpha]_D$-31° (H_2O), and that hydrolysis of this material caused the rotation to change from α_D-0·19° (initial)→ -0·03 (4 hours) → ±0° (14 hr.)→+0·11° (22 hours, const.), indicating the presence of L(+) tartaric acid. The possibility that the positive rotation was due to a small quantity of mannurone which had arisen from unoxidized mannuronic acid residues was considered. Paper chromatography of the hydrolysate in an acid eluant gave a spot identical with tartaric acid. Ionophoresis (borate buffer, pH 10) showed the absence of any mannurone. Furthermore, the addition of crystalline ammonium molybdate to a portion of the hydrolysate greatly enhanced the rotation, a property characteristic of L(+)tartaric acid.[2] Addition of an equivalent quantity of ammonium molybdate to an aqueous solution of mannurone reduced the rotation to a negative value. Hence these results have provided additional evidence for the presence of L-guluronic acid residues in alginic acid. This work is proceeding and will be published in more detail later.

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